AMELIORATIVE EFFECTS OF A MULTI HERBAL FORMULATION UPON SOME CHOSEN DISEASE MODELS IN MICE

A thesis submitted for the degree of Doctor of Philosophy (Ph.D) in Science of Jadavpur University

By

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2022


To my beloved parents

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Certificate from the supervisor

This is to certify that the thesis entitled "**Ameliorative effects of a multi herbal formulation upon some chosen disease models in mice**" submitted by Sri Soumendra Darbar who got her name registered on 04/08/2016 (Registration Index no: 159/16/Life Sc./25) for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Prof. (Dr.) Kausikisankar Pramanik and Dr. Atiskumar Chattopadhyay and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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SYNOPSIS

Title: Ameliorative Effects of a Multi Herbal Formulation upon Some Chosen Disease Models in Mice

Submitted by: Soumendra Darbar

Treatment of different disease with a single molecule is becoming a new challenge towards the Scientists as well as researchers throughout the globe due to different reasons. Which are massive environmental changes, for global warming, changing behaviour of the agents of the disease, manifestation of the disease itself. Rapid industrialization, stressful life, uncontrolled modern life style and day to day environmental imbalance good health and long life are being threatened. Now a day's people both developing and developed countries are suffering from some non-communicable diseases like obesity, gastric ulcer, diabetes and chronic kidney disease. In this context fight against these diseases through new medicines (i.e single molecule, mixture of different drug, formulations etc) is a great challenge.

Use of formulations using medicinal plants have burgeoned in recent times due to increased efficiency of drugs derived from plants, as a result in natural products and interest in concerns about the side effects of conventional medicine. Herbal mixture combinations have shown that they possess better efficacy and reduced side-effects in comparison with single drug as in many cases, particularly in combination with single drug or mixture of allopathic drug.

Use of formulations using medicinal plants have burgeoned in recent times due to increased efficiency of drugs derived from plants, as a result also in natural products. Concerns about the side effects of conventional medicine have also become a game changer in this transitional field. Herbal mixtures and combinations have shown that they possess better efficacy and reduced side-effects in comparison with single drug as in many cases, particularly in combination with single drug or mixture of allopathic drug.

Multi herbal complex contain a combination of botanicals; each of these contains a number of chemical compounds that may give the anticipated activity in combination. These complexes may exert synergistic, potentiative, agonistic or antagonistic actions by virtue of its associated diverse active principles. Moreover, combination of herbals may act on multiple targets at the same time to provide a thorough relief. Due to synergism, polyherbalism offers some great benefits which lacks in single herbal formulation. It is evident that better therapeutic effect can be reached with a single multi-constituent formulation. Multi herbal formulation also having multiple types of molecules against a disease complication, so different molecules may cure a disease by different mechanism acting on different target molecules involves in the etiology of a disease and provide a better therapy against a disease condition.

Nanomedicine, raises high expectations for millions of patients for better, more efficient and affordable healthcare and has the potential of delivering promising solutions to many illnesses. From diagnosis to disease monitoring, going through surgery and chemotherapy or regenerative medicine,

nanotechnologies virtually impacts all fields of current medicine. Several areas of medical care are already benefiting from the advantages that nanotechnology can offer. The first nanotechnology-based targeted drug delivery systems are already in the market, others are in clinical trials or, by far the largest part, are under development.

The thesis work proposes synthesis of novel herbal formulation and evaluation of its toxicological, anti-ulcer, anti-obese, chronic kidney disease and non-alcoholic steatohepatic therapeutic action in murine model. The thesis will also encompass green synthesized herbal conjugate with *Andrographis paniculata* and metal oxide nanoparticle like Ag, depicting their ameliorative potencies upon disease models like hepatic fibrosis, diabetic nephropathy and hepatic injuries. Their toxicological signatures are observed prior to their therapeutic applications. The nanoparticles are synthesized using cost effective and facile green routes for better bioavailability. Both in-vitro and in-vivo studies of affected organs along with their histopathological signatures are taken into account and will aim to look into the molecular mechanism for better efficacy.

Our *in-vivo* studies on experimental animal model have addressed the following questions on the medicinal/toxicological effects of the herbal formulations/ nanoconjugates:

1) General toxicological studies and histopathological changes in mouse model
2) Effects of hepatobilary, renal and alimentary system
3) Several parameters including some important biochemical parameters responsible for free radical scavenging and antioxidant activity were monitored
4) Significant ameliorative changes were observed with some findings.

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Soumendra Darbar

LIST OF PUBLICATIONS

1. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Antioxidant and immunomodulatory effect of AKSS16-LIV01–a multi herbal formulation against ethanol induced liver dysfunction in mice. Clinical Phytoscience. 2021; Volume 7, Issue 1:1-20.
2. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Sanative effect of multiherbal formulation–akss16-liv01 on ccl4-induced hepatic dysfunction in mice. Asian Journal of Pharmaceutical and Clinical Research. 2021; Volume 14, Issue 1:101-106.
3. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Ameliorative Effect of Multi Herbal Formulation on Lipid Peroxidation and Redox Dysfunction in Ethanol Induced Hepatic Imbalance. Indian Journal of Pharmaceutical Education and Research. 2021; Volume 55, Issue 1:215-223.
4. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Preliminary assessment of acute and 28-day repeated dose oral toxicity of a newly developed herbal mixture on experimental animal. Indian Journal of Pharmaceutical education and Research. 2000; Volume 54, Issue 1:135-142.
5. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Ameliorative efficacy of novel multi herbal formulation (AKSS16-LIV01) upon Haematological modulations induced by fixed dose combination of tramadol hydrochloride/paracetamol (THP). Journal of Drug Delivery and Therapeutics. 2020; Volume 10, Issue 6:11-17.
6. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Therapeutic Application of Novel Multi Herbal Formulation (AKSS16LIV01) against the inductive influence of Carbon Tetrachloride (CCl4) upon Tissue and serum protein in Experimental animals. Bulletin of Environment, Pharmacology and Life Sciences. 2020; Volume 9, Issue 12: 142-148.
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10. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Unprecedented redox scavenging signature along with antioxidant action of silver nanoparticle coupled with Andrographis paniculata (AP-Ag NP) against carbon tetrachloride (CCl4) induced toxicity in mice. In Biotechnology and Biological Sciences. 2019; Nov 20 (pp. 64-68). CRC Press.

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List of Publications (Associate Author)

Atiskumar Chattopadhyay., **Soumendra Darbar**, Srimoyee Saha, Parimal Karmakar. Effects of alprazolam administration on the vital organs of adult wister albino rats, biochemical and toxicological studies. Indian Journal of Pharmaceutical education and Research. 2019; 53(1):127-132.

Paper Presented in the International Seminar

1. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. A Novel Green Synthetized Silver Nanoparticle act as a Nonozyme and Potentially Useful against Oxidative Stress: Challenges and Opportunities. 3rd Series of the Emerging Scientists, Asian Council of Scientific editors. 20th & 21st February, 2022. Deira Dubai, UAE (Virtual). **Oral Presentation**
2. **Soumendra Darbar**, Srimoyee Saha and Atiskumar Chattopadhyay; Redox scavenging signature along with antioxidant action of silver nanoparticle coupled with Andrographispaniculata (AP-Ag NP) against carbon tetrachloride (CCl_4) induced toxicity in mice. 2nd World Nanotechnology Webinar. Magnus Group-USA. 19th & 20th April, 2021. **Oral Presentation**
3. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Free radical scavenging potential and Antioxidant activity of Silver Nanoparticle Coupled with *Andrographis paniculata* (AP-Ag NP) Against Carbon tetrachloride (CCl4) induced toxicity in mice. International conference on nanotechnology and nanomaterials. 2nd and 3rd March, 2020; Rome, Italy. **Oral Presentation**
4. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Free radical scavenging potential and Antioxidant activity of Silver Nanoparticle Coupled with *Andrographis paniculata* (AP-Ag NP) Against Carbon tetrachloride (CCl_4) induced toxicity in mice. Biospectrum, 8th to 10th 2019. **Oral Presentation**
5. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Therapeutic Potential of AgNPs coupled with *Andrographis paniculata* against Chronic Liver Diseases in Experimental Murine Model. ACS publications forum expanding frontiers in chemical sciences. November 1, 2018; Banaras Hindu University, U.P. **Oral Presentation**
6. Soumendra Darbar, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Therapeutic Potential of AgNPs coupled with *Andrographis paniculata* against Chronic Liver Diseases in Experimental Murine Model. International Seminar on "Recent Advances in Molecules and Materials. 2nd and 3rd August, 2018 at School of Applied Science & Humanities Haldia Institute of Technology Haldia. **Oral Presentation**

Paper Presented in the National Seminar

1. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Sanative effects observed through immunomodulatory and antioxidant activity of AKSS16-LIV01 – a multi herbal formulation against ethanol induced liver dysfunction in mice. Two-Day National

Conference on Sustainable Health Science for Future Generations. 28th and 29th April, 2022, University of Kalyani, West Bengal. **Invited Talk.**

2. **Soumendra Darbar** and Atiskumar Chattopadhyay. Novel Green Synthesis Silver Nanoparticle from *Andrographis paniculata* act as a Nonozyme and Potentially Useful against Oxidative Stress: Challenges and Opportunities. XXXIIth Annual Conference of The Physiological Society of India. 4th to 6th March, 2022, Berhampore Girls College, Murshidabad, West Bengal. **Invited Talk**
3. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Therapeutic Efficacy of a Newly Developed Herbal Formulation Against CCl_4 Induced Hepatic Fibrosis. Indian Science Congress. 3rd to 7th January, 2019, Lavly professional University, Jalandhar, Punjab. **Oral Presentation**
4. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Safety and Efficacy Study of Silver Nanoparticle Synthesized through Green Route Using Andrographis *paniculata* in Experimental Model. 3rd Pharm. Tech. IAPST International Conference; 19th and 20th January, 2019, Centurian university, Bhubaneswar, Odisa. **Oral Presentation**
5. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Evaluation of Acute Toxicity Study of AgNPs coupled with Andrographis paniculata in Experimental Murine Model. Indian Pharmaceutical Congress. Delhi NCR, 21-23 December 2018. **Poster Presentation**
6. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. AKSS-16101 attenuate pathological changes in non-alcoholic steatohepatitis (NASH) mouse model. Indian Science Congress. 3rd to 7th January, 2018, Manipur University, Manipur. **Oral Presentation**

Award and Honour

Best Presentation Award

1. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Free radical scavenging potential and Antioxidant activity of Silver Nanoparticle Coupled with *Andrographis paniculata* (AP-Ag NP) Against Carbon tetrachloride (CCl_4) induced toxicity in mice. Biospectrum, 8th to 10th 2019.
2. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Protective effect of multi herbal formulation against CCl_4 induced liver damage in mice. National workshop on Pharmacist for Healthy India. Indian Pharmaceutical Association. 25th November, 2018, Jadavpur University, Kolkata.

Book Chapter

1. **Soumendra Darbar**, Srimoyee Saha, Kausikisankar Pramanik and Atiskumar Chattopadhyay. Haematological Modulations by Fixed Dose Combination (FDC) of Tramadol Hydrochloride/Paracetamol (THP). Frontiers in Clinical Drug Research-Hematology: Bentham Science Publishers, 2022; Volume 5, Mar 8;5:154.

Workshop Attended

1. Selected and Participated in the two days training programme for Nominees of CPCSEA organized by CPCSEA conducted from 18th September 2018 to 19th September 2018.
2. Participated in the ACS Publication Forum, Benaras Hindu University, November 1, 2018.

CONTENTS

ABBREVIATIONS

Sl. No.	Abbreviation	Meaning
1.	WHO	World Health Organization
2.	DILI	Drug induced liver injury
3.	DAMPs	damage associated molecular pattern molecules
4.	FDC	Fixed dose combination
5.	PCD	Programme cell death
6.	ROS	Reactive oxygen species
7.	ALD	Alcoholic liver disease
8.	BR	bilirubin
9.	NAFLD	non-alcoholic fatty liver diseases
10.	CKD	Chronic kidney disease
11.	MHF	Multi-herbal formulation
12.	NO	Nitric oxide
13.	TCA	trichloroacetic acid
14.	PDA	Potato dextrose agar
15.	DM	dichloromethane
16.	AQ	aqueous extract
17.	EA	ethyl alcohol
18.	DPPH	1, 1-diphenyl-2-picrylhydrazyl
19.	TPC	total phenolic component
20.	HSCs	hepatic stellate cells
21.	PDGF	platelet-derived growth factor
22.	TNF-α	tumor necrosis factor-α
23.	TGF-β	transforming growth factor-β
24.	CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals

Sl. No.	Abbreviation	Meaning
25.	IAEC	Institutional Animal Ethics Committee
26.	EDTA	Ethylenediamine tetraacetic acid
27.	Hb	Haemoglobin
28.	RBC	Read Blood corpuscle
29.	RT	Reticulocyte
30.	HCT	Haematocrit
31.	MCV	Mean corpuscular volume
32.	MCH	Mean corpuscular hemoglobin
33.	MCHC	Mean corpuscular hemoglobin concentration
34.	AST	Aspartate aminotransferase
35.	ALT	Alanine aminotransferase
36.	ALP	Serum alkaline phosphatase
37.	GGT	Gamma-glutamyl transferase
38.	TC	Total cholesterol
39.	TG	Triglycerides
40.	FFA	Free fatty acids
41.	HDL	High-density lipoprotein
42.	LDL	low-density lipoprotein
43.	SD	standard deviation
44.	ANOVA	analysis of variance
45.	BUN	Blood urea nitrogen
46.	CAT	Catalase
47.	SOD	Superoxide dismutase
48.	GST	Glutathione S-transferees
49.	GPx	Glutathione peroxidase
50.	DNA	Deoxyribonucleic acid
51.	TBA	Thiobarbituric acid

Sl. No.	Abbreviation	Meaning
52.	MDA	Malonedialdehyde
53.	DCF-DA	Dichlrofluorescein diacetate
54.	THP	tramadol hydrochloride/paracetamol
55.	SNRI	serotonin–norepinephrine reuptake inhibitor
56.	IR	immediate release
57.	HSLs	hormone-sensitive lipases
58.	HFD	high-fat diet
59.	OECD	Organisation for Economic Co-operation and Development
60.	UV-VIS	UV-visible
61.	HRTEM	high resolution transmission electron microscopy
62.	XRD	X-Ray Diffraction
63.	NPs	Nanoparticles
64.	FTIR	Fourier Transform Infrared Spectroscopy
65.	TAA	total antioxidant

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Chapter - I

Introduction

1. INTRODUCTION

1.1 The Burden of Chronic Diseases

The burden of chronic disease in the U.S. and worldwide has sharply increased over the last two to three decades and is now so significant that both the Centers for Disease Control and Prevention and the World Health Organization consider both prevention of chronic disease and prevention of chronic disease complications to be critical goals in improving national and global health [1]. Chronic diseases require a long period of treatment that leads to the increase in demand for healthcare services and changes its nature. Chronic diseases and conditions are the leading cause of death and disability throughout the globe [2-4].

1.1a Liver complications

Liver is the major essential organ of the body. The main roles of the liver include removing toxins, processing food nutrients and regulating body metabolism. Important causes of liver disorders are fatty liver, hepatitis virus infections and alcohol. Cirrhosis (liver scarring), the end-result of many liver disorders, can lead to liver failure [4].

1.1b Hepatic Fibrosis

Treatment upon various liver complication is became a new challenge. Liver disease is the 5th most common cause of death. Every year there are 400 million detected cases of potentially life threatening liver diseases [5]. Hepatic fibrosis ultimately leads to cirrhosis or hepatic carcinoma, causing significant morbidity and mortality. Hepatic fibrosis, increased accumulation of extracellular matrix and activation of hepatic stellate cells, is a common response to chronic liver injury from a number of causes including alcohol, toxin, and persistent viral and helminthic infections which may ultimately lead to hepatic carcinoma and end-organ failure. No drugs because of unacceptable side effects and limited efficacy during long term therapy [6-9].

1.1c Drug induced liver injury (DILI)

Drug-induced hepatotoxicity is one of the major concerns in medical practice. The incidence of DILI in general populations is about 14–19 per 100,000 inhabitants, while frequency estimated in a healthcare system is around 30–33 per 100,000 persons. The reported incidence and severity of DILI varies among drugs Mechanisms in DILI are two-fold: 1) drug/metabolite exposure to a threshold level, determined by the dose and drug handling of the liver, and 2) the adaptive immune response or ‘‘alarm-signalling’’ by the damage associated molecular pattern molecules (DAMPs). Cellular damage occurs at an intricate balance between toxic drug exposure and defence mechanisms [10]. Once cells are damaged, innate and adaptive responses kick-in and play a significant role in driving tissue inflammation and injury [11]. According to the report of World Health Organization (WHO) 14 million worldwide suffered in various hepatic and renal complications out of which 2.38 million deaths were estimated due to consumption of fixed dose combination (FDC). Chronic used and over

dose of FDC break antioxidant defence system in the body resulting programme cell death (PCD). Thus to overcome this deleterious effects upon liver by the FDC is become a new challenge in this era [12].

1.1d Alcoholic liver disease (ALD)

Alcohol, a natural product that has been available for human consumption for thousands of years, is a common cause for ROS insult in the liver Despite the claim that small amount of alcohol consumption may be beneficial for preventing and reducing the mortality rate of coronary heart diseases and ischemic stroke, it should also be noted that alcohol is toxic to almost every organ of the body [13, 14]. Metabolism of alcohol in liver generates excessive free radicals and increased peroxisomal oxidation of fatty acid, which would ultimately affect functionality of the antioxidant systems to eliminate ROS in the body. Therefore, the mechanism to restore hepatic injuries caused by alcoholic oxidative stress is tightly regulated by the antioxidant status of a living system. Drugs which prevent liver damage by alcohol is now really needed [15-17].

1.1e Hyperlipidaemia (Obesity)

Overweight and Obesity is a serious health problem throughout the world, is reaching pandemic levels. According to World Health Organization's (WHO) report more than one billion people are overweight out of which three hundred million meeting the criteria for obesity [18]. Due to the excessive fat accumulation in the body, people both developed and developing countries are suffering for various overweight related complication such as type 2 diabetes, cardiovascular disease, coronary artery disease, stroke, myocardial infarction, dyslipidemia, anxiety, hypertension, mental stress, hepatic disorder and various type of cancer. Another report stated that 26% nonpregnant woman ages between 20 to 39 years are overweight and out of which 29% are obese. World statistics stated that 35% men and 37% woman are overweight and 40% men and 30% women are obese. Developing effective strategies to prevent obesity is therefore of paramount importance [19].

1.1f Hyperbilirubinemia

The term hyperbilirubinemia is defined as increased bilirubin (BR) level (>1.3 mg/dl in human) in blood. Hyperbilirubinemia is caused when there is an imbalance between production of BR (resulting from hemolysis, sepsis, blood extravasation or polycythemia) and decrease in BR excretion due to inadequate hepatic conjugation and increased enterohepatic reabsorption (resulting from pyloric stenosis, delayed bacterial gut colonization, GI tract immobility or obstruction). Although at micromolar concentrations BR acts as an antioxidant for scavenging peroxyl radicals in blood, yet it can be toxic and harmful to cells at higher concentration. Elevated level of BR and its oxidative products in human blood causes various diseases including neonatal jaundice (>10 mg/dl), Gilbert syndrome (with BR level reaching >6 mg/dl), Crigler–Najjar type I disease (>30 mg/dl) and BR-induced neurologic dysfunction. Severe neurotoxicity in case of neonates (Kernicterus) and damage in white matter of adult brain are also the consequences of higher BR level. So invention of safe and symptomatic medication is a new challenge in the 21st century [20].

1.2 Liver Disorders

There are many different types of liver diseases but with any liver disease the liver damage will progress in a similar way. It is important to know that what happens to the liver at each stage of the progression before complete liver failure.

The four major stages of liver failure are as follows:

1.2a Stage 1: Inflammation (Fatty liver / NAFLD)

Inflammation is a sign of preliminary stage of liver diseases: viral hepatitis or immune hepatitis, alcoholic fatty liver or a non-alcoholic fatty liver. The latter, however, can occur without noticeable inflammation. If you have these symptoms, see a doctor right away. At this stage, the damage is potentially reversible [21].

1.2b Stage 2: Scarring (Liver Fibrosis)

Left unchecked the inflammation will start to scar your liver. Scarred tissue won't do the work of healthy tissue, so the more scarring there is the less efficient your liver becomes. This process is known as Fibrosis. If the amount of tissue within the liver is overtaken by Fibrosis, toxins will build up, waiting to be processed. The more this happens, the more the tissue will become fibrotic and incapable of performing its function [22].

1.2c Stage 3: Cirrhosis

At this point, your liver is severely scarred and cannot heal itself. However, developing cirrhosis takes a long time, sometimes decades. So, there's plenty of time to act before things start getting critical. The bad news is this is usually the stage where liver disease makes its presence most felt. Discomfort and tenderness moves to outright pain. You'll also start feeling tired all the time, fatigued easily. You'll eat less because you find you have no appetite. Ironically, you'll still feel bloated even though you're not eating as much. This is caused by the build-up of fluid as a result of the liver not functioning properly [23].

1.2d Stage 4: Liver Failure (Hepatic Carcinoma)

There are two types of liver failure. Acute Liver Failure happens fast, within 48 hours. Usually, this is a reaction to having a poison in your system or a drug overdose. But in the case of alcohol poisoning your liver, you get Chronic Liver Failure. It's the latter that most leads to the need to have a liver transplant [24].

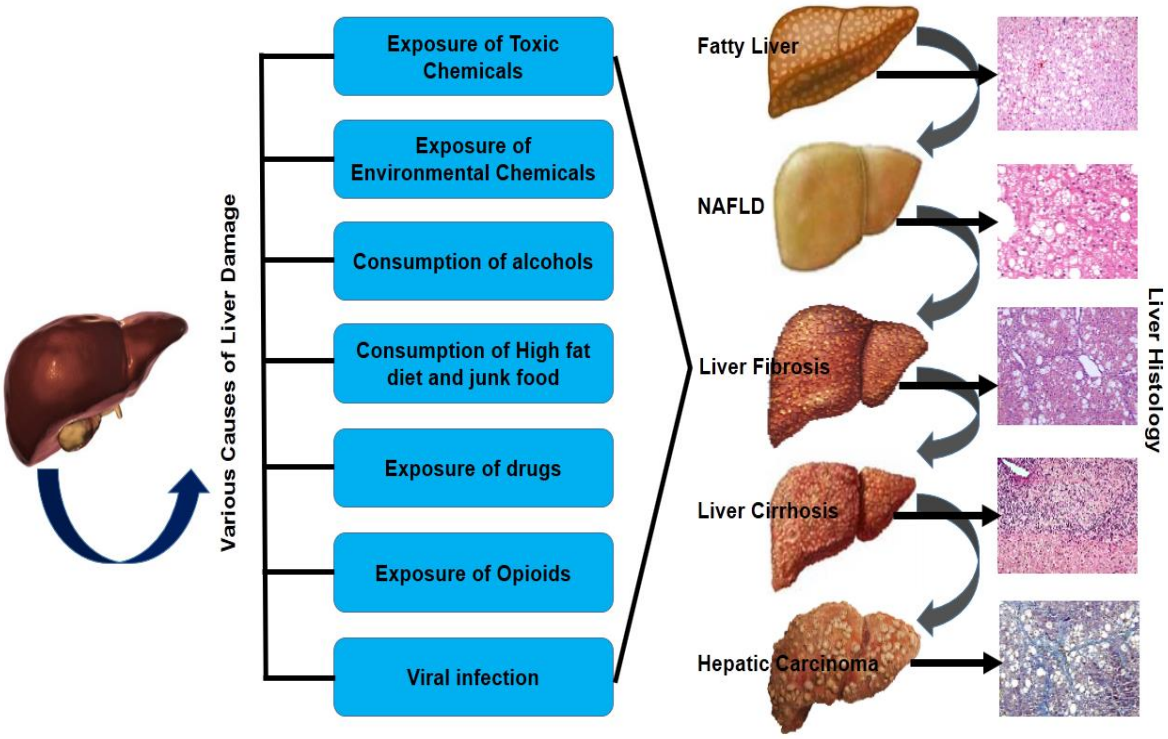

Figure 1.1: Various stages of Liver Complications

1.3 Detoxification

Detoxification, or "detox" for short, is one of the crucial body's process of removing toxic substances and maintain the homeostasis. Liver and Kidneys are the main detoxifying organ those protects our body from the deleterious effects of the various toxic and hazardous substances [25]. It is established that liver enzymatically disassembles the undesired toxic, hazardous and poisonous chemicals and substances by a process called detoxification that occurs in two steps known as Phase-I and Phase-II.

1.3a Phase-I detoxification

Scientific evidence revealed that enzymes which is collectively known a cytochrome P450 (CYP450) is mainly involved in this process. The enzymes either directely neutralised a toxin or modify it to form an active intermediate that is then neutralised by phase-II detoxification process [26]. The activity of the enzymes can very significantly from one individual to another based on their genetic makeup, exposure level of toxin, drugs effects and nutritional status. Without adequate antioxidant defence system, tissue lost their normal functions and oftern damage the cell.

1.3b Phase-II detoxification

In Phase II liver detoxification neutralizes the by-products of Phase I liver detoxification and other remaining toxins. This is done by making the toxins water-soluble. That way they can be excreted from the body. This process is known as conjugation. Glutathione, sulphate, and glycine are the

primary molecules responsible for this process [27]. Under normal conditions, Phase II liver detoxification enzymes produce low levels of glutathione. Under times of high toxic stress the body increases production of glutathione [28].

1.4 Chronic kidney disease

Chronic kidney disease is nowadays increasing at an alarming rate. Chronic kidney disease (CKD), is characterized by sudden loss of the ability of the kidneys to excrete wastes, concentrate urine, conserve electrolytes, and maintains fluid balance. The mortality rate of patients with CKD has remained 25–70% despite the use of various pharmacologic agents. Nephrotoxicity is mostly related to oxidative stress and nowadays much attention has been made towards the possible nephroprotective properties of medicinal plants [29].

1.5 Drug development

Drug is the main weapon that continuously fights against diseases. In this era without taking drug healthy and disease free life practically impossible. Scientists and researchers all over the globe engaged for development of new therapeutic medication for the benefit of mankind's [30]. Apart from age drug is the main tool for the survivability. Scientific study reveled that modern allopathic medicine produced lots of complication as well as makes serious adverse effects. So in point of safety these medicines are not 100% safe in the body. In the 21st century people are engaged to find out some new medications those are safe and cost effective. Doctors and researchers are now focused on phyto and nano medication as there tergated drug delivery action [31].

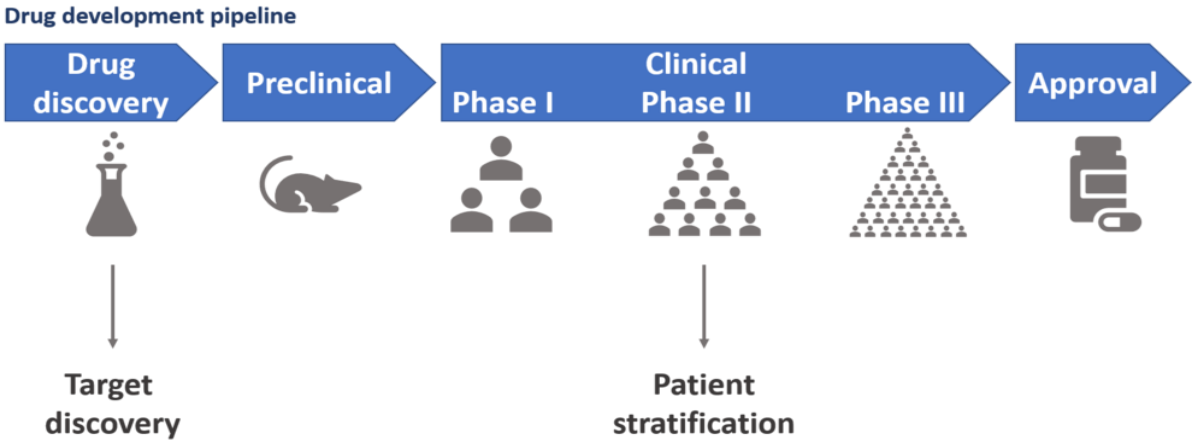

Figure 1.2: Various stages of drug development

1.5a Phytomedicine

Medicine derived from medicinal herbs are enrich with polyphenols and flavonoid, are safe for medication in various diseases. Natural medicine generally acts as an antioxidant and inhibits the oxidative stress and inflammation which gift disease free life. Herbal drug combinations have shown

that they possess better efficacy and reduced side-effects in comparison with single herbal drugs. Drug combination of ten produces a promising effect in treatment of diseases over a single drug [32].

1.5b Nanomedicine

Nano technology possesses several branches including nanomedicine, which is the most promising field in the future medicine and is a probable therapeutic agent in prevention and medication of life threatening diseases through ROS inhibition. *Synthesis of nanomaterials through green route* can be useful for both in vivo and in vitro in biomedical research. Chronic diseases such as diabetes, cardiovascular disease and liver fibrosis increase oxidants and decrease antioxidants in patients [33].

1.6 Natural therapy

In the last two decades therapeutic application of plants and plants related products are extensively popular throughout the globe. Medical practitioners extensively depend on traditional and alternative medicine for prevention, diagnosis and treatment of various illnesses. Peoples really depend on the herbal drug because of their fewer side effects in comparison to modern allopathic drugs. Chronic consumption of conventional medicines produces various complications and became sometimes life threatened [34](34). The World Health Organization's (WHO) report stated that 89% of the world population relies on "alternative" plant-based medicines as their primary medical intervention especially in the developing and in the developed countries [35].

Generally natural products play a dominant role in the development of novel drug leads for the treatment and prevention of diseases. Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. Herbal medicines have attained the widespread acceptability as natural therapeutic agents for various diseases like fibrosis, arthritis, renal and liver diseases, obesity and hyperbilirubenimia. It is proved that herbal combination made up of different herbs which produce maximum therapeutic outcomes than the individual herbs. These combinations are employed for the betterment of various chronic disorders. Currently worldwide there is need to found out the safe, less toxic, cost effective polyherbal remedies that can be effective against various chronic diseases like diabetes, obesity, liver dysfunction [36].

1.6a Role of Natural product in prevention of diseases

Recently, it has been reported that herbal medicines possess various biological activities, such as antinociceptive, antibacterial, antiviral, anti-inflammatory, anticancerous, antifungal, antitumoral, antioxidant, Hepatoprotective, antiulcer, antiaging, Antidiabetes, and immune modulating, properties, the most essential of which is its action against microorganisms. Chemical studies carried out on various poly-herbal extracts revealed the existence of a very complex mixture of different naturally occurring compounds those are actively sound for potential therapeutic effects [37]. Scientific studies identified various compounds present in the herbal formulations those include: flavonoids, prenylated p-coumaric acids and acetophenones, lignans, phenolic compounds, di- and triterpenes, caffeoylquinic acids, sugars, sugar alcohols, hydrocarbons, and mineral elements. It is very true that natural products are helpful in drug development as most clinical drugs originated from natural products including plant secondary metabolites. Although the main role of these secondary metabolites is defences

against plant predators and pathogens, interestingly, there are now a huge number of reports that explore the activity of these natural products present in leaves and many other plant parts for pharmacological applications including the development of antimicrobial drugs [38].

1.6b Mechanism involved for natural product action

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide, and free radicals, plays a crucial role in the development of various ailments such as fibrosis, hyperlipidaemia (Obesity), drug induced liver and renal dysfunctions, alcoholic liver diseases, hyperbilirubenemia etc. Many natural herbs contain antioxidant compounds which protects the cells against the damaging effects of ROS. Though our body is safeguarded by the natural antioxidant defense, there is always a demand for antioxidants from external natural source [39]. In addition, secondary metabolites, such as phenolic compounds, flavonoids, alkaloids, and tannins, are widely distributed in plants and are reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic effect, etc.

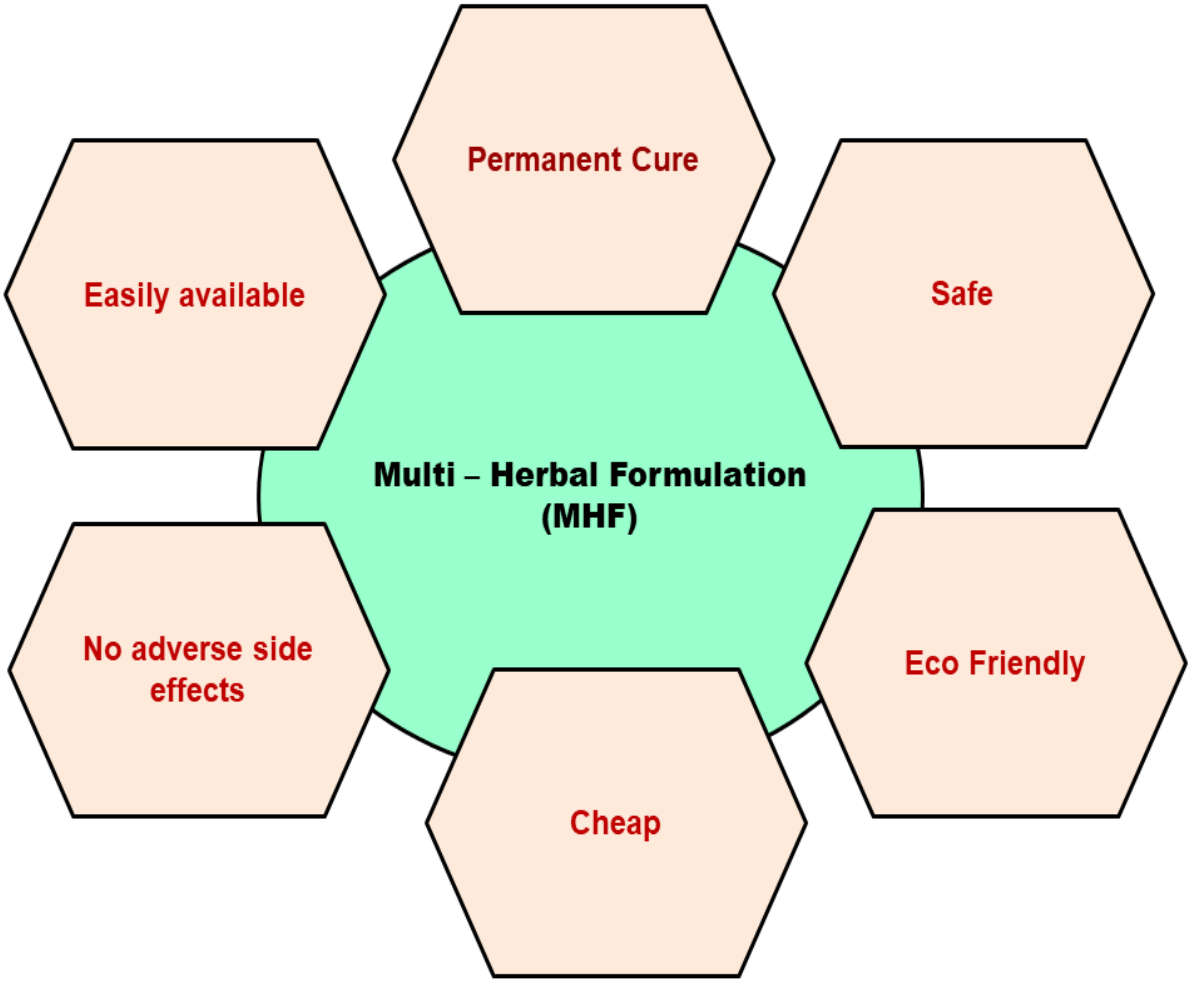

Figure 1.3: Advantages of Multi Herbal Formulation (MHF)

1.6c Concept of Multi herbal formulation

Use of formulations using medicinal plants have burgeoned in recent times due to increased efficiency of drugs derived from plants, as a result in natural products and interest in concerns about the side effects of conventional medicine. Herbal mixture combinations have shown that they possess better efficacy and reduced side-effects in comparison with single drug as in many cases, particularly in combination with single drug or mixture of allopathic drug. Multi herbal formulation also having multiple types of molecules against a disease complication, so different molecules may cure a disease by different mechanism acting on different target molecules involves in the etiology of a disease and provide a better therapy against a disease condition [40]. Multi-herbal formulation (MHF) is the use of more than one herb in a medicinal preparation. It is a dosage form of Indian traditional medicinal system. The concept is found in Ayurvedic and other traditional medicinal systems where multiple herbs in a particular ratio may be used in the treatment of illness. Herbal medicines have existed world-wide with long recorded history and they were used in ancient Chinese, Greek, Egyptian and Indian medicine for various therapies purposes [41]. World Health Organization estimated that 80% of the word's inhabitants still rely mainly on traditional medicines for their health care.

1.6d Why Formulation?

Multi-herbal formulations express high effectiveness in numerous diseases with safe high dose.

- ❖ It is established that MHF have multiple active compounds that together can provide a potentiating effect that may not be achievable by any single compound.

- ❖ Various pharmacodynamics and pharmacokinetic study on MHF proved its better synergistic effect compared to the single one. Due to synergism, polyherbalism offers some great benefits which lacks in single herbal formulation.

Aims and Objective:

In this thesis our main objective is to develop a newly novel multi herbal medication composed of Indian medicinal herbs and medicinal spices first time the globe which is effectively sound for various non-communicable diseases like liver fibrosis, alcohol induced liver damage (AILD), hyperlipidemia (Obesity), Drug induced liver injury (DILI). We also try to apply the medicine against renal dysfunction and noted the therapeutic effects. On the other hand we also developed a novel nano conjugate synthesized through green route and find out there targeted action on liver protection. Apart from this we chose the key herbs from the said formulation and the different extract which is apply for prevention of hyperbilirubinemia. We are committed to provide safe and symptomatic medications which are cast effective for the beneficial to the mankind.

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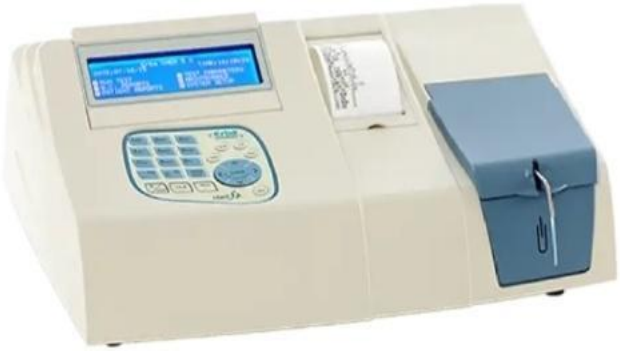
1A Instruments used in this study

1A.1 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a sophisticated instrument for detecting and quantifying peptides, proteins, antibodies, and hormones. In ELISA, an antigen must be immobilized to a solid surface and then complexes with an antibody that is linked to an enzyme. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. The instrument is typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. The binding and immobilization of reagents makes ELISAs simple to design and perform. Having the reactants of the ELISA immobilized to the microplate surface enables easy separation of bound from non-bound material during the assay. In this study the all cytokines assay as well as antioxidant enzymes measurements were determined by ELISA.

1A.2 Semi-automated biochemistry analyzer (SABA)

This is a compact, simple, reliable semi-automated biochemistry analyzer capable to perform tests on whole blood, serum, plasma, cerebrospinal fluid and urine as sample. It is a programmable Analyzer for manual applications, designed for In Vitro Diagnostic. Photometry is the most common method for testing the amount of a specific analyte in a sample. In this technique, the sample undergoes a reaction to produce a color change. Then, a photometer measures the absorbance of the sample to indirectly measure the concentration of analyte present in the sample. The instrument measured properties of blood and other fluids which is useful in the diagnosis of disease. In this study various biochemical parameters, lipid profile & renal parameters were analyzed with this instrument.



1A.3 Automated Cell Counter (ACC)

Automated Cell Counter (ACC) (Hematology analyzers) are used widely in patient and research settings to count and characterize blood cells for disease detection and monitoring. Basic analyzers return a complete blood count (CBC) with a three-part differential white blood cell (WBC) count. Through this modern instrument measured Haemoglobin (Hb), Packed cell volume (PCV), Haematocrite (Hct), Platelet, Red blood cell (RBC) count, White blood cell (WBC) count, Mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC). Here haematological study was carried out with this instrument.

1A.4 Compound Advanced microscope (CM)

Compound microscope is a microscope that uses multiple lenses to enlarge the image of a sample. The instrument is used to view specimens NOT visible to the naked eye such as blood cells and tissues. This microscope provides two dimensional image. In this study all the photographs of different tissue sections and blood cell image was carried out with this instrument. Histological sections photographs were taken with this microscope.

1A.5 UV Visible Spectrometers (UV-VIS)

Ultra violet visible spectrophotometer is a instrument used to quantitative measure how much a chemical substance absorbs light. This is done by measuring the intensity of light that passes through a sample with respect to the intensity of light through a reference sample or blank. The basic principle is when ultraviolet radiations are absorbed; this results in the excitation of the electrons from the ground state towards a higher energy state. The absorption of ultraviolet light by a chemical compound will produce a distinct spectrum which aids in the identification of the compound. With this instrument measured and quantify various biochemical parameters as well as standardized the nanoparticle (APAg-NP).

1A.6 Fourier-transform infrared spectroscopy (FTIR) analyzer

A Fourier Transform InfraRed (FT-IR) Spectrometer is an instrument which acquires broadband Near InfraRed (NIR) to Far InfraRed (FIR) spectra. Unlike a dispersive instrument, i.e. a grating monochromator or spectrograph, FTIR spectrometers collect all wavelengths simultaneously. It is a technique used to obtain an infrared spectrum of absorption or emission of a solid, liquid or gas. An FTIR spectrometer simultaneously collects high-resolution spectral data over a wide spectral range. In this study FTIR is used for standardization of nanoparticle (APAg-NP).

1A.7 Soxhlet Extraction Apparatus (SEA)

The Soxhlet extractor is placed onto a flask containing the extraction solvent. The Soxhlet is then equipped with a condenser. The solvent is heated to reflux. The solvent vapour travels up a distillation arm and floods into the chamber housing the thimble of solid. Apparatus designed for extraction and concentration where the desired compound has a limited solubility in a solvent and the impurity is insoluble in that solvent; may be operated without strict monitoring or management. The extraction was prepared by this apparatus.

1A.8 Transmission electron microscope (TEM),

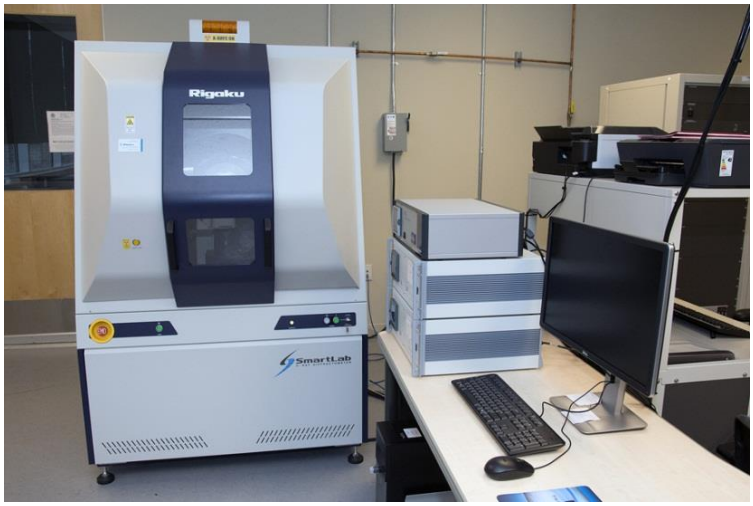
Transmission electron microscope (TEM)**,** type of electron microscope that has three essential systems: (1) an electron gun, which produces the electron beam, and the condenser system, which focuses the beam onto the object, (2) the image-producing system, consisting of the objective lens, movable specimen stage, and intermediate and projector lenses, which focus the electrons passing through the specimen to form a real, highly magnified image, and (3) the image-recording system, which converts the electron image into some form perceptible to the human eye. The image-recording system usually consists of a fluorescent screen for viewing and focusing the image and a digital camera for permanent records. In addition, a vacuum system, consisting of pumps and their associated gauges and valves, and power supplies. For capture nanoparticle (APAg-NP) image TEM is used.

1A.9 Scanning electron microscope (SEM)

Scanning electron microscope is a type of electron microscope that produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the surface topography and composition of the sample. The Scanning Electron Microscope (SEM) introduced here utilizes an electron beam whose wavelength is shorter than that of light and therefore observing a structure down to several nm in scale becomes possible. The instrument is utilized in various fields such as medical, biological, metals, semiconductors and ceramics, is broadening its application frontier. With abundant attachments and devices being combined, its capability is expanding. For capture nanoparticle (APAg-NP) image of SEM is used.

1A.10 X-ray diffraction (XRD)

X-ray diffraction (XRD) is a powerful nondestructive technique for characterizing crystalline materials. It provides information on structures, phases, preferred crystal orientations (texture), and other structural parameters, such as average grain size, crystallinity, strain, and crystal defects. For characterization of nanoparticle (APAg-NP) the instrument is used.



Chapter - II

Development of a Novel Multi Herbal Formulation (AKSS16-LIV01) and its Standardization, In-vitro analysis and Safety & Efficacy Study

2A Study of in vitro phytochemical constituents, antimicrobial and antifungal activity of a low cost novel multi herbal formulation (AKSS16-LIV01)

2A.1 INTRODUCTION

Therapy by traditional herbal medicine extensively used from the ancient age and have more or less very minimum adverse effects from compared with synthetic modern medicines [1]. As per world health organization's (WHO) report 25% of the crude modern drugs used in last decade are derived from plants, out of which approximately 5-15% have been explored for bioactive compounds [2]. It is noted that last few decades use of multi-herbal remedies extensively increase due to their therapeutic effects upon various diseases. In the traditional herbal medical system India is the pioneer country from the ancient time. From the time of Charaka and Susutra it is proved that this medicinal plants used prevent various life threatening human diseases [3]. Alkaloids, flavonoids, tannins, polyphenols, terpenoids, resins, steroids etc. are the major key phytochemicals found in medicinal plants responsible for preventing the various chronic and life threatening diseases without any adverse side effects. Theses phytochemicals have various antimicrobial and antifungal properties. Bacteria including, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris* and *Bacillus subtilis*, were mostly responsible for different infections [4, 5]. These phytochemicals have capable to prevent various infectious diseases as either suppression of bacterial growth or destroys the bacterial membranous lipids [6].

Now a day's approximately 85 to 87% people throughout the globe depend upon herbal remedies either as crude extract or drugs for the medications of lots of infectious & non infectious diseases, wound healing activity, inflammations, etc. It is established that mostly 95% medicinal plants those are used in ayurvedic medicines are less toxic, cost effective and produce optimum sanative effects in compared to allopathic modern medicine [7, 8]. So, over the globe scientist and researchers are engaged to formulate some novel herbal medicine those are potent for various diseases.

Here, we developed a novel, low cost, safe and symptomatic herbal formulation (AKSS16-LIV01) containing nine Indian medicinal plants out of which six were medicinal plants and three were medicinal spices. The present work highlighted to screen the in-vitro phytochemicals analysis and antimicrobial and antifungal study of the developed formulation.

2A.2 MATERIALS AND METHODS

2A.2.1 Collection and Authentication of herbs –

All raw medicinal plants were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by an expert taxonomist of the Jadavpur University, and kept as a voucher specimen. The identification was based on Ayurvedic parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti (shape) and Parimana (size). The plants and plant parts used in the preparation of the extract are depicted in Table 1.

Table 2A.1: Details ingredient(s) present in the newly developed multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Family	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	10 mg

* Amount depicted in the table are required for preparation of 5 ml extract.

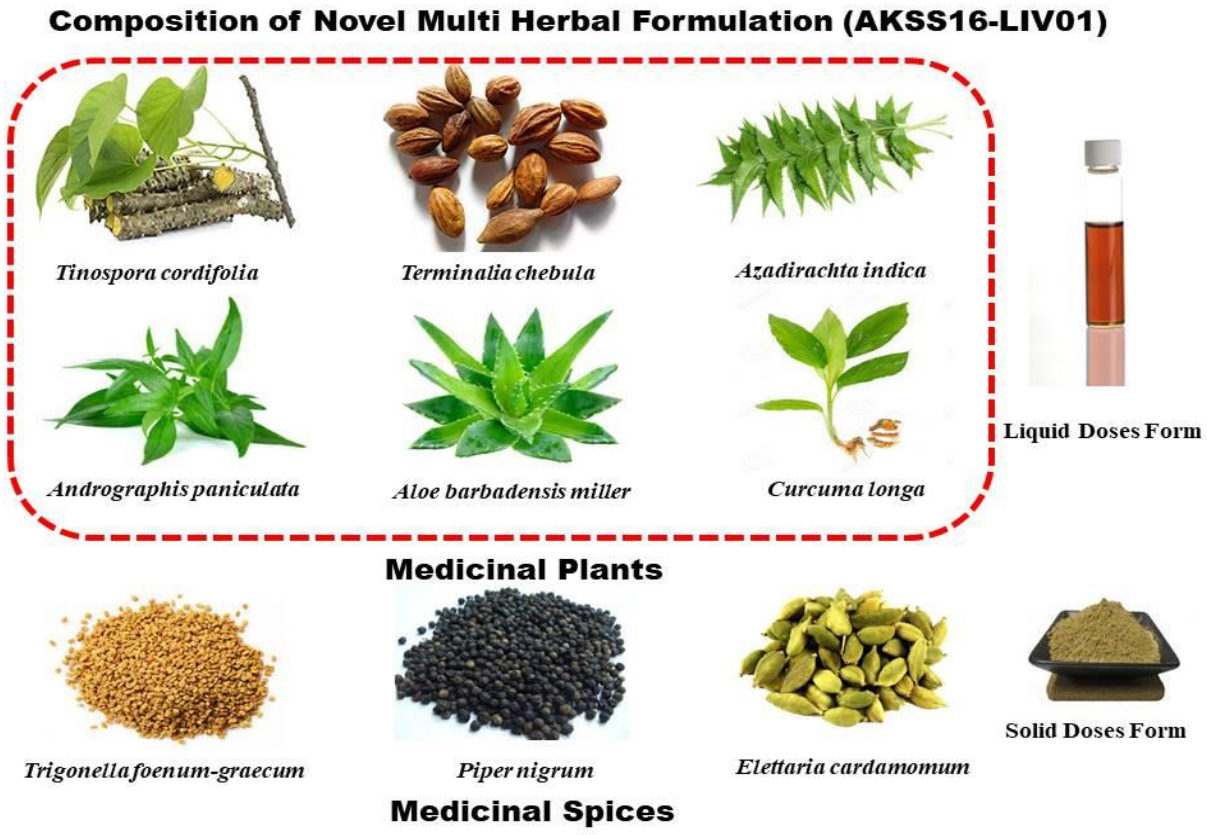


Figure 2A.1: Composition of a novel multi herbal formulation (ASKK16-LIV01) has containing six medicinal herbs and three medicinal spices.

2A.2.1a Ingredients present in the multi herbal formulation (ASKK16-LIV01)

Our developed formulation contains nine plants out of which six are Indian medicinal herbs and three medicinal spices. This is the only developed formulation which blends medicinal herbs and spices with minimum required quantity as compared with marketed products (figure 1). The formulation contains medicinal plants such as *Tinospora cordifolia, Terminalia chebula, Azadirachta indica, Andrographis paniculata, Aloe barbadensis miller, Curcuma longa* and medicinal spices as *Trigonella foenum-graecum, Piper nigrum and Elettaria cardamomum.* This plants and spices had lots of active ingredients which produces medicinal effects and beneficial for curing various chronic diseases. The major two active chemical constituents present in each plant are presented in Figure 2A.2 and 2A.3.

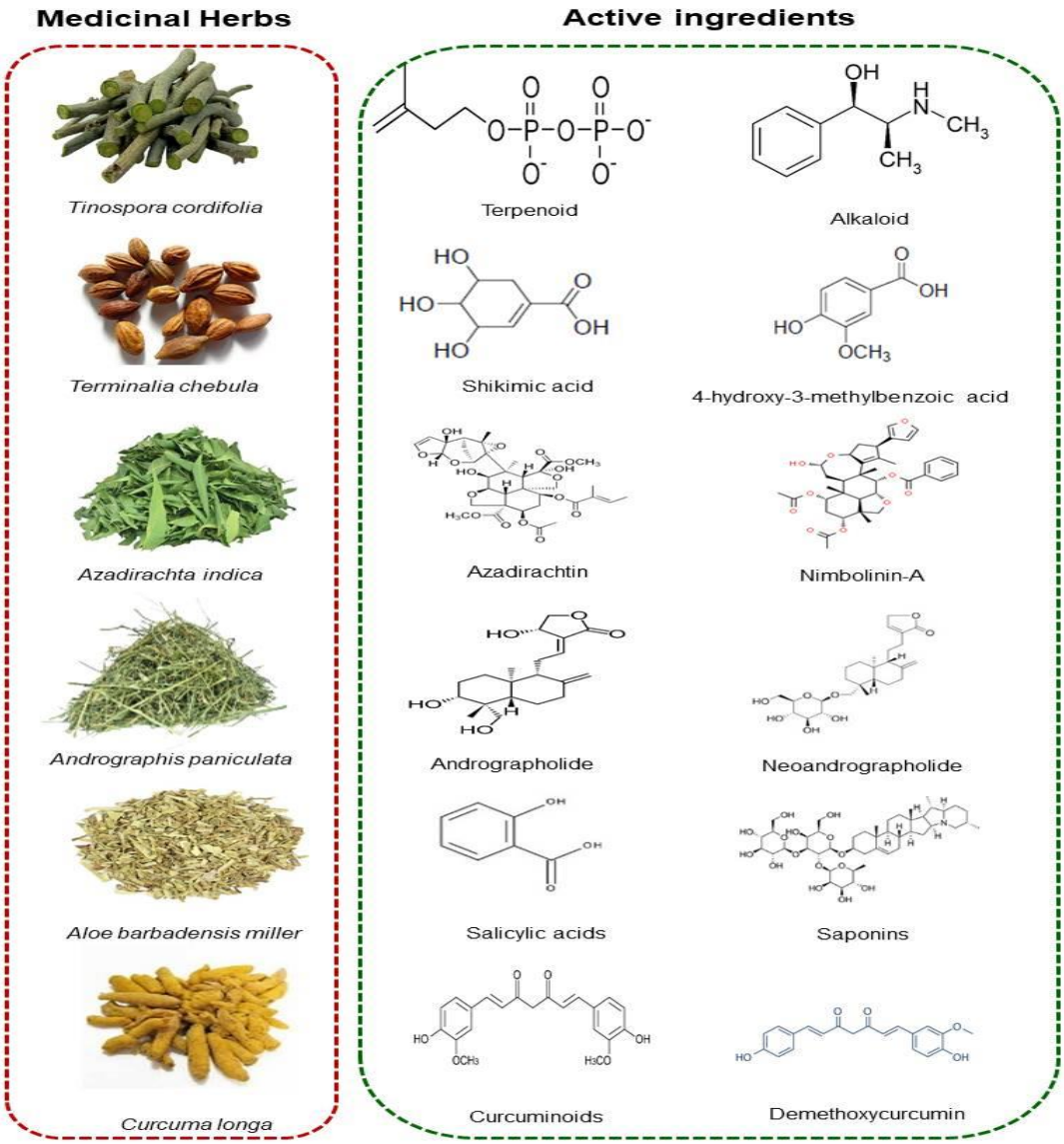


Figure 2A.2: Major two active chemical constituents present in the medicinal plants those are used to prepare the multi-herbal formulation AKSS16-LIV01.

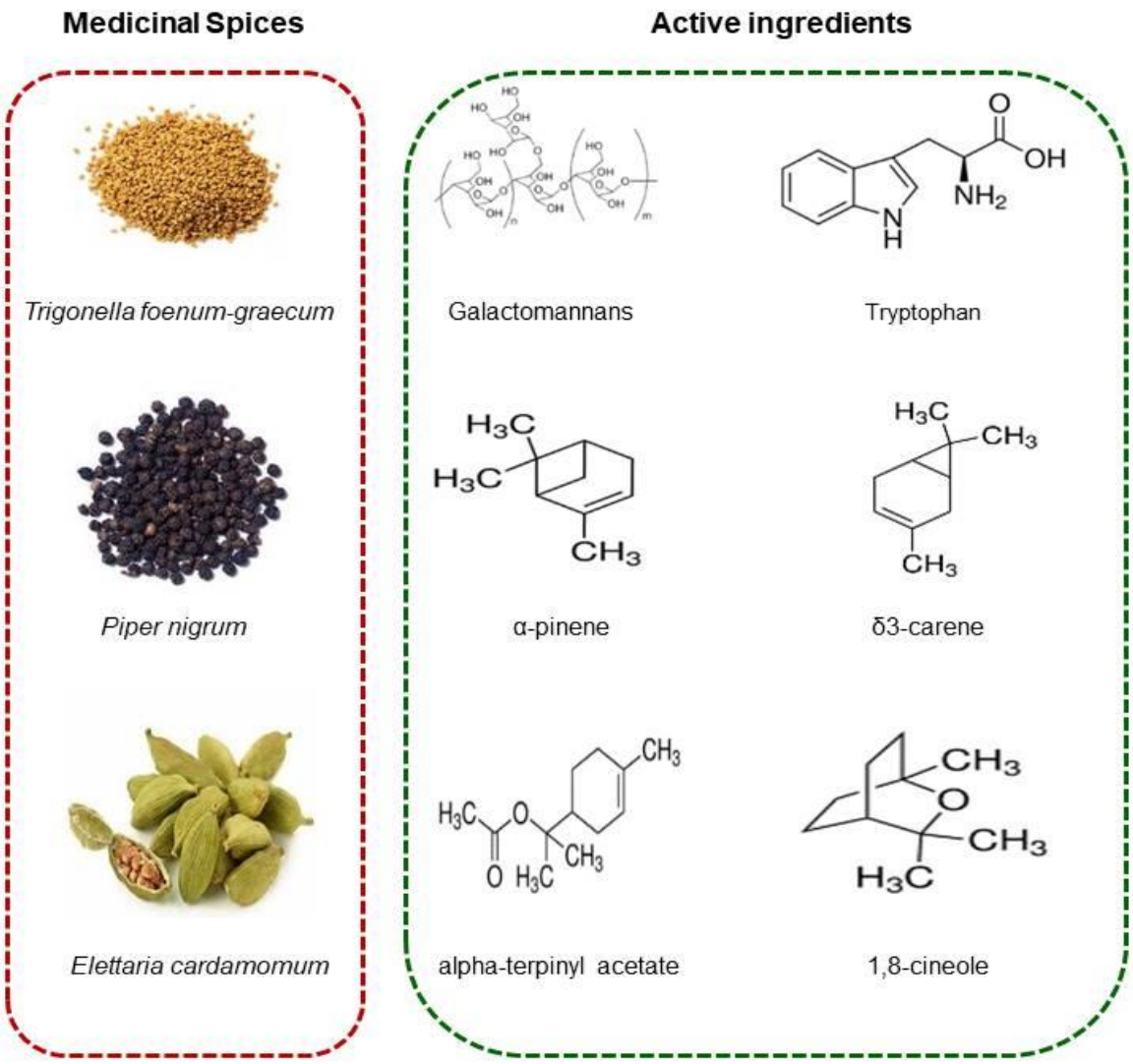


Figure 2A.3: Major two active chemical constituents present in the medicinal spices those are used to prepare the multi-herbal formulation AKSS16-LIV01.

2A.2.2 Preparation of Extract

Fresh parts of the medicinal plants were first air-dried after cleaning with double distilled water and kept in an oven at 80ºC for 10 min and 60ºC for 30 min and grounded by a blade mill to a fine powder. After that the polar fraction was extracted by modified method of Adhikari *et al.* (2018) [9].

2A.2.2a Quality control analysis

Wt. per ml, pH, homogeneity, total ash, LOD and bacterial limit tests were carried out according to the standard pharmaceutical protocol (IP-2014).

2A.2.2b Phytohemical screening

Various essential plant secondary metabolites such as sterols and triterpenes, Mg^{2+} turning test of flavonoids, alkaloids, saponins, glycosides, tannins, phenolic content, total flavonoids content in the developed multi-herbal formulation (AKSS16-LIV01) were detected through quantitative analysis with slight modification as described by Evans and Gueverra [10].

2A.2.3 In vitro study

2A.2.3a DPPH activity

Free radical scavenging activity of AKSS16-LIV01 was determined by using DPPH method.

Percentage Inhibition= Abs Control - Abs Test Abs Control ×100%

Effective 50% the concentration value that scavenged 50% of the DPPH radicals and antiradical power (ARP or AE) is the reciprocal of it (AE=1/EC50). Quercetin and ascorbic acid were used as reference standard [11].

2A.2.3b Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was carried out according to the standard established method of Erica et. al. [12].. Intensity of the developed yellow color formed was measured at 412 nm.

2A.2.3c Superoxide scavenging activity

Superoxide scavenging activity was determined according to standard protocol with slight modification [13]. This activity measured the removal rate of xanthine/xanthine oxidase-generation from the substances.

2A.2.3d Nitric oxide scavenging activity

Established standard method was used for determination of Nitric oxide (NO) scavenging activity [14]. In brief at first we prepared 2.0 ml of 10 mM sodium nitroprusside in 0.1 M phosphate buffer, pH 7.4 and were taken in a conical flask. Then 0.15 ml plant extract of different concentration was added carefully. At room temperature the solution were incubate for two hours. The solution was placed for cooling and then gently add 5 ml Griess reagent. The absorbance of chromophore was measured at 546 nm.

2A.2.3e Reducing power assay

Reducing power activity of AKSS16-LIV01 was carried out by the method of Abdullahi with slight modification [15]. Reaction was initiated by adding 2.5 ml of the extract, 2.5 ml of phosphate buffer and 1% potassium ferricyanide followed by gentle shaking. It was then placed in a water bath for 20 min at 50°C for initiation to complete. The solution was cooled and 2.5 ml of 10% trichloroacetic acid (TCA) was added subsequently. It was then centrifuged at 3,000 rpm for 10 min..5 ml of distilled water was mixed with 5.0 ml fraction from the supernatant and 1ml of 1% ferric chloride was added to

it and resulting solution was placed for incubation at room temperature for 10 min. The absorbance was noted at 700nm.

2A.2.4 Antimicrobial activity of AKSS16-LIV01

Antimicrobial activities of developed formulation AKSS16-LIV01 were determined according to the standard method with slight modification. Five bacterial pathogens namely *Staphylococcus pyogenes*, *Escherichia coli*, *Streptococcus aureus*, *Staphylococcus typhi*, and *Klebsiella pneumonia* were chosen for this study [16]. Tetracycline was used as a positive control. Nutrient broth was used for culturing the microbes and then placed for incubation at 37°C for 24 hours and seeded in Mueller-Hinton sterile agar plates. The plates were left undisturbed for about 10 minutes to enhance the culture. A set of four dilutions (25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml) of the herbal extract AKSS16-LIV01 was used for measured the activity. The whole procedure was repeated for three times for obtaining the precise result.

2A.2.5 Antifungal activity of AKSS16-LIV01

To determine the in-vitro antifungal activity of AKSS16-LIV01 we used three fungal pathogens namely *A. niger*, *Aspergillus oryzae*, and *Candida albicans* [17]. The plates were prepared with Potato dextrose agar (PDA) media and inoculated carefully with the fungal pathogens after the solidification of PDA. Five wells of size 5 mm were cut out on the agar plates. A set of four dilutions such as 25 µg/ml, 50 µg/ml , 75 µg/ml and 100 µg/ml of the newly prepared plant extract AKSS16-LIV01 and antifungal agent (positive control) ketoconazole (20 mg/ml) was introduced in well. At room temperature, the plates were placed for 3 to 4 days incubation. After 3 days, the zone of inhibition obtained was measured.

2A.3 RESULTS

The composition of the polyherbal preparation AKSS16-LIV01 is given in Table 2A.1 and Figure 2A.1. We have extracted the plant materials in seven well-known solvents and evaluated various properties including extraction efficiency, the quantity of various phytochemicals extracted and antioxidant activity to choose the best one. The highest yield was obtained with AQE while with EA the yield was the lowest.

2A.3.1a Quality control analysis

The developed multi herbal formulation (AKSS16-LIV01) showed a clear brown color liquid extract with a characteristic odour (Table 2A.2). Results depict that its Wt. per ml is 1.189 g and pH 6.88. The developed extract is uniform in nature. Total ash content is <5% w/w and LOD is 46 within the IP limit. 243 cfu/ml found in the bacterial Limit Test showed less bioburden and less pathological load. This formulation complies with the entire relevant quality control test as per Indian Pharmacopoeia limit.

Table 2A.2: Routine Quality Control analysis of multi herbal formulation (AKSS16-LIV01)

Sl. No.	Test	Results
1.	Description	A brown colour liquid
2.	Wt. per ml	1.189 g
3.	pH	6.88
4.	Order	Characteristic
5.	Homogeneity	Uniform
6.	Total ash	<5% w/w
7.	LOD	46
8.	Bacterial Limit Test	243 cfu/ml

2A.3.1b Phytochemical constituents

From the qualitative analysis (Table 2A.3) of the plant secondary metabolites of AKSS16-LIV01 multi extract, it is observed that sterols are present in trace amounts, alkaloids and glycosides are in moderate amount, but triterpenes, flavonoids, saponins, and tannins are abundantly available. In the dichloromethane (DM) extract, sterols were abundant flavonoids, alkaloids, and glycosides were moderately present and saponins were detected in trace amount. In the ethyl alcohol (EA) extract triterpenes were not detected. In the aqueous extract (AQ) all the above phytochemical constituents were detected abundantly. We can conclude that more polar secondary metabolites were extracted with the solvents used compared to non-polar metabolites.

Table 2A.3: Qualitative analysis of the phytochemical constituents of multi herbal formulation (AKSS16-LIV01)

Phytochemicals	DM	EA	AQ	ET	ME	AQM	AQE
Alkaloids	(++)	(+)	(++)	(+)	(+)	(+)	(+)
Flavonoids	(++)	(++)	(+++)	(++)	(++)	(+)	(+)
Glycosides	(+)	(-)	(++)	(++)	(++)	(-)	(-)
Tannins	(+)	(+)	(+++)	(+)	(+)	(+)	(+)
Triterpenes	(++)	(-)	(+++)	(-)	(+)	(-)	(-)
Sterols	(+)	(+)	(++)	(++)	(+)	(+)	(+)
Saponins	(+)	(++)	(++)	(+)	(-)	(+)	(+)

(+) = traces, (++) = moderate, (+++) = sufficient, (-) = absence of various constituents
DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

2A.3.1c Total polyphenol and flavonoids content

Flavonoids and total phenolic compounds of the various solvent extract were measured *spectrophotometrically*. The concentrations of total phenolic were higher compared to that of total flavonoids. Table 2A.4 represents the flavonoids and total phenolic content of the various solvent

extracts of AKSS16-LIV01. The flavonoid and total phenolic content of the five different solvent extract of AKSS16-LIV01 were significantly ($P<0.05$) varied from each other's. The total phenolic content in EA (2.29±0.41) gGAE/100g of raw material and in AQ was highest (8.59±0.45) gGAE/100g of raw material. The total flavonoids content was lowest in EA (0.45±0.03) gQE/100g of raw material but highest in AQ (1.48±0.05) gQE/100g of raw material.

Table 2A.4: Flavonoids and total polyphenol content of multi herbal formulation (AKSS16-LIV01)

Fraction	Total Polyphenols (gGAE/100g raw material)	Total flavonoids (gQE/100g raw material)
DM	2.42 ± 0.73^{a}	0.84 ± 0.04^{b}
EA	2.32 ± 0.37^{a}	0.48 ± 0.02^{a}
AQ	8.64 ± 0.47^{d}	1.21 ± 0.03^{d}
ET	4.51 ± 0.51^{b}	1.52 ± 0.06^{e}
ME	5.70 ± 0.53^{c}	1.10 ± 0.04^{c}
AQM	4.11 ± 0.44^{c}	0.91 ± 0.05^{b}
AQE	5.89 ± 0.51^{c}	0.97 ± 0.04^{c}

DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

2A.3.1d In vitro study

The purpose of the study was to confirm the high antioxidant content of the multi herbal extracts. For this, we evaluated DPPH, hydroxyl, superoxide and nitric oxide scavenging activity of the multi herbal formulation. The results are depicted in Figure 2A.4 to 8. In organic fractions of the extract, it was observed that increasing polarity of the solvent increased the DPPH radical scavenging capacities and higher DPPH scavenging activities was seen in all aqueous fractions which had a positive correlation with total phenolic component (TPC). In case of AQE fraction, the observed EC50 and ARP (or AE) values (EC50=0.065 mg/mg DPPH; AE=15.4) found to be comparable to quercetin (EC50=0.0652 mg/mg DPPH; AE=16.4) and even better than Trolox (EC50=0.0958 mg/mg DPPH; AE=10.6), the two well-known standards frequently used to compare antioxidant efficacy. In aqueous fractions higher superoxide scavenging activity was observed in comparison to organic fractions. The neutralization of O_2- radicals via hydrogen donation and inhibition of xanthine oxidase by various phenols present are responsible for the O_2- scavenging properties of the extracts. Significant higher activity was found in AQE fractions ($p<0.05$ compared to other solvents) in terms of hydroxyl free radical and nitric oxide scavenging.

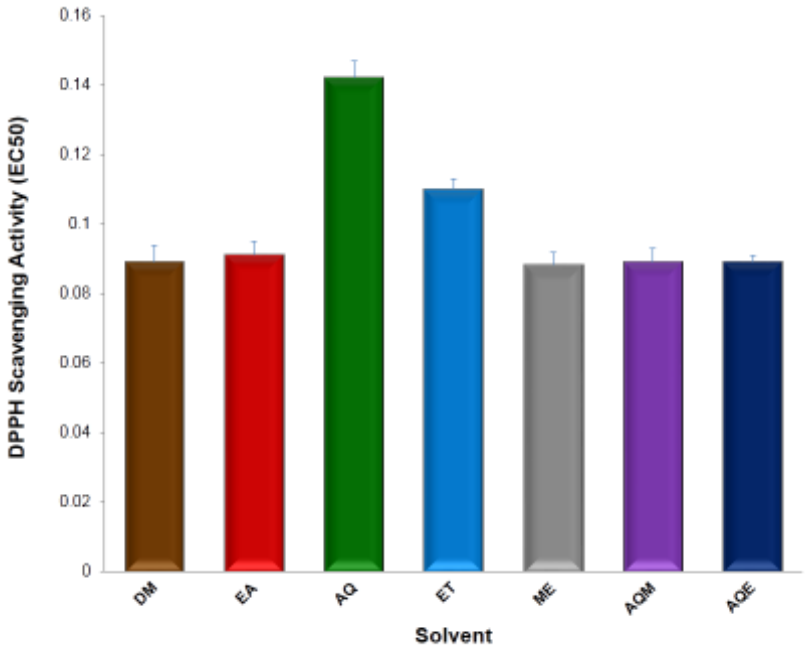


Figure 2A.4: DPPH Assay DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

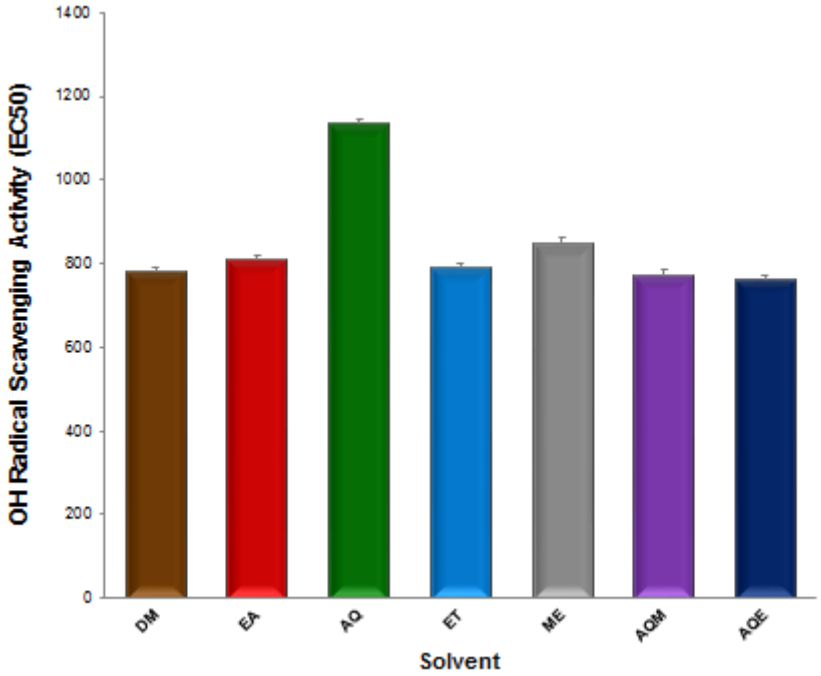


Figure 2A.5: Observation of OH radical activity. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

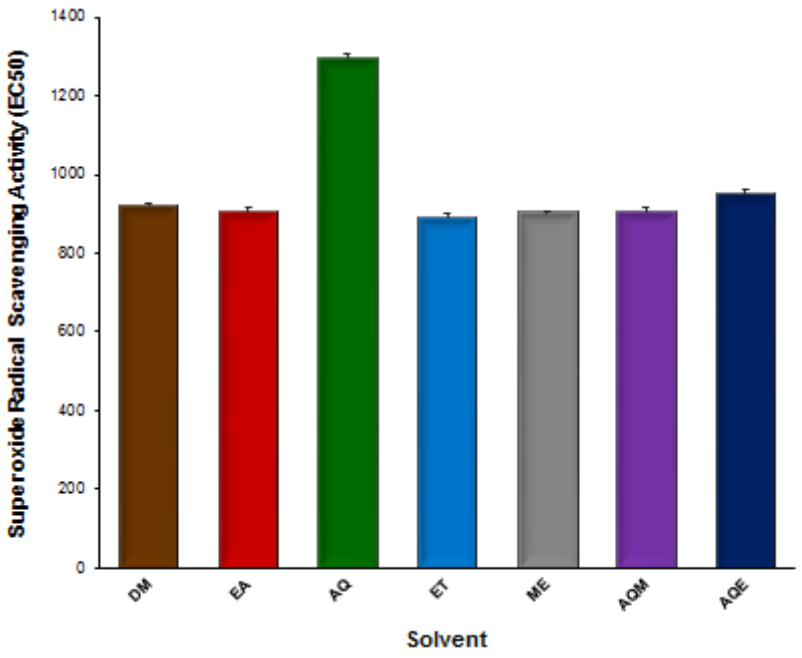


Figure 2A.6: Superoxide radical scavenging activityDM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

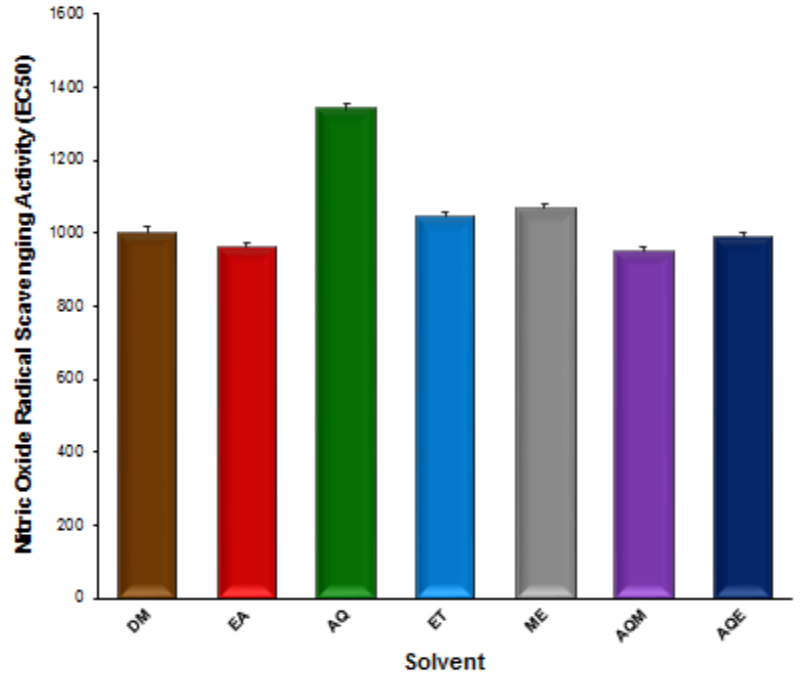


Figure 2A.7: Nitric oxide radical assay. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

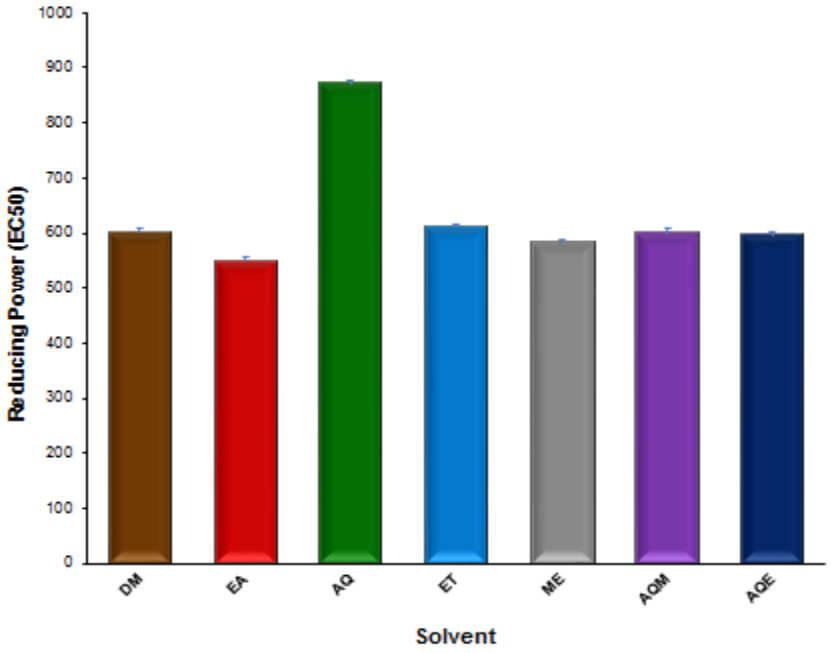


Figure 2A.8: In-vitro Reducing power activity of AKSS16-LIV01. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

2A.3.1e Antibacterial and Antifungal activity

The data from in vitro antibacterial study revealed that the inhibitory action of herbal extract AKSS16-LIV01 was dependent on the dose, increasing with an increase in concentration. The inhibitory action on different pathogens was also variable. Significant activity was seen in the case of *Escherichia coli* which were followed by *Streptococcus aureus* and *Klebsiella pneumonia*, *Staphylococcus pyogenes* and *Staphylococcus typhi* showed less inhibition as seen from Figure 2A.9. The Figure 2A.9 reveals that the different concentrations of herbal extract AKSS16-LIV01 showed efficient antifungal activity for three fungal pathogens taken. The antifungal activity was more for *C. albicans* and was least for *A.niger*. We can conclude that the herbal extract possesses the antimicrobial property.

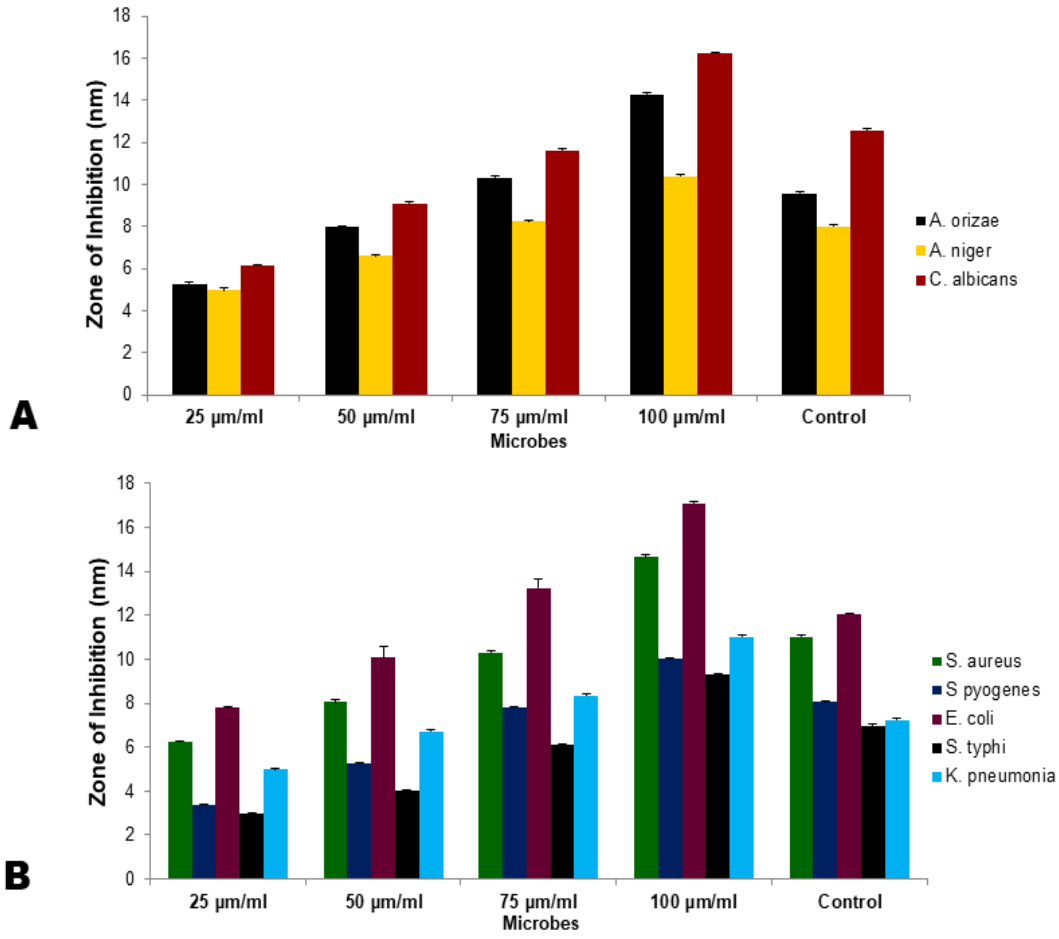


Figure 2A.9: A. Antifungal activity of AKSS16-LIV01. Data represent mean ± standard deviation. B. Antibacterial activity of AKSS16-LIV01. Data represent mean ± standard deviation.

2A.4 DISCUSSION

Scientific reports [18, 19] confirmed that secondary metabolites of the plants were associated with various bioactivities and showed inhibitory action against microorganisms and pathogens. Upon these different metabolites, alkaloids have extensive antimicrobial and antiviral activities [20]. On the other hand research established that other metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols have potent anti-pathogenic activity [21, 22].

In-vitro study showed that our newly developed multi herbal combination (AKSS16-LIV01) have different plant secondary metabolites upon seven solvents, namely, DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous

ethanol (80%). In this experiment aqueous (AQ) extracts of the multi herbal extract have optimum secondary metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols. Other solvent extracts like DM, EA, ET and ME showed moderate secondary metabolites. Sterols and glycosides were completely absent in AQM (80%), and AQE (80%) solvent extract. The stated phyto-constituents present in the aqueous (AQ) extracts have significant bioactivity against microorganisms and pathogens.

Scientific study reports that aqueous extract of the medicinal plants have enormous flavonoids and phenolic compounds are responsible for antioxidant activity [23]. Hydroxyl constituents and their aromatic structure are mainly responsible for antioxidant activity as free radicals scavengers [24]. It is established that biological activities mainly depends upon the flavonoid content of the plants which improve the extracts quality [25]. It is evident from Table 4 the phenolic and flavonoids compounds are chief components of AKSS16-LIV01and are responsible for antioxidant properties. Data of the study represents that flavonoids and phenolic contents are remarkably higher in the aqueous extract compared to others solvent extracts [26]. The routine Quality Control analysis of multi herbal formulation (AKSS16-LIV01) indicates that the developed drug complies with all the relevant parameters as per IP 2014 specification. Medicines from plant sources have gained global importance because of medicinal and economic importance [27]. The widespread sale of adulterated products and misleading health claims of herbal products require stringent regulations [28]. Generally, most of the plants have more than one key ingredient which carries antimicrobial property. Herbs containing in AKSS16-LIV01also have some antimicrobial activity which shows its medicinal values against diseases. AKSS16-LIV01at concentration of 75 µg/ml and 100 µg/ml showed higher antimicrobial activity than the standard drug Tetracycline in the inhibiting the growth of *Klebsiella pneumonia, Salmonella typhimurium, Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pyogenes*. Thus this superior antimicrobial activity was because the various active ingredients released form medicinal plants penetrated and disrupted the cell membrane of bacteria [29].

CONCLUSION

We can conclude from this preliminary study that the developed novel multi-herbal formulation AKSS16-LIV01showed promising results in in-vitro studies. The developed formulation has antioxidant and antimicrobial properties. Further studies are required to explore its efficacy in humans and to find out the key marker ingredient responsible for biological effects. The developed novel formulation may be a low cost, safe and symptomatic medication in future.

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2B Preliminary Assessment of Acute and 28-Day Repeated Dose Oral Toxicity of a Newly Developed Multi Herbal Formulation (MHF) on Experimental Animal

2B.1 INTRODUCTION

Medicinal plants have burgeoned in recent times due to increased efficiency of drugs derived from plants, growing interest in natural products and raising concerns about the side effects of conventional medicine [30]. Herbal drug combinations have shown that they possess better efficacy and reduced side-effects in comparison with single herbal drugs. The World Health Organization (WHO) estimates that 82 % of the world's population relies on these “alternative” plant-based medicines as their primary medical intervention especially in the developing and in the developed countries where modern medicines are predominantly used [31]. Over the years, the use of herbs in the treatment of illnesses has been very successful and its historic usage has been useful in drug discovery development. Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases [32, 33].

Toxicology may be defined as the study of harmful /poisonous effects of drugs and other chemicals with emphasis on detection, prevention and treatment of poisonings. After gaining relevant information on the harmful effects of a compound, the levels for its safe usage or the degree of its safety is established, this is known as its (compound) Biosafety level [34]. Acute toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effect characteristics of a test substance, and its primary purpose is to provide information on potential health hazards that may result from a short term exposure.

Traditional and alternative medicine is extensively practiced in the prevention, diagnosis, and treatment of various illnesses. It has attracted increasing public attention over the past 20 years as this type of medicine is easily accessible in some regions [35]. Medicinal plants contribute great importance in daily life by providing a wide range of nutrients, vitamins and other compounds which widen the therapeutic arsenal. In general, natural products play a dominant role in the development of novel drug leads for the treatment and prevention of diseases [36]. Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. Determination of efficacy and safety of herbal remedies is necessary because many people use these agents as self-medication. Since, there is limited data available about the safety of the commonly used herbal remedies, therefore, efforts to elucidate health benefits and risks of herbal medicines should be intensified1. It is the need of the hour to evaluate acute and chronic toxicities of herbal drugs [37].

Herbal formulations available with a wide range of indications like protective to liver, appetite and growth promoters, gastrointestinal and hepatic regulator, as treatment for hepatic dysfunction, for hepatic regeneration as well as liver stimulant and tonic. Despite the widespread use, there is a lack of scientific evidence on their efficacy and safety. In fact, there is a lack of evidence on quality, safety and efficacy of many herbal preparations. Although many herbal preparations are non-toxic, many plants currently used for medicines have been shown to be highly toxic when given either acutely or

sub-chronically [38, 39]. The increasing number of plant based medication users around the globe and lack of experimental reports on their safety make it basic to direct toxicological investigation on natural herbal products [37, 40, 41].

2B.2 MATERIALS AND METHODS

2B.2.1Experimental animals

Swiss albino wistar mice (30– 40 g) were obtained from the animal house. The room was well ventilated and maintained on light for 12 hours and 12 hour darkness. Temperatures were maintained at 27– 30 °C. The rats were provided with the standard rat pellets and clean water ad libitum. The experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee's (IAEC) rules and regulation of this institute and the experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2B.2.2 Composition of herbal formulation

The composition of each 5ml of Multi Herbal Formulation (MHF) compose of *Tinospora cordifolia* (Guduchi) 20 mg; *Terminalia chebula* (Haritaki) 20mg; *Azadirachta indica* (Neem) 50 mg; *Andrographis paniculata* (Kalmegh) 50 mg; *Aloe barbadensis miller* (Aloe vera) 50mg; *Curcuma longa*(Turmeric/Haldi) 20mg; *Trigonellafoenum graecum* (Methi)10mg; *Piper nigrum* (Black pepper)10mg; *Elettariaca rdamomum* (cardamom)10mg.

2B.2.3 Assessment of Acute toxicity test

Acute toxicity study was performed in healthy swiss albino mice (30-40gm) as per guidelines (AOT 425) suggested by the Organization for Economical Co-operation and Development (OECD). The animals were randomly assigned into two groups of 5 mice each and kept overnight fasting prior to extract administration. Group 1 served as the control and the mice were orally administered with 2ml distilled water. Single concentrations of the polyherbal extract 2000 mg/kg body weight was constituted in 5ml distilled water through a mice gavage. Food was withheld for further 3 hours.

The mice were observed after every 30 minutes post extract administration for the first 2 hours and latter once a day up to the 14^{th} for changes in skin and fur, eyes and mucus membranes, behavior pattern, tremors , salivation, diarrhea, sleep, coma, mortality, moribund, ill health or any visible reaction to treatment. Weight recording was done before extract administration, a t 48 hours, day 7 and day 14 using a sensitive balance.

2B.2.4 Clinical Observation

The treated animals were observed for mortality (twice daily) and the clinical signs were recorded to note the onset, duration and reversal (if any) of toxic effect at 2, 4, 6 and 8 hours after the administration of last substances and once daily thereafter for 14 days. The routine cage side observation s included changes in skin and fur, eye and mucus membrane, somatomotor activity,

general behavior pattern were noted. Miscellaneous sings like arching of the back, alopecia, wound, nasal discharge, lacrimation and loose stool were also recorded during the observation.

2B.2.5 Body weight

Body weight data of individual animals were recorded following the period of fasting on the day of dosing, weekly thereafter and at termination on day 15. Weekly changes in body weight gain were calculated and recorded.

2B.2.6 Repeated dose 28-day oral toxicity study

Sub-acute (Repeated dose 28-day oral toxicity study) was carried out as per OECD guidelines Guideline-407 2.Healthy Swiss albino mice were used for the study. The Animals were divided into four groups of 6 animals each. Group-1 animals served as a control animals. Group II Animals received low dose of test drug (Herbal formulation) 100 mg/kg orally. Group III animals received middle dose of test drug 200 mg/kg orally. Group IV animals received high dose of test drug 400 mg/kg orally. The animals were administrated with the study drug once daily for 28 days. All the experimental animals were observed for clinical signs of mortality and morbidity once a day, preferably at the same time each day, till the completion of treatment.

2B.2.7 Haematological study

On the last day of dose administration all the animals were kept for overnight fasting (water ad libitum). The overnight fasted animals were anaesthetized under general anaesthesia using isoflourane, blood samples were collected using heparinised microhematocrit tubes by retro-orbital puncture into a potassium EDTA containing blood collection tubes (for haematological) and 11% w/v tri-sodium citrate (TSC) containing tubes (for biochemical analysis). Blood smear was prepared from the EDTA containing blood sample, air dried and stained (Hemacolor rapid staining of blood smear, E. Merck, Mumbai, India) for differential leukocyte count (DLC). Haematological analysis were performed using automated haematology analyzer (Model PE 6000 Rapid Diagnostics Pvt Ltd, New Delhi, India), which includes analysis of haemoglobin (HGB), red blood cell count (RBC), white blood cell count (WBC], platelet count and hematocrit (HCT).

2B.2.8 Liver function test

The plasma thus collected was analysed for glucose, triglyceride, cholesterol, alkaline phosphatase (ALP) aspartate transaminase (AST) alanine transaminase (ALT) lactate dehydrogenase (LDH), total bilirubin creatinine, urea, protein and albumin levels by using biochemical kits (Accurex Biomedical Pvt. Ltd, Thane, India) in semi-automated biochemical analyzer (Model: Star 21 Plus, Rapid Diagnostics Pvt Ltd, New Delhi, India).

2B.2.9 Statistical analysis

Data were expressed as mean ± standard error mean. Data obtained from repeated dose studies were analysed by Student's ttest using GraphPad prism 5.0 to determine significant difference between the means of control and test groups. p value 0.05 was considered significant.

2B.3 RESULTS AND DISCUSSION

Traditional medicine has maintained greater popularity all over developing world and the use is rapidly on the increase. Despite this, the safety of herbal medicine use has recently been questioned due to reports of illness and fatalities; hepatotoxicity and nephrotoxicity. Although there are many traditional herbal medicines available, only a few have been verified by clinical trials, their efficacy and safety are still questioned by consumers [42, 43].

Table 2B.1: Effect of Multi Herbal Formulation (MHF) on the body weight of male mice at 2,000 mg/kg dose

Swiss Albino Mice	Weight in grams		
	Day 1	Day 7	Day 14
1.	25.5	27.0	30.5
2.	25.0	28.5	31.4
3.	25.0	27.6	30.5
4.	26.0	28.7	31.0
5.	25.4	27.5	30.5
6.	25.5	27.0	30.0

Observation included the change in skin, fur, eyes and mucus membrane. Appearance of toxicity related to central nervous system, Cardiovascular system and Autonomic nervous system such as tremors, convulsions, sedation, stereotypic behavior, respiratory distress, cardiovascular collapse, response to sensory stimuli, salivation, diarrhea, , pilo erection, Muscular co-ordination, Muscular grip, posture, gait, limb paralysis, lethargy, sleep, coma and mortality were observed with special attention (Table 4). The results revealed no treatment related death or signs of toxicity in the treated animals in all the doses throughout the study. Body weight gain of both male and female mice was also observed (Table 2B.1 & 2) when compared with before and after treatment of the observed groups (Figure 2B.1). Further, there were no gross pathological abnormalities (Table 2B.3) which prove the LD50 value was found to be greater than 2000mg/kg b.wt.

Table 2B.2: Effect of Multi Herbal Formulation (MHF) on the food consumption of female mice at 2,000 mg/kg dose

Swiss Albino Mice	Weight in grams		
	Day 1	Day 7	Day 14
1.	5.13	5.88	5.90
2.	5.22	5.43	6.01
3.	5.28	5.63	5.83
4.	5.28	5.77	6.24
5.	5.02	5.48	6.11
6.	4.96	5.61	6.08

Table 2B.3: Effect of Multi Herbal Formulation (MHF) on the Necropsy of mice at 2,000 mg/kg dose

Experimental Animals	Observed lesions during study
1.	Nil
2.	Nil
3.	Nil
4.	Nil
5.	Nil
6.	Nil

Table 2B.4: Clinical observations of mice at 2,000 mg/kg dose of Herbal Formulation (HF)

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

There were no treatment-related toxicity signs and mortality observed in mice treated at 100mg, 200mg and 400 mg/kg orally for a period of 28 days and in the satellite group of mice. Bodyweight gain was observed between control and treated groups during the study. Food and water consumption (Figure 2B.2 & 3) of treated groups were found to be insignificant in both the sexes when compared to the control groups. Since there is no significance decrease in mean body weight and there is considerable increase in mean body weight of control and treatment groups

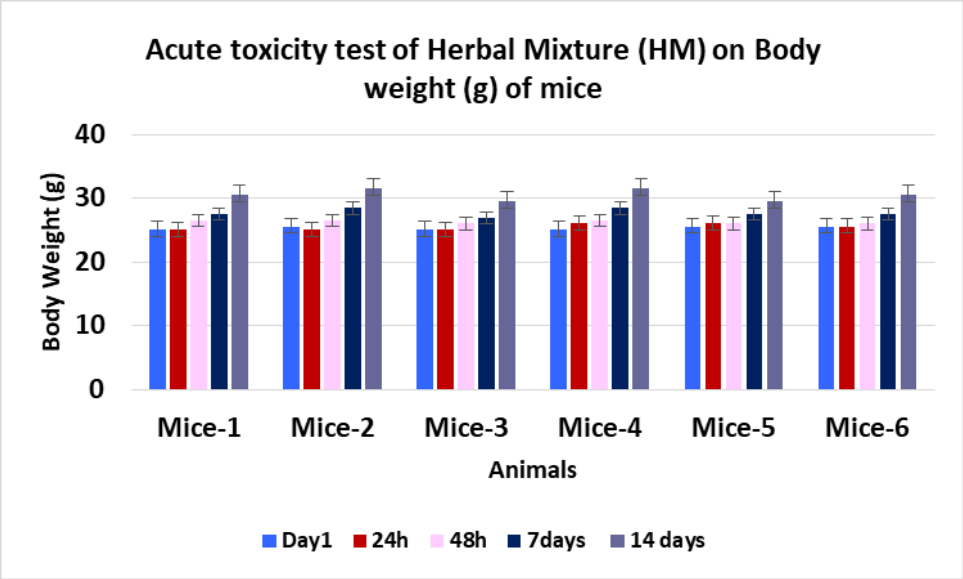


Figure 2B.1: Acute Toxicity Test of HM on Body Weight

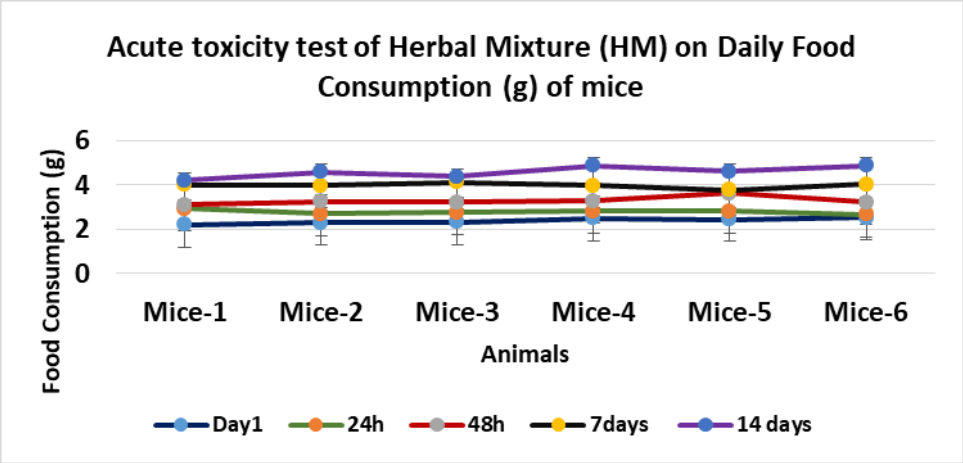


Figure 2B.2: Acute Toxicity Test of HM on Daily Food intake

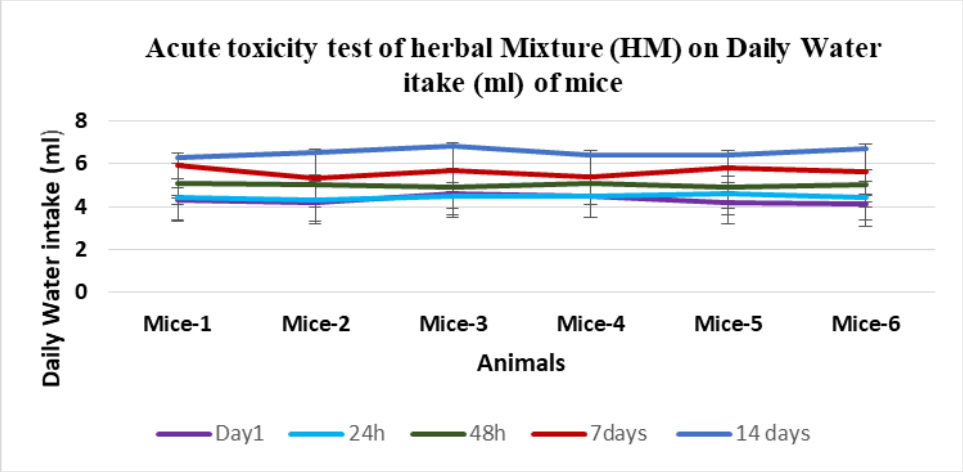


Figure 2B.3: Acute Toxicity Test of HM on Daily Water Consumption

Lipid profiles such as HDL, LDL, VLDL, TGL, and Total Cholesterol did not show any significant changes. The main product of protein metabolism is urea and an increased level of urea in the blood is an indicator of renal impairment (Table 2B.5). The present study showed no significant changes pertaining to renal parameters (BUN and creatinine level). Blood sugar level also not altered after application of herbal formulation (Table 2B.5). Serum marker enzymes are biochemical parameters associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health. Alanine amino transaminase (ALT) and Aspartate amino transaminase (AST) are largely used in the assessment of liver damage by drugs or any other hepatotoxin (Table 2B.6). So, to elucidate the toxicity produced during liver metabolism of drug, transaminase markers play a vital role. Aspartate Transaminase (AST), Alanine amino Transaminase (ALT) which are the indicators of hepatocellular injury also did not show any significant alterations in the multi herbal formulation treated groups and control groups.

Table-2B.5: Effect on biochemical parameters-Sub acute toxicity study

Parameters	Control	HM 100mg/kg	HM 200mg/kg	HM 400mg/kg
Blood sugar (mg/dl)	82.33±12.05	79.33±11.29	82.5±13.64	81.02±11.05
BUN (mg/dl)	19.67±6.87	19.32±5.14	18.96±4.29	18.57±3.58
Creatinine (mg/dl)	0.7856±0.56	0.6667±0.44	0.7667±0.32	0.7549±0.41
Total cholesterol (mg/dl)	122.7±14.02	118.2±14.78	115.6±12.54	116.3±12.35
Triglycerides (mg/dl)	74.5±9.65	77.5±8.55	78.5±5.21	74.5±8.47
HDL (mg/dl)	59.17±4.21	61.6±3.22	60.7±6.01	60.9±6.05
LDL l (mg/dl)	55±6.54	31.1±5.97	45.2±5.49	55.1±3.64
VLDL (mg/dl)	14.43±2.51	17.32±2.64	15.8±1.23	16.2±2.11

Values are mean ± S.D (n = 6 per group). Control and treatment groups were compared statistically using one way ANOVA followed by Dunnett's test.

Table 2B.6: Effect on Liver function parameters-Sub acute toxicity study

Parameters	Control	HM 100mg/kg	HM 200mg/kg	HM 400mg/kg
(AST (IU/L)	101.3±12.05	106.6±12.56	100.5±11.27	102.4±11.58
ALT (IU/L)	19.46±6.87	20.56±5.14	20.64±4.29	21.33±3.58
ALP (IU/L)	140.25±14.25	138.25±16.11	142.57±19.65	145.28±17.54

Hematological profile such as Packed Cell Volume (PCV), Red Blood Cells (RBC) count, White blood cell count (WBC), Platelet Count, Hemoglobin (Hb), Mean cell Haemoglobin Concentration (MCHC), Mean Red Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean platelet volume (MPV), Neutrophils, Eosinophil's, Basophils, Lymphocytes and Monocytes were found to be within the normal physiological limits for rodents and no significant change has been observed in treatment groups when compared with the control groups (Table 2B.7). Hence there are no serious toxicological implications such as destruction of Erythrocytes.

Table 2B.7: Haematological parameters as studies across the group CM (Control); LD (Low dose); MD (Middle dose) and HD (High dose)

Groups	Hb (g%)	RBC ($x10^6$ cm^2)	RT (%)	HCT (%)	MCV (μm^3)	MCH (pg)	MCHC (%)	Platelets	WBC ($x10^5$ $cm^{2)}$)	L	N
CM	11.6±2.3	10.8±2.3	2.8±1.5	34.1±6.2	37.2±1.5	21.4±2.6	40.2±6.5	6.5±4.2	9.1±2.2	72±5.1	25±2.5
LD	12.6±2.0	11.5±1.0	4.8±2.6	36.4±5.4	31.7±2.6	21.9±3.3	36.5±6.2	5.5±4.1	9.9±3.0	79±6.5	18±2.6
MD	10.8±1.1	9.1±1.1	3.1±3.9	30.7±3.2	33.7±3.7	21.5±5.4	38.2±5.9	3.9±5.0	9.5±0.9	74±4.9	23±3.4
HD	13.7±3.0	10.4±3.2	2.5±2.4	38.6±2.6	37.1±2.9	22.6±2.6	32.4±5.2	4.8±6.1	11.5±1.6	74±7.5	23±2.1

Data are expressed as mean ± standard deviation (N=6)

Hb: Haemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White Blood corpuscle

Serum and liver MDA level did not show any significant alterations in the high dose (HD) treated group in compared with control group (Table 2B.8). The histopathological studies revealed no significant weight changes and normal architectural changes in the vital organs such as heart, brain, lungs, spleen, kidneys, liver, stomach, testes, and ovary suggesting that the preparation is devoid of serious organ degenerative potential both dose levels (Figure 2B.4).

Table 2B.8: Serum MDA level as studied across the group C (Control); LD (Low dose); MD (Middle dose); HD (High dose).

Groups	Lipid peroxidation (µmoles MDA)	Lipid peroxidation (µmoles MDA/ g liver)
C	21.32 ±2.55	38.74 ±3.25
LD	24.15 ±5.64	39.22 ±2.62
MD	23.67 ±3.44	40.54 ±4.01
HD	32.47 ±2.99	52.16 ±3.62

Data are expressed as mean ± standard deviation (N=6). LD-Low Dose; MD-Middle Dose and HD-High Dose

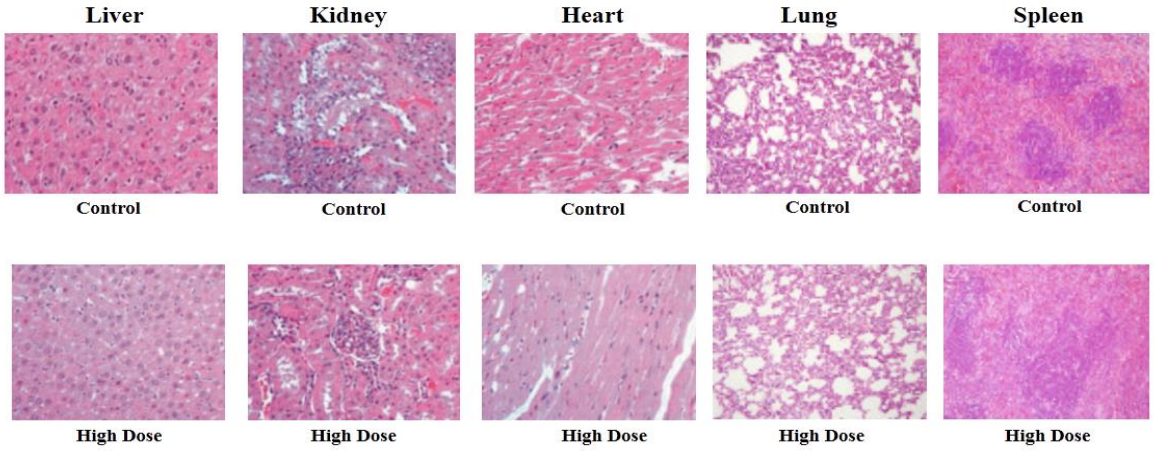
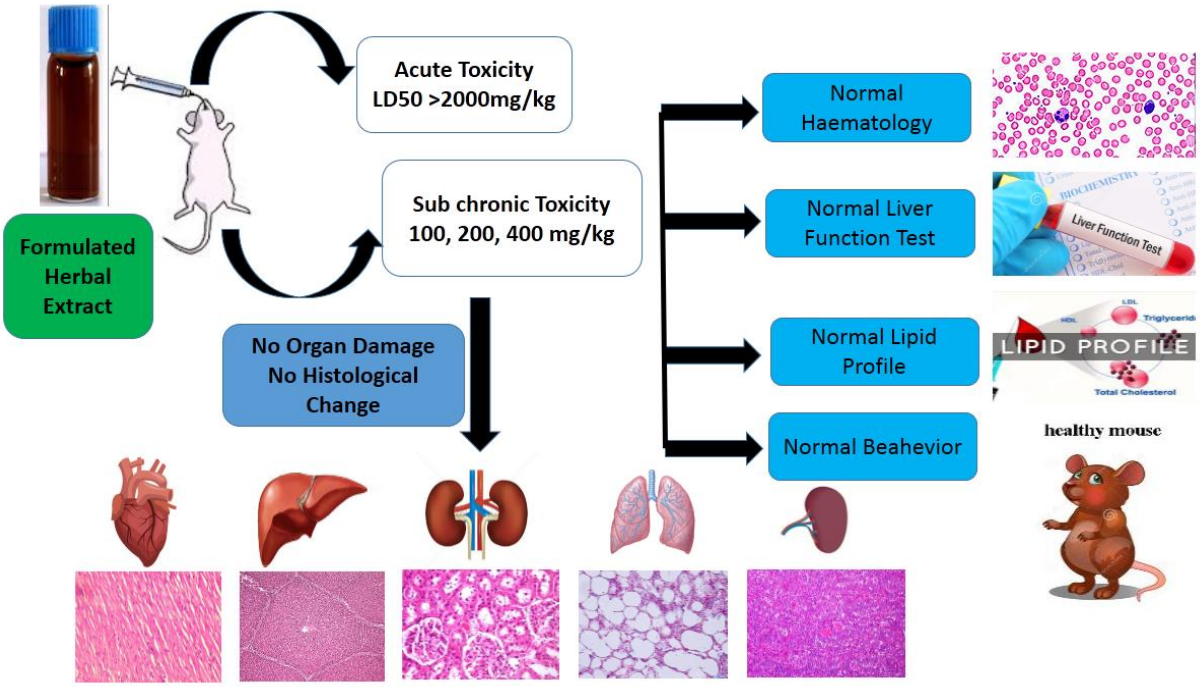


Figure 2B.4: Histological study

CONCLUSION

The present Acute and sub-acute toxicity results suggest that LD50 of developed formulation is >2000mg/kg. Repeated dose toxicity study also established that developed formulation does not produce any behavioral, biochemical and haematological effects in mice. It also stated that AKSS16-LIV01 did not change organs architecture which strongly established that the formulation is safe in mammalian system. Further studies on long term toxicity and clinical trials may be may be rational to substantiate the study results.

SUMMARY



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Chapter - III

Protective Effect of a Novel Multi Herbal Formulation (AKSS16-LIV01) against CCl_4 induced Liver dysfunction, redox inhibition and hepatic Fibrosis

3A Sanative effect of a multi herbal formulation–AKSS16-LIV01 upon CCl_4 induced hepatic dysfunction in mice

3A.1 INTRODUCTION

Hepatocytes, sinusoidal cells, Kupffer cells and HSCs are the main cells those are involved in normal liver functions. Several pathogens when attack the liver cells they alter the normal liver functions and damage the liver cells produce liver fibrosis [1, 2]. Inflammatory reactions take place when hepatocytes cause damage which lead to the activation of hepatic stellate cells (HSCs) [3].During the abnormal situation activated Kupffer cells release a number of soluble agents, including cytokines, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α), generate reactive oxygen species (ROS), and other factors causing inflammation and damage to hepatocytes leads to liver fibrosis [4, 5]. In an experimental setting, carbon tetrachloride (CCl_4) is able to induce hepatic fibrosis by stimulating the formation and generation of various free radicals, ROSs and lipid peroxidation products [6] and thus is widely used to generate hepatic fibrosis in model animals [7, 8].

Presently global attention has been paid to the protective effects of natural antioxidants against chemically induced toxicities [9]. Herbal extracts could significantly contribute to recovery processes of the intoxicated liver and kidney [10-14]. The World Health Organization defines traditional medicine as “diverse health practices, approaches, knowledge, and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual technique, and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness” [15, 16]. In third world countries including India, up to 90% of the populace still relies entirely on plants as a resource of medicines [17].

Herbal medicines have progressively become prevalent due to rising costs of treatments with synthetic western medicine, numerous side effects of allopathic drugs, drug resistance, unregulated purchase options for consumers on most herbal drugs and easy availability of these medicines [18]. Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Natural remedies from medicinal plant are considered to be effective and alternative treatment for hepatotoxicity [19].

In the present study we formulated a new phytomedicine composed of eight indigenous medicinal and dietary herbs which were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and happiness.. The results of the present study are expected to provide a clear picture about the role of our newly formulated AKSS16-LIV01 in CCl_4-induced hepatic damage, and may shed light on an achievable ethno-botany driven solution to the serious liver problems.

3A.2 MATERIALS AND METHODS

3A.2.1 Chemicals

Carbon tetrachloride, trichloroacetic acids, TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. ALT, AST, GGT, ALP and Lipid profile kits were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

3A.2.2 Animals

Healthy adult Swiss albino mice weighing 28 ± 5 g taken from our registered animal house were divided in to 6 experimental groups with 6 animals per group. The animals were maintained at 12 h light/dark cycle, at constant temperature (22±2°C) and humidity (55±5%). Mice were feed standard pellet diet (Purchase from Hind liver India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture, and 2.6% by weight of lipids and water *ad libitum*. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. All the experimental procedure were carried out according to the guidelines of CPCSEA, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC) of Jadavpur University having approval number 261/JU/s/IAEC/Pharma/2018 (Figure 1).

3A.2.3 Experimental Design

Male adult mice were divided into 6 groups as follow: Group I served as normal control received only the vehicle (1ml/kg olive oil twice a week for 6 weeks), Group II treated with multi herbal formulation (MHF) AKSS16-LIV01 (400 mg/kg bw/day all over the experiment), Group III received 1 ml/kg bw of CCl_4 diluted 20% in olive oil twice a week for 6 weeks, Group IV pre-treated with herbal formulation AKSS16-LIV01 (200 mg/kg bw/day) low dose (HF) for four weeks after two weeks CCl_4 induction and ensure occurrence of liver injury, Group V pre-treated with multi herbal formulation (MHF) AKSS16-LIV01 high dose (400 mg/kg bw) for four weeks after two weeks CCl_4 induction and ensure occurrence of liver injury. Group VI pre-treated with Silymarin standard hepatoprotective drug at a dose (100 mg/kg bw) for two weeks after CCl_4 induction and ensure occurrence of injury.

3A.2.4 Body weight gains and feed efficiency

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

3A.2.5 Blood Collection

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without

EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

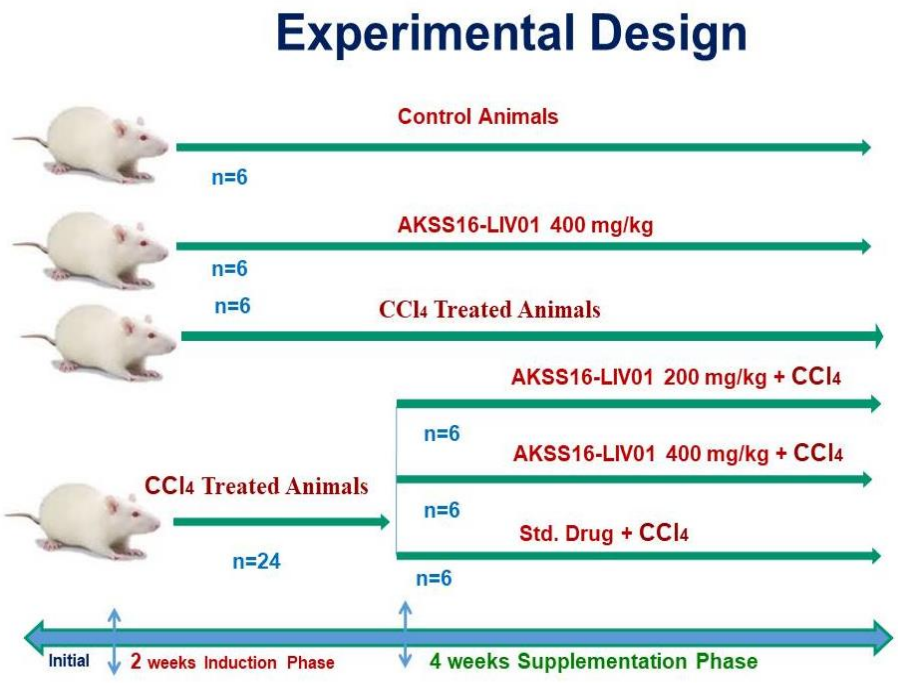

Figure 3A.1: Experimental design study of the

3A.2.6 Hematological Parameters

For hematological studies, the blood was collected in heparinized tubes. Blood-cell count was done using blood smears in Sysmax-K1000 Cell Counter. Parameters studied were hemoglobin, total red blood cell, reticulocyte, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, total white blood cell and differential count.

3A.2.7 Assessment of Liver Function Parameters

The biochemical parameters like serum enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), albumin, globulin, total and direct bilirubin and blood urea nitrogen (BUN) along were assayed using assay kits (Thermo Scientific, USA) following the protocol prescribed by manufacturers. Total protein concentration was determined in the serum by the method of Lowry et al. [20].

3A.2.8 Lipid profile

Serum levels of total cholesterol (TC), triglycerides (TG), phospholipids, free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL) were assayed using assay kits (ELITech Diagnostic, France) following the protocol prescribed by manufacturers with slight modification.

3A.2.9 Hematoxylin and Eosin staining

Five micron paraffin-embedded liver sections were deparaffinised and washed with water. Hydrated tissue sections were incubated with Mayer's hematoxylin for 5 min followed by vigorous washing in running tap water. Sections were counter stained with 1% eosin for 2 min. Eosin stained sections were washed with water and dehydrated with alcohol. Dehydrated sections were washed with xylene. Images were taken with a microscope (Olympus BX51 fluorescence microscope).

3A.2.10 Statistical Analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

3A.3 RESULTS

3A.3.1 Body weight, Liver weight, Liver index, food consumption and water intake

In this study we determined the body weight, liver weight, liver index, food consumption and water intake of mice treated with CCl_4 and pretreated with multi herbal formulation-AKSS16-LIV01. Body weight and liver index of CCl_4 treated animals were significantly reduced ($p<0.001$) when compared with normal control animals. Liver weight of CCl_4 treated animals were significantly elevated ($p<0.001$) when compared with normal control animals. Treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly increased ($p<0.05$, $p<0.001$) body weight and liver index whereas significantly decrease the liver weight ($p<0.001$) when compared with CCl_4 treated animals. Sylimarin showed less positive effect in comparison with multi herbal formulation. On the other hand food consumption and water intake capacity of CCl_4 treated animals were significantly reduced ($p<0.001$) when compared with normal control animals. Treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly increased ($p<0.05$, $p<0.001$) the food consumption and water intake capacity when compared with CCl_4 treated animals. Treatment with multi herbal formulation - AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity (Table 3A.1).

Table 3A.1: Summary of the body weight, liver weight and liver index of mice

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + Silymarin 100
Body Weight (g)	36.52±1.18	35.01±1.34	26.94±1.22$^{\#}$	34.62±1.26*	38.01±2.21**	33.26±2.17*
Liver Weight (g)	1.94±0.82	1.98±0.18	3.50±0.28$^{\#}$	1.99±0.52*	1.86±0.64**	2.03±0.64*
Liver Index	4.02±0.12	5.65±0.17	7.72±0.15$^{\#}$	5.51±0.24*	4.95±0.17**	5.22±0.18*
Food consumption (g)	3.98±0.05	3.95±0.04	2.91±0.05$^{\#}$	3.74±0.06*	3.99±0.02**	3.92±0.05*
Water intake (ml)	4.11±0.02	4.02±0.04	2.95±0.05$^{\#}$	3.99±0.02*	4.04±0.05**	3.91±0.03*

Values are mean of six individual observations in each group ± S.D. *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 *****($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by

3A.3.2 Haematological Study

All the different haematological parameters such as Hb: Haemoglobin (Hb), Read Blood corpuscle (RBC), Reticulocyte (RT), Haematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), and White Blood corpuscle (WBC) were studied in mice treated with CCl_4 and pretreated with AKSS16-LIV01. Hemoglobin level was significantly reduced ($p<0.05$) and WBC count was significantly elevated ($p<0.05$) in CCl_4 group as compared with normal control group. The Pretreatment with AKSS16-LIV01 (400 mg/kg) was recovered theses alteration when compared with CCl_4 group (Table 3A.2).

Table 3A.2: Summary of the haematological parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + Silymarin 100
Hb (g %)	12.1± 1.05	11.21±0.82	9.03± 0.89$^{\#}$	11.05±0.99	12.51±0.95*	10.96±0.74
RBC ($x10^6$ cm^2)	10.8±0.82	9.62±0.84	9.1±0.71	9.44±0.71	10.02±0.85	9.85±0.79
RT (%)	2.7±0.12	3.6±0.16	4.9±0.26	3.1±0.14	2.8±0.15	3.0±0.12
HCT (%)	34.6±0.48	35.1±0.77	39.4±0.55	35.8±0.51	34.9±0.56	34.4±0.51
MCV (μm^3)	37.8±0.32	35.5±0.36	31.0±0.68	36.5±0.44	35.9±0.79	36.2±0.43
MCH (pg)	21.2±0.15	21.1±0.12	22.2±0.14	21.1±0.12	21.4±0.11	21.2±0.14
MCHC (%)	41.2±1.06	36.2±0.91	32.4±0.95	37.1±0.92	39.6±0.87	38.6±0.99
Platelets	6.5±0.02	5.4±0.06	5.5±0.03	5.8±0.05	6.1±0.07	5.5±0.05
WBC ($x10^5$ $cm^{2)}$)	9.2±0.09	10.7±0.11	12.4±0.11$^{\#}$	10.8±0.12	9.2±0.11*	10.1±0.13
Lymphocyte	74±2.98	71±3.11	79±3.04	73±3.06	71±2.58	72±3.08
Neutrophil	26±1.12	21±0.55	15±0.49	20±0.56	25±0.69	24±0.51

Data are expressed as mean ± standard deviation (N=6). Hb: Haemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White Blood corpuscle. Values are mean of six individual observations in each group ± S.D. *Significantly different from control group $^{\#}$($p<0.05$) and significantly different from CCl_4 group *****($p<0.05$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

3A.3.3 Liver function test

Liver function parameters such as serum AST, ALT, GGT, ALP, BUN, total bilirubin, direct bilirubin, total protein, albumin and albumin-globulin ratio were measured in mice treated with CCl_4 and pretreated with AKSS16-LIV01 after CCl_4 induction were presented in Table 3A.3. AST, ALT, GGT, ALP, BUN, total bilirubin and direct bilirubin of CCl_4 treated animals were significantly elevated ($p<0.001$) when compared with normal control animals. Serum total protein, albumin and albumin globulin ratio of CCl_4 treated animals were significantly reduced ($p<0.001$) when compared with normal control animals. The Pretreatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly reduced ($p<0.05$, $p<0.001$) AST, ALT, GGT, ALP, BUN, total bilirubin and direct bilirubin whereas significantly increased ($p<0.05$, $p<0.001$) the serum total protein, albumin and albumin-globulin ratio when compared with CCl_4 treated animals. Sylimarin showed less positive effect in comparison with multi herbal formulation. Results of the biochemical study stated that treatment with multi herbal formulation - AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity.

Table 3A.3: Summary of the liver function test parameters studied across the groups

Parameters	**Normal**	**AKSS16-LIV01 (400)**	**CCl_4**	**CCl_4 + AKSS16-LIV01 (200)**	**CCl_4 + AKSS16-LIV01 (400)**	**CCl_4 + Silymarin 100**
AST(IU/l/min/mg protein)	137.25±2.62	135.25±3.02	255.49±1.98$^{\#}$	166.27±2.19*	140.27±2.02**	164.02±1.44*
ALT(IU/l/min/mg protein)	55.16±2.22	58.61±1.81	118.03±3.16$^{\#}$	82.58±1.02*	61.29±1.63**	72.58±1.85*
GGT(IU/l/min/mg protein)	0.26±0.02	0.26±0.09	2.11± 0.51$^{\#}$	1.06±0.08*	0.34±0.12**	0.41±0.02*
ALP(IU/l/min/mg protein)	232.05±3.11	237.84±2.91	461.27±4.96$^{\#}$	279.55±2.06*	250.44±2.71**	262.11±2.04*
BUN(mg/dl)	0.41±0.02	0.46±0.03	0.72±0.04$^{\#}$	0.67±0.02*	0.46±0.02**	0.55±0.03*
Total Bilirubin(mg/dl)	0.12 ± 0.2	0.19±0.11	0.62 ± 0.11$^{\#}$	0.34±0.08*	0.18±0.09**	0.25±0.08*
Direct Bilirubin (mg/dl)	0.06±0.001	0.07±0.002	0.33 ± 0.07$^{\#}$	0.09±0.003*	0.09±0.004**	0.24±0.005*
Total Protein	6.51±0.65	627±0.21	2.56±0.32$^{\#}$	4.58±0.41*	6.22±0.27**	5.39±0.21*
Alb (gr/dL)	3.48±0.186	3.16±0.13	1.97±0.036$^{\#}$	3.70±0.11*	4.12±0.12**	3.30±0.15*
Alb/globulin	1.18±0.141	0.98±0.135	0.54±0.013$^{\#}$	0.91±0.096*	1.23±0.95**	1.06±0.091*

Values are mean of six individual observations in each group ± S.D. *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

3A.3.4 Lipid Profile Test

Lipid profile parameters such as, serum total cholesterol (TC), total triglyceride (TG), phospholipids, free fatty acid (FFA), LDL and HDL were measured in mice treated with CCl_4 and pretreated with AKSS16-LIV01 were presented in Table 3A.4. Cholesterol triglyceride, phospholipids, free fatty acid

and LDL of CCl_4 treated animals were significantly elevated ($p<0.001$) when compared with normal control animals. Serum HDL level of CCl_4 treated animals were significantly reduced ($p<0.001$) when compared with normal control animals. The Pretreatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly reduced ($p<0.05$, $p<0.001$) serum cholesterol triglyceride, phospholipids, free fatty acid and LDL level and significantly increased ($p<0.05$, $p<0.001$) the serum HDL when compared with CCl_4 treated animals. Sylimarin showed less positive effect in comparison with multi herbal formulation. Results of the serum lipid profile study stated that treatment with multi herbal formulation - AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity.

Table 3A.4: Summary of the lipid profile parameters studied across the groups

Parameters	**Normal**	**AKSS16-LIV01 (400)**	**CCl_4**	**CCl_4 + AKSS16-LIV01 (200)**	**CCl_4 + AKSS16-LIV01 (400)**	**CCl_4 + Silymarin 100**
Cholesterol (mg/dL)	81.03 ± 5.02	88.05±3.16	135.69 ± 6.15$^{\#}$	91.47 ± 2.37*	79.36 ± 4.35**	85.11±2.98*
Triglyceride(mg/dL)	40.58 ± 2.05	51.23±3.01	72.58 ± 3.28$^{\#}$	47.98 ± 1.97*	37.25 ± 1.87**	41.75±3.25*
Phospgolipids (mg/dL)	76.59 ± 6.28	92.37±2.66	142.97 ± 4.69$^{\#}$	98.51 ± 3.28*	82.69 ± 4.87**	88.03±2.84*
Free fatty acids (mg/dL)	15.97 ± 0.58	20.14±1.96	31.87 ± 1.67$^{\#}$	21.22 ± 1.69*	14.09 ± 1.22**	16.27±2.01*
LDL-cholesterol (mg/dL)	39.65 ± 1.96	43.61±1.88	76.94 ± 1.77$^{\#}$	45.78±1.65*	36.85 ± 1.25**	44.52 ± 2.28*
HDL-cholesterol (mg/dL)	19.58 ± 0.69	15.28±0.55	10.28 ± 0.28$^{\#}$	16.24±0.41*	10.28 ± 0.28**	22.67 ± 0.99*

Values are mean of six individual observations in each group ± S.D. *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

3A.3.5 Histology

3A.3.5a H&E staining

Figure 3A.3 showed histological photographs of the liver tissue both control and different experimental groups. The normal control group animals showed the typical architecture of liver tissue with a central vein (CV) and chords of hepatocytes radiating whereas CCl_4 treatment produced extensive necrosis of hepatocytes which was more pronounced in the centrizonal (zone 3) area. The fatty changes were of macrovesicular type which was evident in centrizonal and portal areas with inflammatory reactions (Figure 3A.2). Partial hepatic protection with reduction in the extent of hepatic necrotic areas, fatty infiltration, and mild portal inflammation was visualized in the liver section of AKSS16-LIV01 (200 mg/kg) treated animals. On the other hand administered with AKSS16-LIV01 (400 mg/kg) completely protected the liver as evidenced by restoration of a normal histoarchitecture of the liver.

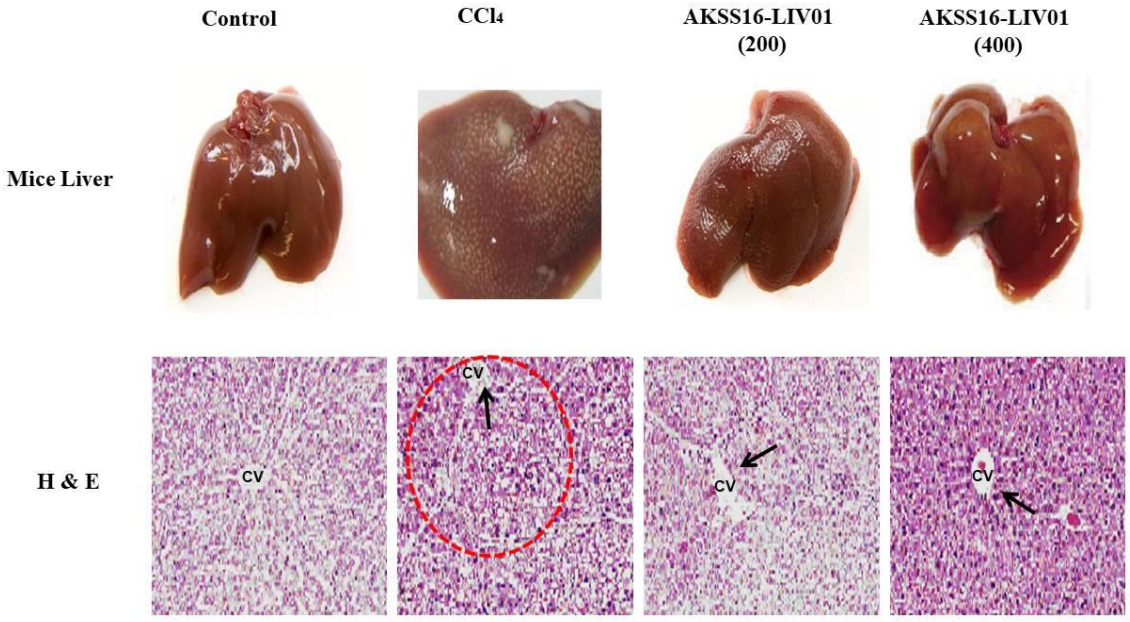

Figure 3A.2: Morphological and histopathological photographs of liver tissue. Lane 1: Morphological photographs of whole liver tissue; Lane 2: H & E staining section of liver tissue

3A.4 DISCUSSION

CCl_4 is a well-known hepatotoxin which is widely used to induce toxic liver injury and to study the cellular mechanisms behind oxidative damage in laboratory animals [21]. Fight against various liver dysfunctions such as liver fibrosis, fatty liver, liver cirrhosis etc. through safe and symptomatic medicine is a new challenge. Currently, there is no effective treatment for hepatic dysfunctions. To overcome this worldwide health complication, we formulated a novel herbal drug composed of nine indigenous medicinal and dietary herbs those were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and happiness. Our previous reports stated that the formulation have no adverse side effect and no toxicity in mice [22, 23].

AST and ALT are the most sensitive indicators for the diagnosis of liver cell damage. During amino acid synthesis and catabolism, AST and ALT play vital roles as endo-enzymes in hepatocytes. Under the circumstance of the normal working condition of the body, ALT and AST levels in the blood are very low and, thus, the activity of these two enzymes in normal serum is very low. When the liver tissue is damaged and the cell membrane permeability increases, these two enzymes penetrate into the blood in large quantities, leading to a significant increase in the activity of the enzymes in the sera. Therefore, an increase in serum AST and ALT can reflect the extent of liver cell damage [24]. When CCl_4 enters the animal body, liver microsomal lipids and hepatocyte membrane phospholipid molecules are attacked by free radicals generated by CCl_4, which in turn trigger changes in the TC and

TG levels in the liver [25, 26]. The increase in the AST, ALT, ALP, GGT and bilirubin levels indicates an exaggeration of liver damage. The experimental data from this study also confirmed that CCl_4 resulted in an increase in the AST, ALT, ALP, GGT and bilirubin levels in mice. On the other hand serum lipid profile i.e. total cholesterol, triglyceride, free fatty acids, phospholipids, LDL significantly elevated by the deleterious action of CCl_4.

The damage to the hepatic parenchymal cells due to accumulated toxins act on the liver leading to the formation of reactive oxygen species (ROS) which further leads to oxidative stress augmenting hepatic damage and dysfunction altering the liver transaminase enzyme parameters [27, 28].

Therapeutic application of novel multi herbal formulation AKSS16-LIV01 significantly reduced the serum biochemical and lipid profile levels in the serum and thus exerted a preventive effect on liver damage. This is further confirmed by our histopathological analysis of liver tissues.

CONCLUSION

The protective effect of multi herbal formulation (AKSS16-LIV01) in CCl_4 induced liver injury was established in this study in mice. The newly developed multi herbal formulation AKSS16-LIV01 normalized biochemical enzymes levels, lipid profile parameters; blood parameters from CCl_4 induced liver injury. Liver histology strongly supported that AKSS16-LIV01 (44mg/kg) able to protect liver cell form CCl_4 induced liver damage. Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of liver dysfunction.

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3B Multi Herbal Formulation of AKSS16-LIV01 ameliorates carbon tetrachloride-induced liver fibrosis in mice via redox inhibition route

3B.1 INTRODUCTION

Hepatic fibrosis is a programmed response to parenchymal injury orchestrated primarily by the hepatic stellate cell (HSC), a pleiotropic non-parenchymal cell that resides in the perisinusoidal space of Disse [29]. When the liver is injured, several signaling pathways converge upon the stellate cell, which promotes transdifferentiation to an activated myofibroblast, and the acquisition of phenotypic changes that culminate in extracellular matrix deposition. If the injury persists, hepatic fibrosis develops and may progress to cirrhosis, liver failure, hepatocellular cancer, and death. With HSCs positioned at the nexus of hepatic fibrosis, a greater appreciation of its complex biology may hasten our progress toward developing effective anti-fibrotic therapies [30]. Scientifically, it is characterized by extracellular matrix accumulation and is promoted by exposure to oxidative stress, including reactive oxygen and reactive nitrogen species (ROS, RNS). It was almost 50 years since oxidative stress, indicated by lipid peroxidation, was discovered in chemical-induced liver injury in an animal model, and now oxidative stress is the common factor among chronic liver diseases, such as CCl_4, iron overload, virus infection, and alcoholic and nonalcoholic liver diseases [31, 32]. There is a fine line between fibrosis and cirrhosis [33, 34]. Reactive oxygen species (ROS) are related to chronic liver damage and fibrogenesis [35]. ROS stimulates the expression of cytokines, hormones, and growth factors, and ROS-generated cytokines like platelet-derived growth factor (PDGF) and TGF-β1 are closely related to hepatic fibrogenesis [36].

Complementary and alternative medicine (CAM) is used in medical treatment but it is not a component of the mainstream medical system. Extensive use of CAM is highlighted among people with chronic diseases since it helps to avoid malaise often associated with conventional health care and empowers people to manage their chronic condition [2]. Complementary and alternative medicine is classified by National Center for Complementary and Alternative Medicine (NCCAM), USA into five categories: whole medical system, mind-body medicine, manipulative and body-based practices, energy medicine, and biologically based practices. On record, biologically-based practices such as herbal remedies continue to play a highly significant role in health care. About 80% of the world's population relies mainly on CAM, especially herbal medications for their primary health care [3]. Herbal remedies are growing in popularity throughout the world because of many reasons like long-lasting curative effects, efficacy, safety, and natural way of healing and lesser side effects [5, 37]. The World Health Organization defines traditional medicine as "diverse health practices, approaches, knowledge, and beliefs incorporating plant, animal and/or mineral-based medicines, spiritual therapies, manual technique, and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness" [15, 16]. In third world countries including India, up to 90% of the populace still relies entirely on plants as a resource of medicines [17]. Treatment with medicinal herbal-concentrates fortifies the natural healing process and adds to the feeling of wellness [38]. Several herbal derivatives show promising effects against hepatic fibrosis either experimentally in cell culture (*in vitro*), in animal models (*in vivo*), or even in clinical trials. Several researchers have described the mechanism of attenuation of liver fibrosis in experimental models [6].

Currently, there is no effective safe and symptomatic treatment for hepatic fibrosis and many patients develop a progressive form of hepatic cirrhosis. So, in the present study, we formulated a new phytomedicine (AKSS16-LIV01) composed of six indigenous medicinal herbs and three Indian medicinal spices which were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and happiness. Our previous study clearly showed that the developed formulation is safe and had no adverse effects on preclinical animal models [22, 39]. In the present study, our primary aim was to evaluate the therapeutic efficacy of newly formulated Phyto extract (AKSS16-LIV01) against Carbon tetrachloride (CCl_4) induced acute liver fibrogenesis in mice model and to explore the molecular mechanism underlying its protective action.

3B.2 MATERIAL AND METHODS

3B.2.1 Chemicals

Trichloroacetic acid, TRIS buffer, and PBS buffer (pH 7.4) were obtained from Merck, India. Carbon Tetrachloride (CCl_4) from Sigma Chemical Co. (St. Louis, MO, USA). Biochemical determination kits i.e. ALT, AST, GGT, ALP, bilirubin, BUN, and protein kits were procured from Thermo Scientific, USA. Biochemical assay kits of total cholesterol (TC), triglycerides (TG), phospholipids, free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL) were purchased from ELITech Diagnostic, France. Antioxidant enzyme kits i.e. SOD, CAT, GSH, and GPx were obtained from Boehringer, USA. ELISA kits of TNF-α and TGF-β_1 were procured from Sigma Aldrich, USA. All other reagents used in this study are laboratory grade.

3B.2.2 Collection and Authentication of the Herbs

Six medicinal plant and three medicinal spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by taxonomists of the Department of Botany, Uluberia College, University of Calcutta, India, and kept as voucher specimens with the number (Table 2). Ayurvedic identification parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti (shape), and Parimana (size). The plants and plant parts used in the preparation of the extract are listed in Figure 1.

3B.2.3 Preparation of Extract

At first, collected plant parts were air-dried and then clean with double distilled water and kept in a hot air oven at 80ºC for 10 min and 60ºC for 30 min. All the plants and spices were placed in a blade mill to obtained fine powder. Aqueous extract of the polar fraction was performed according to the method of Adhikari et al. (2018) with slight modifications. 5 gm of dry plant parts were taken and dissolved by using 10 ml of methanol. The extract then sonicated at room temperature for 30 min using an ultrasonic bath, centrifuged at 4000 rpm for 20 min, and finally, the supernatant was removed. This procedure was repeated four times, collecting all the supernatants, which were finally evaporated in a rotary evaporator under reduced pressure at 35°C. Finally, the residue was reconstituted in 3 ml of methanol, filtered using Whatman filter papers (GE Healthcare and Life Sciences, MA, USA), and kept at 4ºC for further use [40].

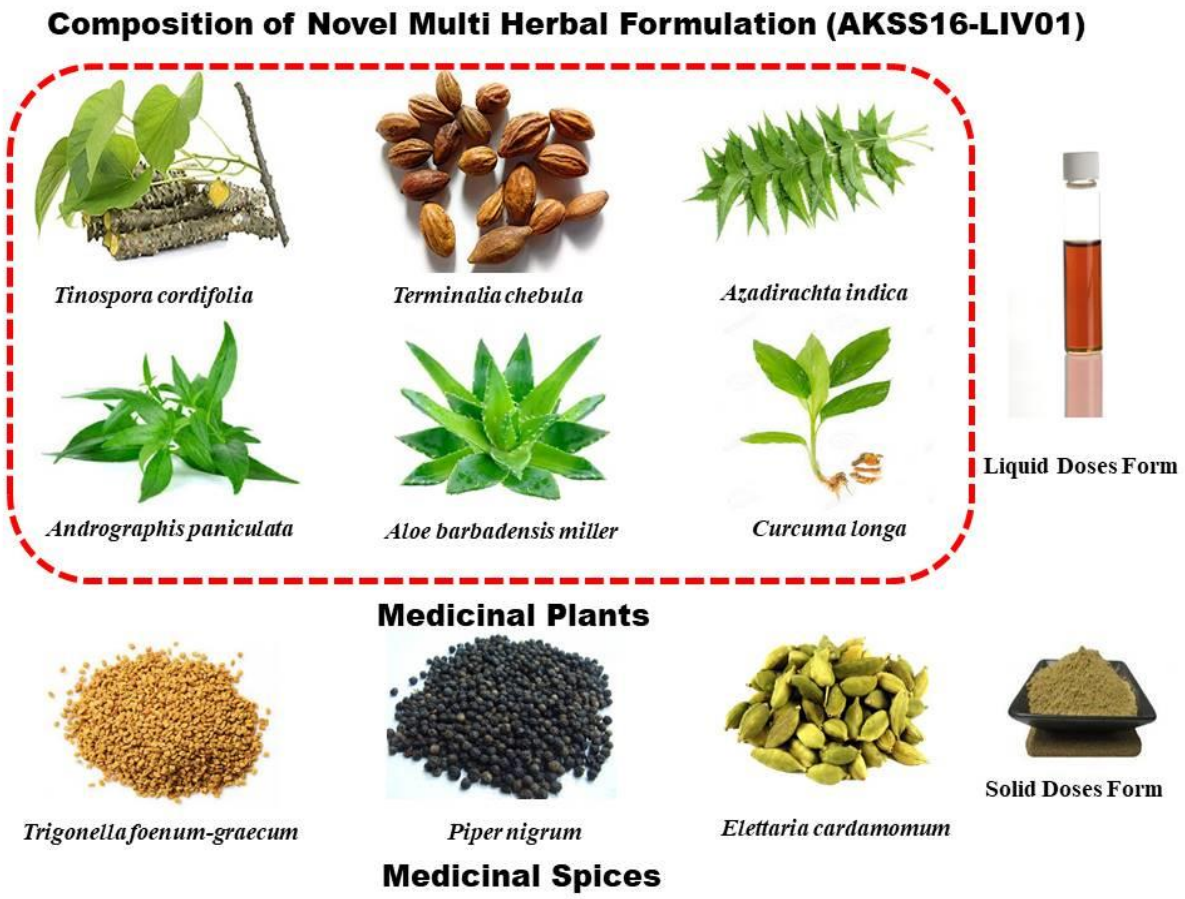


Figure 3B.1: Composition of multi herbal formulation (ASKK16-LIV01) containing six medicinal herbs and three medicinal spices.

3B.2.4 Animal experiments

Adult Swiss albino mice weighing between 25 ± 5 g were procured from the central animal house, Jadavpur University. All healthy mice were housed in standard polypropylene cages, each of 6 mice. They were given free access to a standard pellet diet (Purchase from Hind liver India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture, and water *ad libitum*. The animal house was maintained under a controlled condition of temperature at 24 ± 1 °C, the relative humidity of 60–70%, and 12 h light 12 h dark cycle. All the experimental procedures were carried out according to the guidelines of CPCSEA, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC) of Jadavpur University having approval number 261/JU/s/IAEC/Pharma/2018.

After acclimatization, the mice were randomly divided into six groups with 6 mice each: Group I served as normal control received only the vehicle (1ml/kg olive oil twice a week for 6 weeks), Group II treated with multi herbal formulation (MHF) AKSS16-LIV01 (400 mg/kg BW/day all over the

experiment), Group III received 1 ml/kg BW of CCl_4 diluted 20% in olive oil twice a week for 6 weeks, Group IV pre-treated with herbal formulation AKSS16-LIV01 (200 mg/kg BW/day) low dose (HF) for four weeks after two weeks CCl_4 induction and ensure occurrence of fibrosis, Group V pre-treated with multi herbal formulation (MHF) AKSS16-LIV01 high dose (400 mg/kg BW) for four weeks after two weeks CCl_4 induction and ensure occurrence of fibrosis. Group VI pre-treated with Silymarin standard hepatoprotective drug at a dose (100 mg/kg BW) for two weeks after CCl_4 induction and ensure the occurrence of fibrosis (Table 3B.1).

Table 3B.1: Experimental Design

Groups	Treatment
I	Normal control received only the vehicle (1ml/kg olive oil twice a week for 6 weeks)
II	Treated with multi herbal formulation (MHF) AKSS16-LIV01 (400 mg/kg BW/day all over the experiment).
III	Received 1 ml/kg BW of CCl_4 diluted 20% in olive oil twice a week for 6 weeks.
IV	Pre-treated with herbal formulation AKSS16-LIV01 (200 mg/kg BW/day) low dose (HF) for two weeks after CCl_4 induction.
V	Pre-treated with multi herbal formulation (MHF) AKSS16-LIV01 high dose (400 mg/kg BW) for two weeks after CCl_4 induction.
VI	Pre-treated with Silymarin standard hepatoprotective drug at a dose (100 mg/kg BW) for two weeks after CCl_4 induction.

3B.2.5 Bodyweight gains and feed efficiency

Body weights were measured on weekly basis from the initial day to the final day of the experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining water found in the drinking bottle from the initial water given to the animals.

3B.2.6 Blood Collection

At the end of the respective fasting period, blood was collected from each mouse by retro-orbital venous puncture. 200 µL of blood samples were collected into micro-centrifuge tubes with and without EDTA (2%). Collected blood was placed in a slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow color serum was separated and used for further analyses.

3B.2.7 Assessment of Liver Function Parameters

The biochemical parameters like serum enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total and direct bilirubin, and blood urea nitrogen (BUN) were assayed using assay kits (Thermo Scientific, USA) following the protocol prescribed by manufacturers. Total protein (23) concentration was determined in the serum by the method of Lowry et al. (1951) [41].

3B.2.8 Lipid profile

Serum levels of total cholesterol (TC), triglycerides (TG), phospholipids, free fatty acids (FFA), high-density lipoprotein (HDL), low-density lipoprotein (LDL) were assayed using assay kits (ELITech Diagnostic, France) following the protocol prescribed by manufacturers.

3B.2.9 Preparation of Tissue Homogenate

Before biochemical analysis, liver samples (100 mg/mL) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10000 rpm for 15 mins and the supernatant obtained was collected and used for further studies. Protein concentrations of liver supernatant were determined using the commercially available kit (Thermo Scientific, USA) following the procedure prescribed by the manufacturer.

3B.2.10 Hydroxyproline assay

Hydroxyproline assay to quantify collagen content was performed using a colorimetric method described by the manufacturer (Bio Vision, Milpitas, CA), In brief, 10 mg of liver tissue were excised and homogenized in 100 ml of sterile MQ water followed by hydrolysis in 12N HCl (100 ml) at 120uC. After 3 h, 5 ml of tissue lysate was transferred to a 96-well plate and incubated at 37uC for 16 h to evaporate the acid (Adhikari *et al.*, 2017) [21]. Samples were incubated with equal amounts of chloramine T and Ehrlich's reagents for 30 min at 65uC. Absorbance was recorded at 560 nm with an ELISA plate reader (Synergy BioTek, Winooski, VT).

3B.2.11 Assessment of Lipid Peroxidation, NO and iNOS

The level of malondialdehyde, as a substance that reacts with thiobarbituric acid (TBARS), was determined in homogenates of the organs according to the standard method [42]. The level of NO and iNOS activity was determined from the liver homogenate [43] through the ELISA method using the commercial kit (Sigma Aldrich, USA).

3B.2.12 Determination of TNF-α & TGF 1β

Levels of TNF and TGF in the liver were determined using ELISA kits (Sigma Aldrich, USA) according to the manufacturer's guideline. Protein concentrations were measured according to the manufacturer's guidelines [44].

3B.2.13 Determination of Superoxide Dismutase (SOD)

The activity of SOD was measured according to a reported method [45]. In brief, 2.8 mL reagent solution (xanthine 0.3 mM, EDTA 0.67 mM, 150 µM NBT), sodium carbonate 0.4 M, and bovine albumin (30 mg/30 mL) was added to 0.1 mL sample and 50 µL xanthine oxidase (10 µL in 2 M ammonium sulphate), incubated at 25ºC for 20 min and mixed with,0.1 mL 8 M copper chloride. The color reaction was measured at 560 nm.

3B.2.14 Determination Catalase (CAT)

Catalase activity was measured according to the established method [46]. Briefly, after the addition of 5µL liver homogenate to 0.995 mL, 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm was monitored for 1 min to determine catalase activity. The enzyme activity was expressed as U/mg protein.

3B.2.15 Determination of Glutathione (GSH) and GPx Content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method. Glutathione peroxidase (GPx) activity was assayed using a modified method of Lawrence and Burk [47, 48].

3B.2.16 Hematoxylin and Eosin staining

Five-micron paraffin-embedded liver sections were deparaffinized and washed with water [49]. Hydrated tissue sections were incubated with Mayer's hematoxylin for 5 min followed by vigorous washing in running tap water. Sections were counterstained with 1% eosin for 2 min. Eosin stained sections were washed with water and dehydrated with alcohol. Dehydrated sections were washed with xylene. Images were taken with a microscope (Olympus BX51 fluorescence microscope).

3B.2.17 Sirius red staining

After the experimental period portion of the liver, slices were fixed in 10% neutral buffered formalin overnight and then transferred to 70% ethanol before embedding in paraffin blocks. Paraffin-embedded liver tissues were cut into 5 mm thick sections. Deparaffinized sections were incubated for 60 min with Pico-Sirius red solution (Abcam, Cambridge, MA) followed by a brief rinse with acetic acid (0.05%). Sections were dehydrated by washing with absolute alcohol [50]. Sections were observed with a light microscope (Olympus BX51 fluorescence microscope).

3B.2.18 Immunohistochemical staining of alpha-SMA

A formalin-fixed and paraffin-embedded liver section (5 mm) was subjected to antigen retrieval by heating in a microwave oven for 10 min in citrate buffer (pH 6.0). Sections were blocked for 1 h with 5% BSA in TBST followed by incubation with monoclonal anti- a-smooth muscle actin (a-SMA) primary antibody (dilution 1:50; Pierce, Rockford, IL) at 4uC overnight. Immunostaining was performed using a DAB substrate kit according to manufacturer instructions (Abcam, Cambridge,

MA). Sections were counterstained with Mayer's hematoxylin. The image was scan by using a Leica Microsystem scanner (Leica Microsystem Inc. Buffalo Grove, IL) [51].

3B.2.19 Statistical Analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

3B.3 RESULTS

3B.3.1 Determination of Body weight, food consumption, water intake, and relative Liver weight

Table 3B.2 shows the composition of AKSS16-LIV01 a novel multi herbal formulation containing six Indian medicinal herbs and three Indian medicinal spices. The formulation had potent antifibrotic activity and be an effective medication against liver fibrosis. Figure 3B.2 depicted the body weight, food consumption, water intake, and relative liver weight of mice treated with CCl_4 and pretreated with AKSS16-LIV01. In comparison with standard drug silymarin, we observed that AKSS16-LIV01 has probable protective capacity against deleterious effects caused by CCl_4.

Table 3B.2: Composition of ingredient(s) present in AKSS16-LIV01

Sl. No.	Botanical Name	Common Name	Family	Part Used	The quantity used in extract	Voucher Specimen number
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	Stem	20 mg	MHFJU-11/16
2.	*Terminalia chebula*	Haritaki	Combretaceae	Fruit	20 mg	MHFJU-12/16
3.	*Azadirachta indica*	Neem	Meliaceae	Leaves	50 mg	MHFJU-13/16
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	Leaves & Steam	50 mg	MHFJU-14/16
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	Leaves & Steam	50 mg	MHFJU-15/16
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	Rhizome	20 mg	MHFJU-16/16
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	Seed	10 mg	MHFJU-17/16
8.	*Piper nigrum*	Black pepper	Piperaceae	Seed	10 mg	MHFJU-18/16
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	Seed	10 mg	MHFJU-19/16

* Amount required for preparation of 5 ml extract.

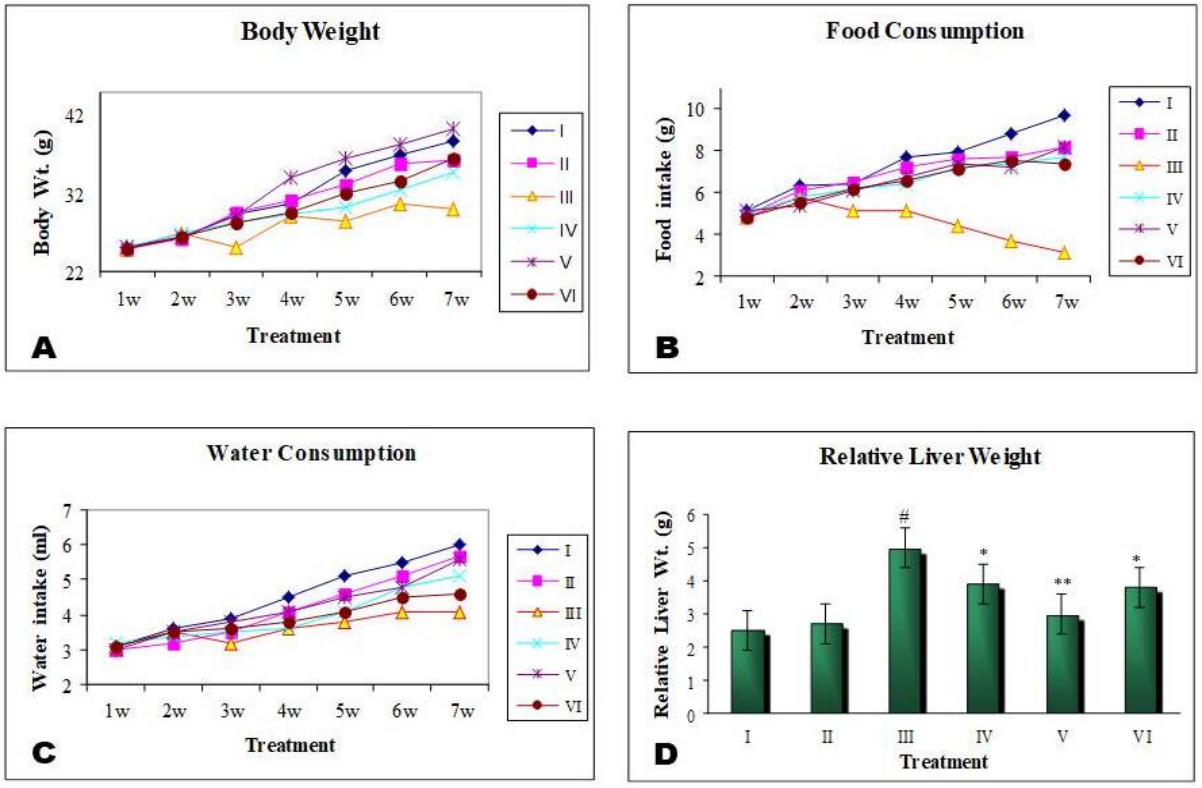


Figure 3B.2: Effect of ASKK16-LIV01 on experimental mice model. A. Relative body weight, B. Average food consumption, C. Average water consumption, D. Relative liver weight of mice after the experimental period. The values are expressed as the mean ± SEM. $^{\#}$Indicates significance difference P< 0.001 compared to the untreated control group and *P< 0.001, **P< 0.001 significance difference compared to the ethanol-treated group using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Group-I: Control; Group-II: treated with AKSS16-LIV01 (400 mg/kg); Group-III: CCl_4 treatment; Group-IV: ASKK16-LIV01 (200 mg/kg) after 2 weeks induction of CCl_4; Group-V: ASKK16-LIV01 (400 mg/kg) after 2 weeks induction of CCl_4; Group-VI: Silymarin (100 mg/kg) after 2 weeks induction of CCl_4.

3B.3.2 Determination of serum AST, ALT, total cholesterol, and triglyceride

Figure 3B.3 shows liver function parameters AST &ALT and lipid profile of total cholesterol and triglyceride of mice treated with CCl_4 and with AKSS16-LIV01 after CCl_4 induction. Results of the biochemical study stated that treatment with multi herbal formulation - AKSS16-LIV01 at a dose of 400 mg/kg showed optimum protective capacity.

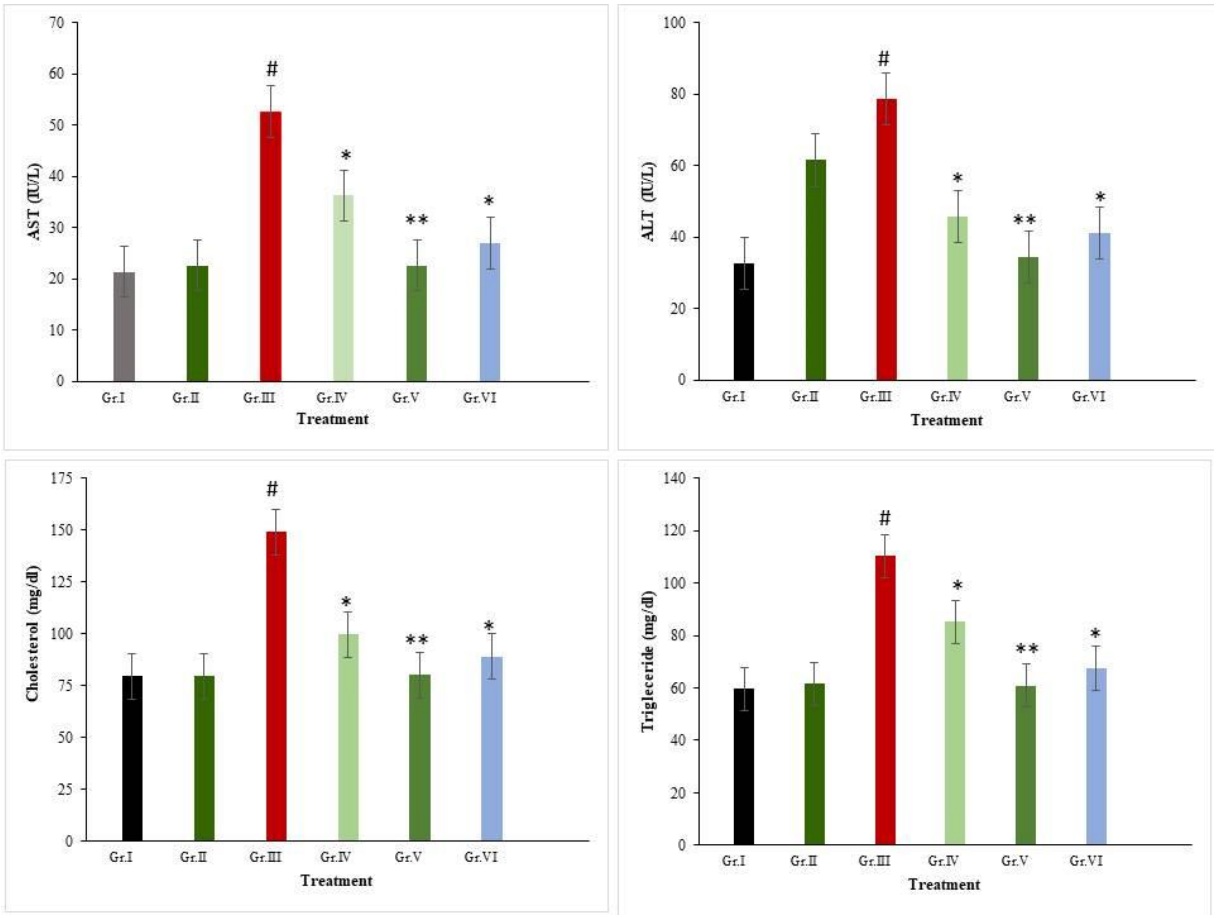


Figure 3B.3: Determination of the biochemical parameters on experimental mice model. A. Aspartate aminotransferase (AST) level, B. Alanine aminotransferase (ALT) level, C. Total Cholesterol, D. Triglyceride content. The values are expressed as the mean ± SEM. # Indicates significance difference P< 0.001 compared to the untreated control group and * P< 0.001, ** P< 0.001 significance difference compared to the ethanol-treated group using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Group-I: Control; Group-II: treated with AKSS16-LIV01 (400 mg/kg); Group-III: CCl_4 treatment; Group-IV: ASKK16-LIV01 (200 mg/kg) after 2 weeks induction of CCl_4; Group-V: ASKK16-LIV01 (400 mg/kg) after 2 weeks induction of CCl_4; Group-VI: Silymarin (100 mg/kg) after 2 weeks induction of CCl_4.

3B.3.3 Determination of serum GGT, ALP, BUN, total bilirubin, direct bilirubin, total protein

Table 3B.3 showed Serum GGT, ALP, BUN, total bilirubin, direct bilirubin, and total protein levels of mice treated with CCl_4 and pre-treated with AKSS16-LIV01 after CCl_4 induction. In comparison with standard drug silymarin, our developed formulation showed better therapeutic effects against CCl_4 induced liver fibrosis by reducing elevated liver markers.

Table 3B.3: Summary of the liver function test parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + Silymarin 100
GGT(IU/l/min/mg protein)	0.26±0.02	0.26±0.09	2.11± 0.51$^{\#}$	1.06±0.08*	0.34±0.12**	0.41±0.02*
ALP(IU/l/min/mg protein)	232.05±3.11	237.84±2.91	461.27±4.96$^{\#}$	279.55±2.06*	250.44±2.71**	262.11±2.04*
BUN(mg/dl)	0.41±0.02	0.46±0.03	0.72±0.04$^{\#}$	0.67±0.02*	0.46±0.02**	0.55±0.03*
Total Bilirubin(mg/dl)	0.12 ± 0.2	0.19±0.11	0.62 ± 0.11$^{\#}$	0.34±0.08*	0.18±0.09**	0.25±0.08*
Direct Bilirubin (mg/dl)	0.06±0.001	0.07±0.002	0.33 ± 0.07$^{\#}$	0.09±0.003*	0.09±0.004**	0.24±0.005*
Total Protein	6.51±0.65	627±0.21	2.56±0.32$^{\#}$	4.58±0.41*	6.22±0.27**	5.39±0.21*

Values are the mean of six individual observations in each group ± S.D. Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

3B.3.4 Determination of Phospholipids, Free fatty acids, LDL-cholesterol, HDL-cholesterol

Table 3B.4 showed Serum Phospholipids, Free fatty acids, LDL-cholesterol, HDL-cholesterol levels of mice treated with CCl_4, and pre-treated with AKSS16-LIV01 after CCl_4 induction. Serum Phospholipids, Free fatty acids, LDL-cholesterol levels were significantly increased whereas total HDL-cholesterol was significantly decreased when compared with normal control animals. In comparison with standard drug silymarin, our developed formulation showed better therapeutic effects against CCl_4 induced liver fibrosis.

Table 3B.4: Summary of the lipid profile parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + Silymarin 100
Phospgolipids (mg/dL)	76.59 ± 6.28	92.37±2.66	142.97 ± 4.69$^{\#}$	98.51 ± 3.28*	82.69 ± 4.87**	88.03±2.84*
Free fatty acids (mg/dL)	15.97 ± 0.58	20.14±1.96	31.87 ± 1.67$^{\#}$	21.22 ± 1.69*	14.09 ± 1.22**	16.27±2.01*
LDL (mg/dL)	39.65 ± 1.96	43.61±1.88	76.94 ± 1.77$^{\#}$	45.78±1.65*	36.85 ± 1.25**	44.52 ± 2.28*
HDL (mg/dL)	19.58 ± 0.69	15.28±0.55	10.28 ± 0.28$^{\#}$	16.24±0.41*	10.28 ± 0.28**	22.67 ± 0.99*

Values are the mean of six individual observations in each group ± S.D. *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

3B.3.5 Determination of hepatic MDA, Hydroxyproline, NO, and iNOS

Figure 3B.4 showed Hepatic MDA level, Hydroxyproline level, nitric oxide (NO), and nitric oxide synthase (iNOS) of mice treated with CCl_4 and pre-treated with AKSS16-LIV01 after CCl_4 induction. Liver MDA level, Hydroxyproline level, NO, and iNOS were significantly increased in the CCl_4 treated animals when compared with normal control animals. Treatment with multi herbal formulation (AKSS16-LIV01) both 200 mg/kg and 400 mg/kg significantly decreased the hepatic MDA level, Hydroxyproline level, NO, and iNOS, when compared with CCl_4, treated animals. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at at a dose of 400 mg/kg showed optimum protective potential against CCl_4 induced liver fibrosis.

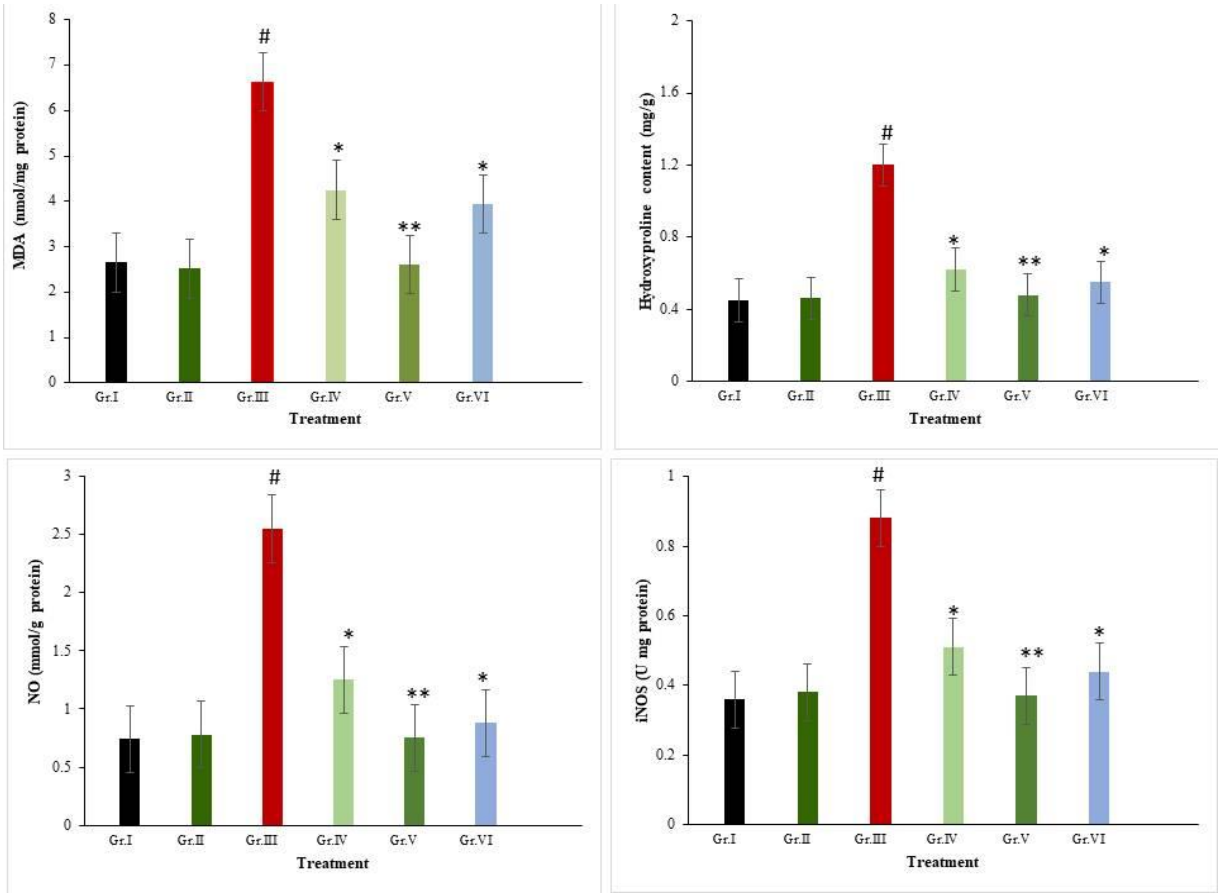


Figure 3B.4: Determination of lipid peroxidation, hydroxyproline, nitric oxide, and nitric oxide synthase content on experimental mice model. A. MDA content, B. hydroxyproline level, C. nitric oxide content, D. iNOS content. The values are expressed as the mean ± SEM. $^{\#}$Indicates significance difference P< 0.001 compared to the untreated control group and *P< 0.001, **P< 0.001 significance difference compared to the ethanol-treated group using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Group-I: Control; Group-II: treated with AKSS16-LIV01 (400 mg/kg); Group-III: CCl_4 treatment; Group-IV: ASKK16-LIV01 (200 mg/kg) after 2 weeks induction of CCl_4; Group-V: ASKK16-LIV01 (400 mg/kg) after 2 weeks induction of CCl_4; Group-VI: Silymarin (100 mg/kg) after 2 weeks induction of CCl_4.

3B.3.6 Determination of hepatic Antioxidant enzymes

Various antioxidant enzymes in liver tissue as superoxide dismutase (SOD), catalase (CAT), reduces glutathione (GSH) and glutathione peroxidases (GPx) were also determined (Figure 3B.5). Liver SOD, CAT, GSH, and GPx were significantly reduced ($p<0.001$) in the CCl_4 treated animals when compared with normal control animals. Treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly increased ($p<0.05$, $p<0.001$) the hepatic antioxidant enzyme levels when compared with CCl_4 treated animals. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at at a dose of 400 mg/kg showed optimum protective potential against CCl_4 induced liver fibrosis.

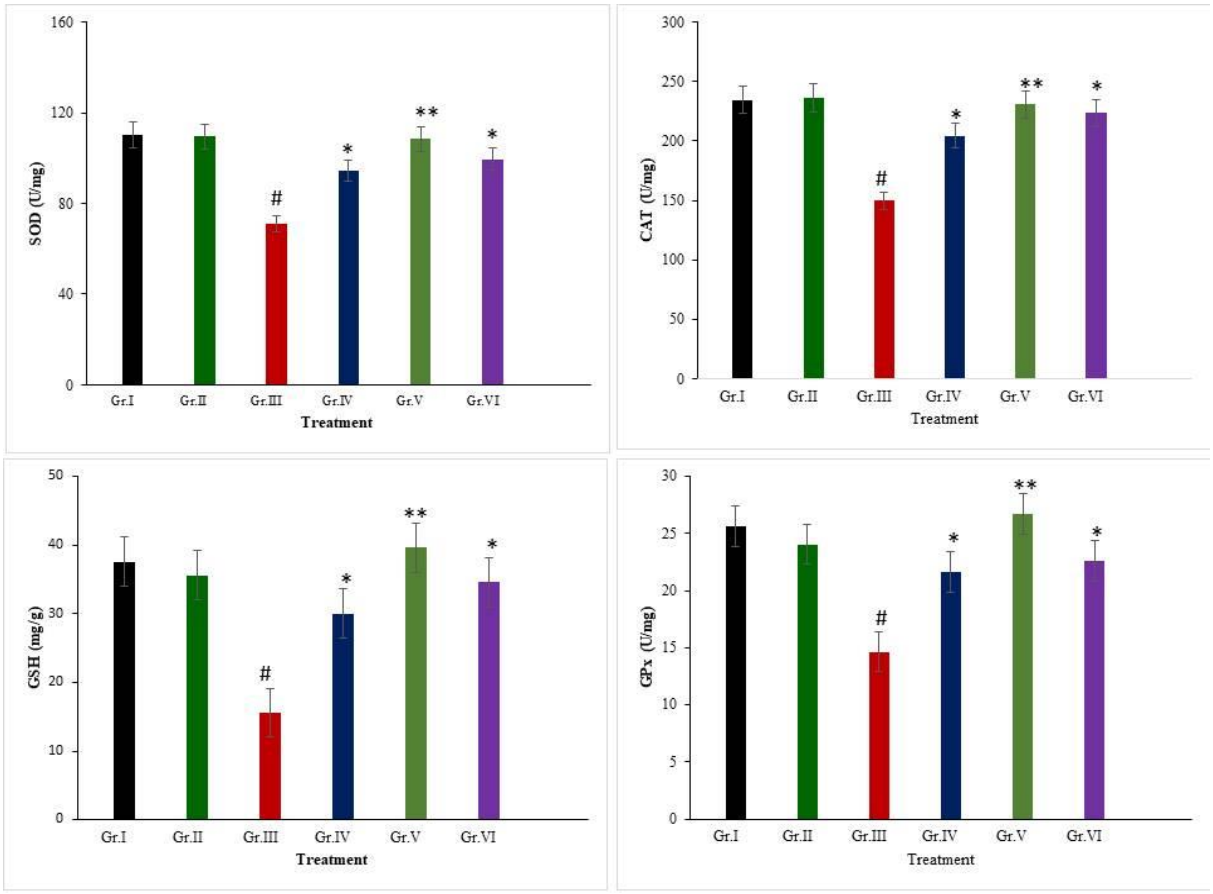


Figure 3B.5: Determination of the antioxidant enzyme levels on experimental mice model. A. Superoxide dismutase (SOD), B. Catalase (CAT), C. Glutathione (GSH), D. Glutathione peroxidase (GSH-Px). The values are expressed as the mean ± SEM. $^{\#}$Indicates significance difference $P< 0.001$ compared to the untreated control group and $^{*}P< 0.001$, $^{**}P< 0.001$ significance difference compared to the ethanol-treated group using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Group-I: Control; Group-II: treated with AKSS16-LIV01 (400 mg/kg); Group-III: CCl_4 treatment; Group-IV: ASKK16-LIV01 (200 mg/kg) after 2 weeks induction of CCl_4; Group-V: ASKK16-LIV01 (400 mg/kg) after 2 weeks induction of CCl_4; Group-VI: Silymarin (100 mg/kg) after 2 weeks induction of CCl_4.

3B.3.7 Determination of the hepatic level of TNF-α

Inflammation is commonly associated with liver fibrosis during chronic liver injury. The levels of proinflammatory cytokines TNF-α were determined in the liver. Table 3B.5 shows a significant increase of TNF-α in CCl_4 treated mice when compared to control untreated animals which are significantly inhibited by the application of multi herbal formulation (AKSS16-LIV01) in a dose-dependent manner. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at at a dose of 400 mg/kg showed optimum protective potential against CCl_4 induced liver fibrosis.

3B.3.8 Determination of the hepatic level of TGF 1β

TGF 1β is the major profibrogenic cytokine. As shown in Table 3B.5 significant increased levels of TGF 1β were observed in CCl_4 treated mice when compared to control untreated animals. On the other hand, treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly decreased the level of TGF 1β when compared with CCl_4 treated animals. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at at a dose of 400 mg/kg showed optimum protective potential against CCl_4 induced liver fibrosis.

Table 3B.5: Summary of the liver TNF α and TGF 1β levels across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + Silymarin 100
TNF-α (pg/mg protein)	1412.96±226.53	1536.77±220.97	2354.87±376.98$^{\#}$	1672.25±236.93*	1440.11±287.83**	1478.26±288.17*
TGF-1β (pg/mg protein)	487.90±92.34	502.38±98.77	1246.05±126.78$^{\#}$	698.11±212.45*	478.03±101.76**	552.03±203.84*

Values are the mean of six individual observations in each group ± S.D. *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from CCl_4 ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

3B.3.9 Histology

3B.3.9a H&E staining

Figure 3B.6 showed histological photographs of the liver tissue in both control and different experimental groups. The normal control animals (Group I) showed the typical architecture of liver tissue with a prominent central vein (CV) and chords of hepatocytes radiating whereas CCl_4 treatment produced extensive necrosis of hepatocytes which was more pronounced in the centrical (zone 3) area (Group II). The fatty changes were the macrovesicular type which was evident in centrical and portal areas with inflammatory reactions (Figure 3B.6). Partial hepatic protection with the reduction in the extent of hepatic necrotic areas, fatty infiltration, and mild portal inflammation was visualized in the liver section of AKSS16-LIV01 (200 mg/kg) treated animals. On the other hand administered with AKSS16-LIV01 (400 mg/kg) completely protected the liver as evidenced by restoration of a normal histoarchitecture of the liver (Group IV).

3B.3.9b Sirius red staining

Sirius red staining of the liver section indicates the fibrotic appearance in the CCl_4 treated mice (Group-II) with large areas of necrotic tissue and a significant number of ballooning hepatocytes (Figure 3B.6). Moreover, the liver sections of CCl_4-intoxicated mice showed massive fatty changes, necrosis, ballooning degeneration, and broad infiltration of the lymphocytes, and the loss of cellular boundaries. Histology of the liver sections of control mice (Group I) showed normal hepatic cells with well-preserved cytoplasm, prominent nucleus, nucleolus, and visible central veins. The histological architecture of liver sections of the mice treated with AKSS16-LIV01 at a dose of 200 mg/kg showed more or less normal lobular pattern compared with control animals. Treatment with AKSS16-LIV01 at a dose of 400 mg/kg showed an almost normal lobular structure of the liver with a mild degree of fatty change, necrosis, and lymphocyte infiltration almost comparable to the control groups.

3B.3.9c The immunohistochemistry

Immunohistochemical staining of α-SMA was used to evaluate the extracellular matrix (ECM) deposition. As shown in Figure 3B.6, the expression of α-SMA was observed in smooth muscle cells of blood vessels in the control group. In the CCl_4-induced model group, α-SMA expression was significantly elevated in the portal areas and collagen fiber deposition areas. In contrast, the expression of α-SMA protein in the AKSS16-LIV01 treatment group was significantly lower than that in the CCl_4-induced model group.

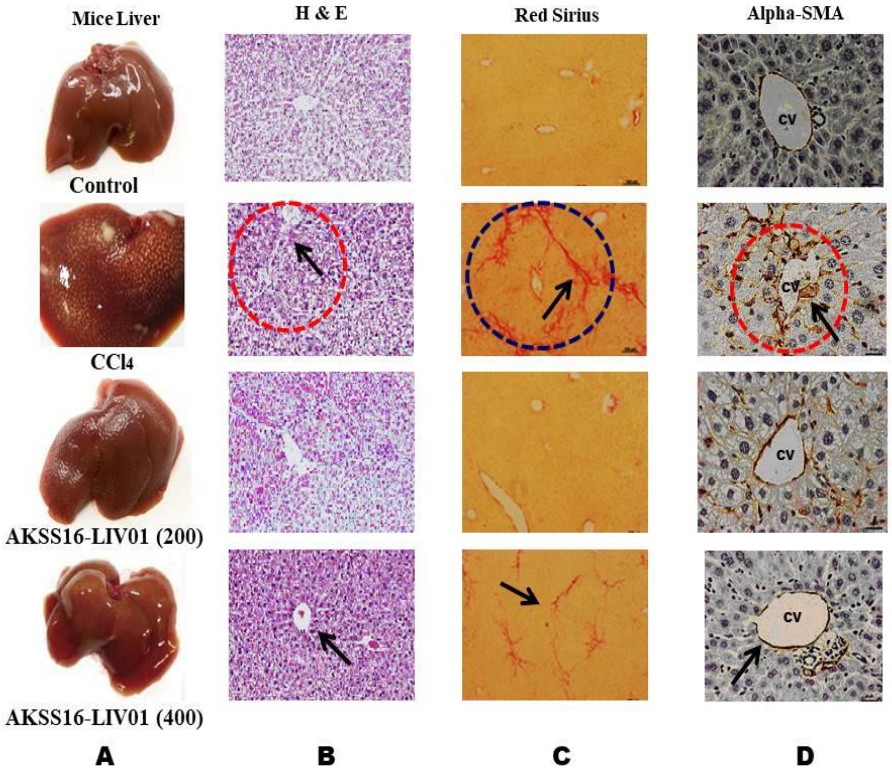


Figure 3B.6: Representative photomicrographs of livers in different experimental groups. A. liver morphology, B. H & E stained liver sections, C. Sirius red-stained liver sections, D. Immunohistochemistry stained liver sections.

3B.4 DISCUSSION

Various scientific studies have already established that chronic exposure to CCl_4 causes liver fibrosis. Consequently, CCl_4, a chemical hepatotoxin, that produces reactive free radicals trichloromethyl radical (CCl_3) and a proxy trichloromethyl radical (CCl_3O_2) when metabolized, has been frequently used to investigate the hepatoprotective effects of drugs and plant extracts as a solvent for induction of hepatic damage in animal models. CCl_4 increases lipid peroxidation and protein oxidation in hepatic cells, as well as induces liver damage and apoptosis [52-55].

In the present study, we investigated the efficacy of oral newly developed multi herbal formulation (AKSS16-LIV01) for treatment of CCl_4-induced hepatic fibrosis in mice. Through a 7-week in vivo experiment, our research demonstrated that therapeutic application of AKSS16-LIV01 was effective in attenuating liver injury and hepatic fibrosis, suggesting that AKSS16-LIV01 has potential value for the prevention and treatment of hepatic fibrosis. Our experiments further suggested that the effectiveness of this treatment may be bestowed upon its antioxidant capacity, anti-inflammatory effect, and its effects on inhibiting TNF-α and TGF-β1 signaling pathway [56].

Liver transaminases like ALT and AST have been reported to be sensitive indicators of liver injury especially fibrosis [57, 58]. ALT and AST levels in the blood are very low and, thus, the activity of these two enzymes in normal serum is very low. When the liver tissue is damaged and the cell membrane permeability increases, these two enzymes penetrate the blood in large quantities, leading to a significant increase in the activity of the enzymes in the sera. When CCl_4 enters the animal body, liver microsomal lipids and hepatocyte membrane phospholipid molecules are attacked by free radicals generated by CCl_4, which in turn trigger changes in the total cholesterol (TC) and triglyceride (TG) levels in the liver [59]. The increase in the AST, ALT, TC, and TG levels indicates an exaggeration of liver damage. The experimental data from this study also confirmed that CCl_4 resulted in an increase in the AST, ALT, TC, and TG levels in mice, whereas pre-treatment with AKSS16-LIV01 significantly reduced the same levels in the serum and thus exerted a preventive effect on liver damage. Apart from this application of this novel multi herbal formulation (AKSS16-LIV01) normalized the serum GGT, ALP, BUN, total bilirubin, and direct bilirubin levels which are other markers of CCl_4 induced liver fibrosis.

Exposure to CCl_4 developed hepatic Oxidative stress causes hepatic injury, which is mediated by the production of free radical derivatives of CCl_4 and is responsible for cell membrane damage and the consequent release of marker enzymes of hepatotoxicity [2, 60]. Oxidative injury induced by CCl_4 can be monitored in experimental animals by detecting oxidative stress parameters, such as MDA, SOD, CAT, GSH, and GSH-Px [61, 62]. The current results showed that SOD, CAT, GSH, and GSH-Px activity were significantly decreased and MDA content significantly increased in the liver in response to CCl_4 treatment, thereby indicating increased oxidative damage to the liver. However, SOD, CAT, and GSH-Px activities were significantly elevated by the pre-administration of AKSS16-LIV01 to CCl_4-intoxicated mice, suggesting the ability of AKSS16-LIV01 to restore and maintain the activities of SOD, CAT, GSH, and GSH-Px in CCl_4-damaged liver. Therefore, the administration of AKSS16-LIV01 can effectively protect against the CCl_4-induced hepatic lipid peroxidation via preventing the decrease of activities of GSH-Px, SOD, GSH, and CAT in mice induced by CCl_4. It is indicated that

AKSS16-LIV01 may improve CCl_4 induced liver injury by alleviating lipid peroxidation and oxidative stress.

Inflammation is another important pathological mechanism propagating CCl_4-induced liver injury (Tak *et al.*, 2001). Accumulating evidence has revealed that CCl_4 and excessive ROS induced by CCl_4 probably activate Kupffer cells, which can mediate the hepatic inflammation process by producing TNF-α, TGF-1β, and other pro-inflammatory cytokines [63]. Our results showed that administration of multi herbal formulation (AKSS16-LIV01) with CCl_4 reduced the level of TNF-α and TGF-1β in the liver as compared to CCl_4 administration alone. These results suggested that AKSS16-LIV01 can alleviate liver injury caused by CCl_4 by suppressing the inflammatory response.

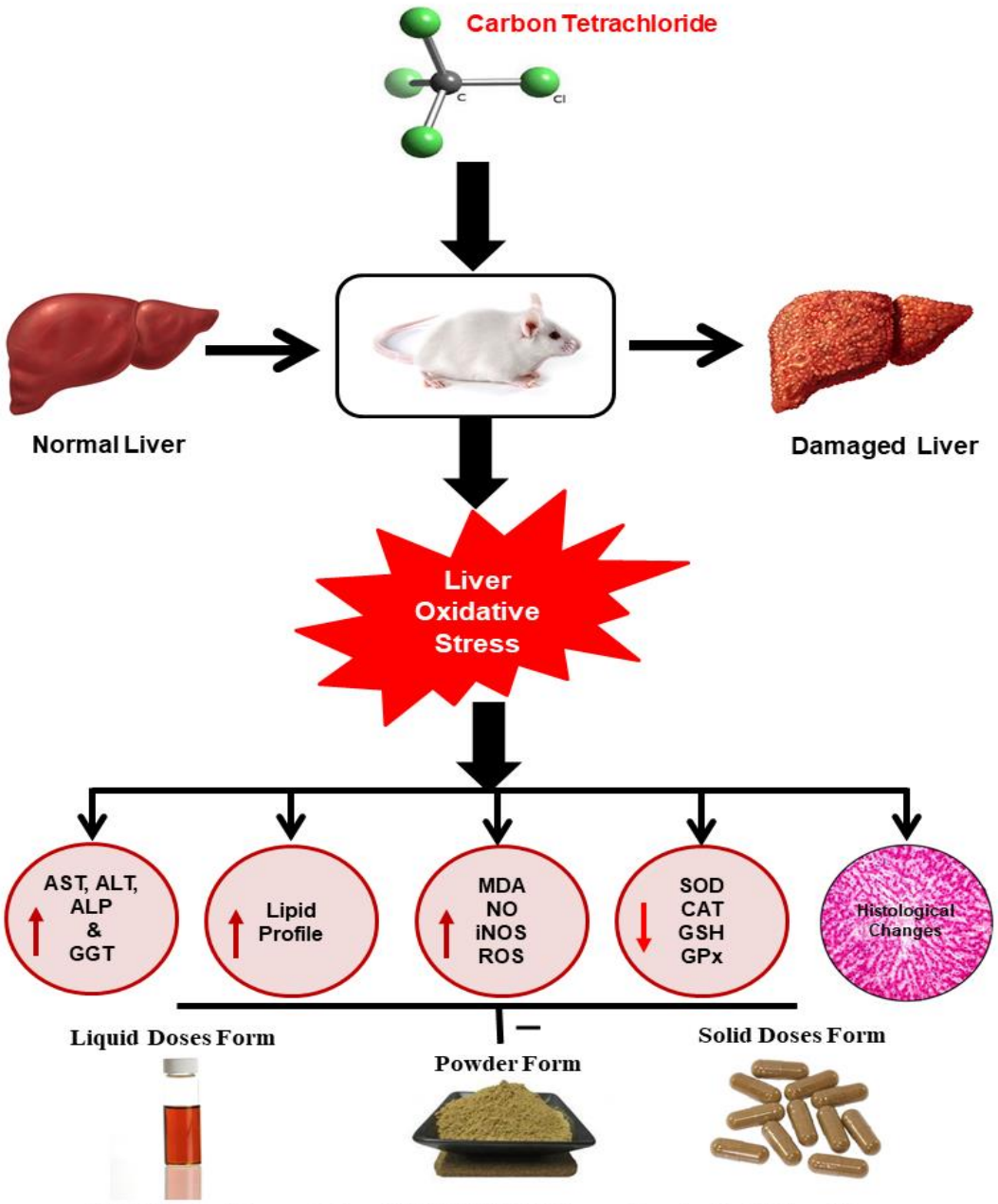
NO, which is produced by iNOS during inflammation and overexpressed in neoplastic lesions, is also an endogenous candidate molecule involved in the development of fibrosis [64, 65]. Our results indicate that CCl_4 is the prime factor that produces hepatic fibrosis and elevates NO production by increasing the iNOS activity. Developed formulation AKSS16-LIV01significantly mitigates these effects and protects the liver from fibrosis. The novel multi herbal formulation also reduced the CCl_4 intoxicated elevated hepatic hydroxyproline level which is a marker enzyme of liver fibrosis. Medicinal herbs and medicinal spices containing this formulation can protect the liver tissue against fibrosis and maintain homeostasis.

The histopathological analysis is used as a direct means of evaluating the protective effects of AKSS16-LIV01. Hepatic fibrosis histopathology is characterized by hepatocyte structural disorder, extensive hepatic steatosis, balloon-like changes, inflammatory necrosis, collagen deposition, and diffuse fibrous septum formation [66, 67]. In the present study, immunohistochemistry results showed that a newly developed multi herbal formulation (AKSS16-LIV01) can reduce liver inflammation, inhibit collagen fiber deposition, and improve liver tissue structure. Immunohistochemical staining of α-SMA further confirmed that AKSS16-LIV01 can also alleviate the deposition of collagen fibers and the formation of pseudolobules, indicating that AKSS16-LIV01can ameliorate liver histopathological changes.

CONCLUSION

The newly developed multi herbal formulation (AKSS16-LIV01) is based on six Indian medicinal plants and three medicinal spices having anti-fibrotic properties and was examined upon mice model. The formulation prevented CCl_4 induced liver fibrosis in mice by reduction of oxidative stress and control the lipid peroxidation, nitric oxide (NO), and nitric oxide synthase (iNOS) concentration. On the other hand decline in antioxidant enzyme levels such as SOD, CAT, GSH, and GPx in fibrotic condition was restored to normalcy by AKSS16-LIV01 due to its medicinal property. AKSS16-LIV01 also can suppress pro-inflammatory cytokines TNF-α as well as by assisting the inhibition of HSC activation by reducing profibrogenic cytokines TGF-β_1. Thus, we are led to conclude that the developed novel formulation composed of medicinal herbs and medicinal spices to be a therapeutic low-cost medication in the future for the prevention of liver fibrosis.

SUMMARY



Multi Herbal Formulation (AKSS16-LIV01) ameliorate Oxidative Stress Induced by Carbon Tetrachloride

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Chapter - IV

Ameliorative Effect of a Novel Multi Herbal Formulation (AKSS16-LIV01) against Alcoholic Liver Disease by inhibition of Oxidative Stress

4A Ameliorative Effect of Multi Herbal Formulation on Lipid Peroxidation and redox dysfunction in Ethanol Induced Hepatic Imbalance

4A.1 INTRODUCTION

Oxidation is a chemical reaction in the body that produces free radicals. These free radicals lead to chain reactions within the body that damage other cells. Commonly the consecutive reduction of oxygen through adding electrons cause the formation of a variety of ROS, which include superoxide (O_2^-), hydroxyl radical (·OH), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2). In general, the harmful effects of ROS come in the form of DNA damage, lipid preoccupation (oxidation of polyunsaturated fatty acids), protein amino acid oxidation, and inactivation of specific enzymes through oxidation of their cofactors [1-3]. These damages can be contributing factor to many general and specific problems and diseases such as Parkinson's, Alzheimer's, asthma, aging, cancer, Rheumatoid Arthritis, Liver disorder etc. Therefore, antioxidant parameters and oxidative stress indices are considered potential biomarkers and are frequently used as screening tools to assess the impacts of environmental stress. Important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione S-transferees (GST) and glutathione peroxidase (GPx). In addition, glutathione, vitamins and carotene also help the organism to mitigate the external pollutants and help the protective enzyme system of the organism [4-6].

Liver is a major organ attacked by ROS [7]. Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. When the ROS is excessive, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders [8]. The oxidative stress not only triggers hepatic damage by inducing irretrievable alteration of lipids, proteins and DNA contents and more importantly, modulating pathways that control normal biological functions. Since these pathways regulate genes transcription, protein expression, cell apoptosis, and hepatic stellate cell activation; oxidative stress is regarded as one of the pathological mechanisms that results in initiation and progression of various liver diseases, such as chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis [9-11]. Moreover, systemic oxidative stress arising during liver disease can cause damage to extra-hepatic organs, such as brain impairment and kidney failure [12].

Worldwide, alcoholic liver disease (ALD) is a major cause of illness and mortality. ALD, a common effect of prolonged and heavy alcohol intake, is one of the leading health problems after cancer and cardiovascular diseases. In the modern way of life, intake of alcoholic beverages is a common characteristic and nowadays alcoholism ranks as a major health problem [13, 14]. Experimental and epidemiologic studies confirmed that the duration and the degree of alcohol consumption promote the progression and genesis of liver damage. The liver is the major site of ethanol metabolism. Liver executes several important mechanisms which play crucial roles in digestion, storage, assimilation, and detoxification [15]. Various study reports illustrate that ethanol causes the accumulation of reactive oxygen species (ROS) such as hydroxyl radical, superoxide radical, and hydrogen peroxide in

the hepatocytes that leads to the oxidation of DNA, protein, and cellular membranes, resulting in the depletion of reduced glutathione and liver damage [16, 17].

Antioxidant is a molecule that inhibits or stops the oxidation of other molecules in the body. So, antioxidant protects the body from cell damage. The potent sources of natural antioxidants are medicinal herbs and spices. Phenolic components in herbs and spices have been reported to be effective as natural antioxidants [18]. Intake of alcohol is associated with increase susceptibility of membranes to peroxidation and an increased requirement of antioxidant. The flavonoids are a large group of naturally occurring compounds that are found in plants and are frequently consumed as part of the human diet. Flavonoids are receiving much attention now a day for their potential pharmacological properties [19]. The antioxidant activity of flavonoids has been demonstrated by their ability to inhibit enzymes such as lipoxygenase, cyclooxygenase, along with chelating metal ions, and scavenging free radicals [20, 21].

Based on previous reports, we developed a multi herbal formulation (MHF) containing nine Indian medicinal plants out of which six were medicinal plants and three were medicinal spices. Therefore, the present study was undertaken to evaluate the oxidative stress induction and tissues injury in liver of adult mice followed exposure to ethanol at different concentrations and its deleterious recovery by the application of multi herbal formulation.

4A.2 MATERIALS AND MTHODS

4A.2.1 Chemicals

Disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and trichloroacetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents used were of analytical grade. 2-thiobarbituric acid (TBA) was obtained from Fluka Chemie (Buchs SG, Switzerland).

4A.2.2 Animals

In the present study 40 male mature Swiss albino mice (25-30 g) were obtained from the CPCSEA approved animal house of Jadavpur University, Kolkata. The animals were maintained at 12 h light/dark cycle, at constant temperature (20±1°C) and humidity (50±5%). Mice were feed standard pellet diet (Purchase from Hind liver India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture, and 2.6% by weight of lipids and water ad libitum. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any pathogenic infection. All the experimental procedure were carried out according to the guidelines of CPCSEA, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC).

4A.2.3 Experimental Design

The mice were divided into four groups (10 per each). The studied groups were as follows: group I as control, group II as sham treated with multi herbal formulation (MHF) (300mg/kg orally, daily), group III as liver damage control, and group IV as ethanol treated with multi herbal formulation (MHF)

(300mg/kg orally, daily) after induce liver damage, respectively (Table 4A.1). Liver damage was induced in the 3rd and 4th groups by ethanol administration (50% v/v).

Table 4A.1: Experimental Design.

Groups	Treatment
I	Normal control received only the normal drinking water for 60 days.
II	Sham treated control received multi herbal formulation (300mg/kg-bw/day) for 60 days.
III	Animals treated with ethanol (50% v/v, single dose/day) for 60 days.
IV	Treatment with ethanol (50% v/v) for 30 days and then pre-treated with multi herbal formulation - MHF (300 mg/kg-bw/day) for next 30 days.

4A.2.4 Blood Collection

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Serum was separated and used for further analyses.

4A.2.5 Preparation of Tissue Homogenate

Prior to biochemical analysis, liver samples (100 mg/mL) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10000 rpm for 15 mins [22] and the supernatant obtained was collected and used for further studies. Protein concentrations of liver supernatant were determined using commercially available kit (Span Diagnostics Ltd, India) following procedure prescribed by manufacturer.

4A.2.6 Levels of Malonedialdehyde (MDA)

Lipid peroxidation was estimated in the serum samples by measuring the malondialdehyde (MDA) formation using the thiobarbituric acid method [23]. Briefly, 100 µL of animals was mixed with 500 µL of 150 mM Tris-HCl and 1.5 mL of 0.375% TBA and vortexed for 10 sec. The reaction mixture was then incubated at 100 °C for 45 min in a water bath. At the end of incubation, the samples were centrifuged at 1000 ×g for 10 min. The MDA content was calculated from the absorbance measurement at 532 nm and using a Shimadzu spectrophotometer (Tokyo, Japan) an absorption coefficient = $1.56 \times 105\ cm^{-1}\ M^{-1}$.

4A.2.7 Determination of ROS activity

Amount of ROS in blood was measured using 2', 7'-dichlrofluorescein diacetate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay

was performed as described by Socci et al.20 Fluorescence was determined at 488 nm excitation and 525 nm [24] emission using a fluorescence plate reader (Tecan Spectra Fluor Plus).

4A.2.8 Determination of Glutathione (GSH) and GPx Content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method. GPx activity was assayed using a modified method of Lawrence and Burk [25].

4A.2.9 Determination of Superoxide Dismutase (SOD)

The activity of SOD was measured according to a reported method [26]. In brief, 2.8 mL reagent solution (xanthine 0.3 mM, EDTA 0.67 mM, 150 µM NBT), sodium carbonate 0.4 M, and bovine albumin (30 mg/30 mL) was added to 0.1 mL sample and 50 µL xanthine oxidase (10 µL in 2 M ammonium sulphate), incubated at 25ºC for 20 min and mixed with,0.1 mL 8 M copper chloride. The color reaction was measured at 560 nm.

4A.2.10 Determination Catalase (CAT)

Catalase activity was measured according to the standard method [27]. Briefly, after addition of 5µL liver homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm was monitored for 1 min to determine catalase activity. The enzyme activity was expressed as U/mg protein.

4A.2.11 Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison t tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), Graph Pad Software, California, USA. P & lt; 0.05 was considered significant.

4A.3 RESULTS

4A.3.1 Determination of serum and liver lipid peroxidation

In the present study we developed a novel multi herbal formulation (MHF) containing six Indian medicinal herbs and three medicinal spices (Figure 4A.1) enrich with high flavonoids and polyphenols. The medicinal effect of multi herbal formulation (MHF) on serum and liver MDA level of mice was determined. The levels of MDA in serum and liver are shown in Figure 4A.2. The level of serum MDA in the untreated liver damage mice was significantly (87%) higher ($P<0.001$) than that of control animals. The level of MDA in the serum of ethanol treated liver dysfunction mice with multi herbal formulation (MHF) was very low, similar to the level (92% recovery) found in the control animals. The treatment of hepatic damage animal with MHF could significantly (92%) inhibit the elevation of MDA in comparison with the untreated experimental animals. The level of liver MDA in the untreated hepatic damage animal was significantly (84%) higher than that of control animals. The treatment of liver damage animal with multi herbal formulation (90%) inhibit the increasing of MDA

(P<0.001) in comparison with the untreated hepatic damage animals. The level of MDA in the serum and liver of sham rats treated with multi herbal formulation were low, similar to the level found in the control animals.

4A.3.2 Determination of serum and liver ROS content

Beneficial effect of multi herbal formulation (MHF) on serum and liver ROS level of mice was determined. The levels of ROS in serum and liver are shown in Figure 4A.1. The level of serum ROS in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of ROS in comparison with the untreated experimental animals. The level of liver ROS in the untreated liver damage mice was significantly (87%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (93%) in ROS level compared with the untreated control animals.

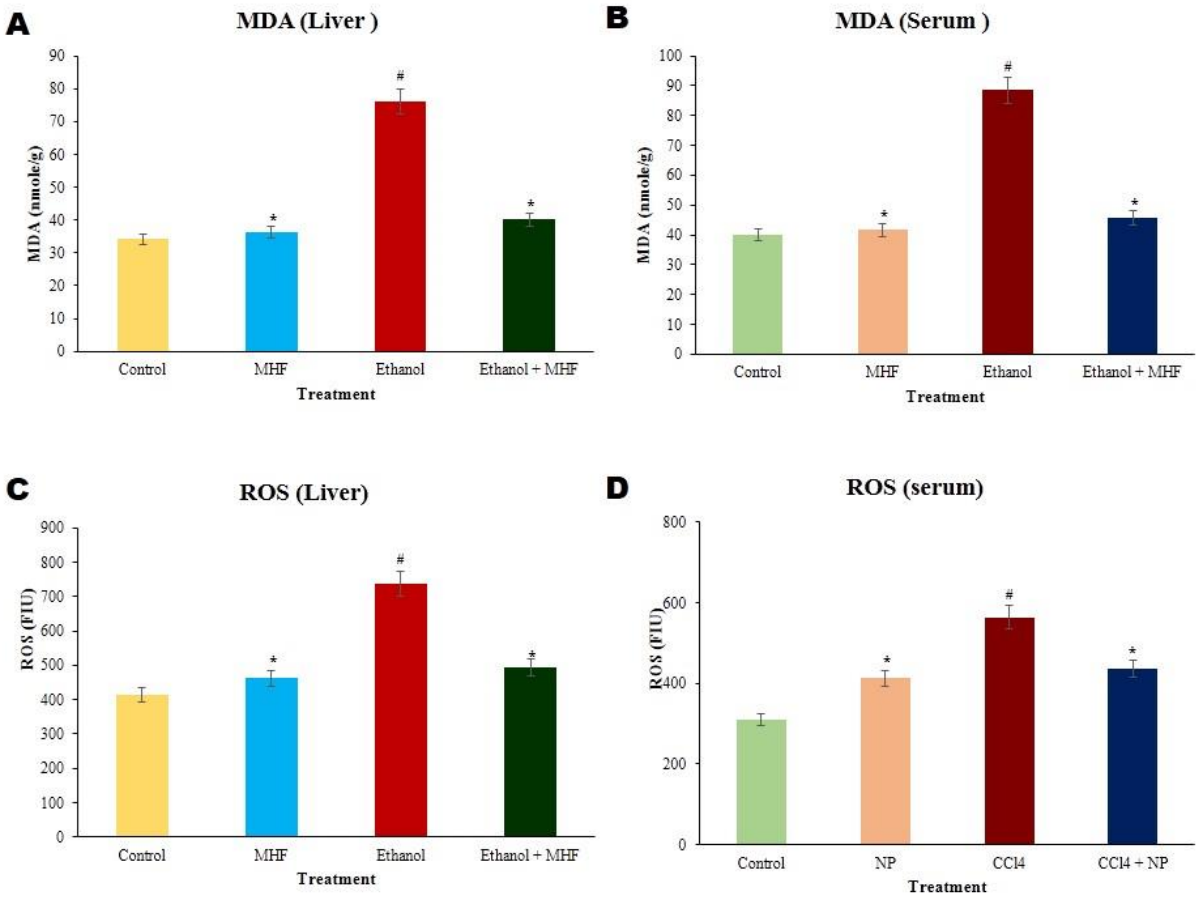


Figure 4A.1: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) MDA content in serum B) MDA content in liver C) Level of ROS in serum D) Level of ROS in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol ***** (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4A.3.3 Determination of serum and liver SOD and CAT content

Beneficial effect of multi herbal formulation (MHF) on serum and liver SOD and CAT level of mice was determined. The levels of SOD and CAT in serum and liver are shown in Figure 4A.2. The level of serum SOD and CAT in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of SOD and CAT in comparison with the untreated experimental animals. The level of liver SOD and CAT in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in SOD and CAT level compared with the untreated control animals.

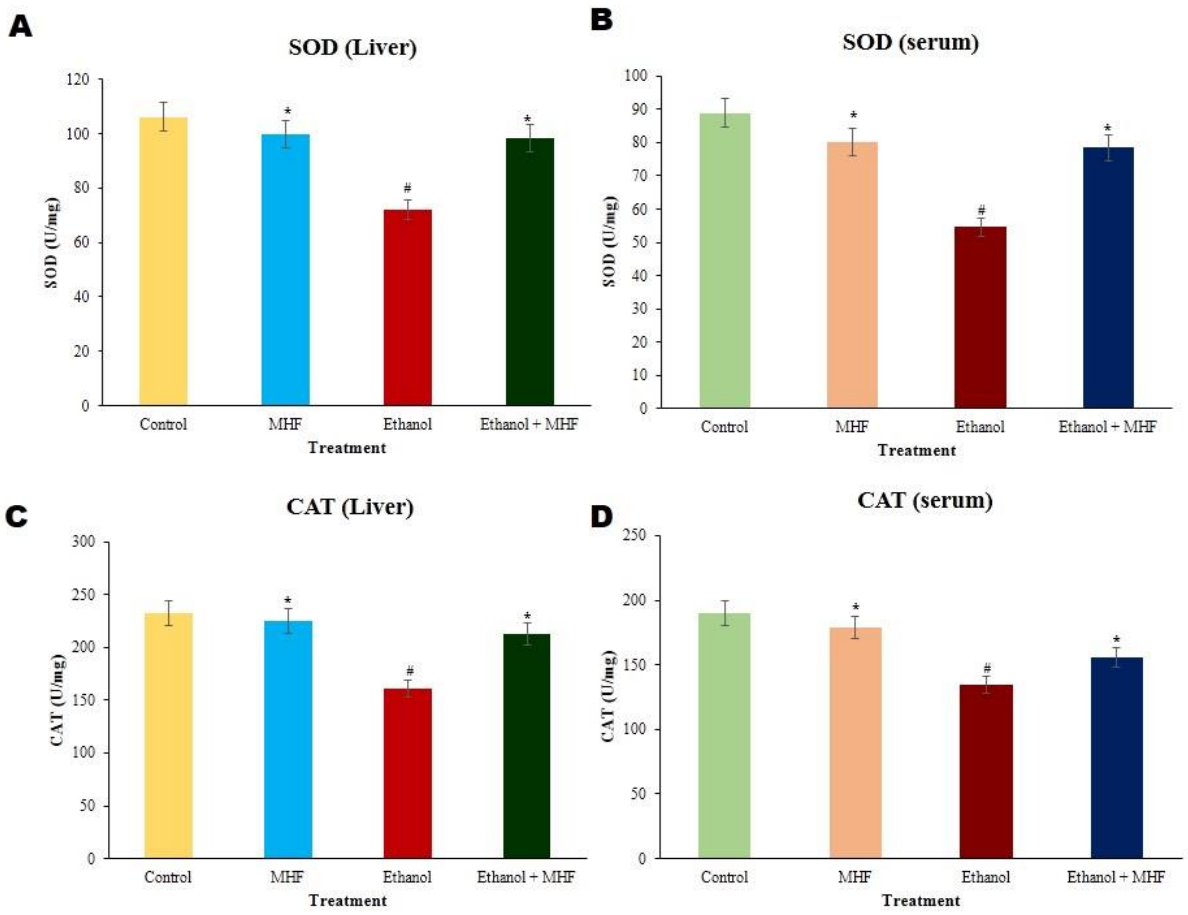


Figure 4A.2: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) SOD content in serum B) SOD content in liver C) Level of CAT in serum D) Level of CAT in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol ***** (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4A.3.4 Determination of serum and liver GSH and GPx content

Effect of multi herbal formulation (MHF) on serum and liver GSH and GPx level of mice was determined. The levels of GSH and GPx in serum and liver are shown in Figure 4A.3. The level of serum GSH and GPx in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of GSH and GPx in comparison with the untreated experimental animals. The level of liver GSH and GPx in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in GSH and GPx level compared with the untreated control animals.

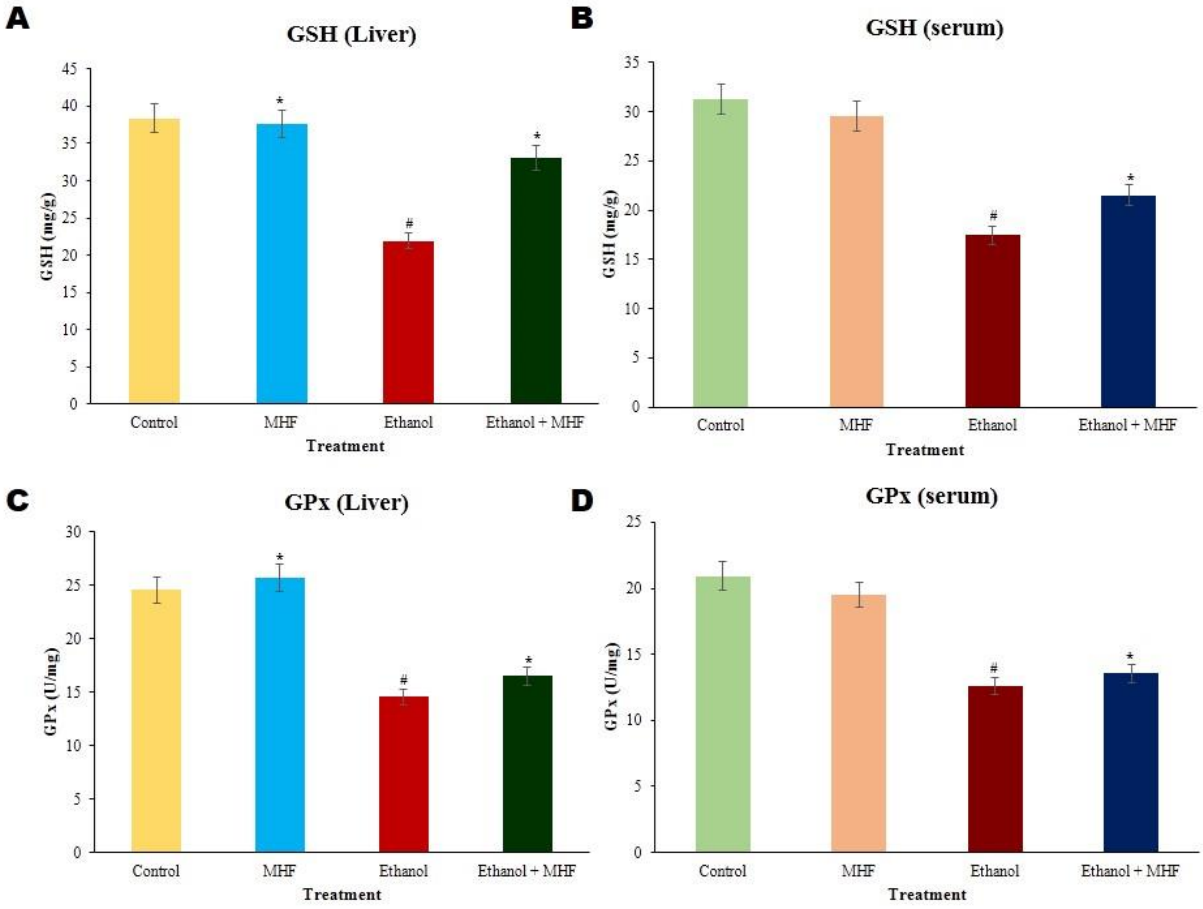


Figure 4A.3: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) GSH content in serum B) GSH content in liver C) Level of GPx in serum D) Level of GPx in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control $^{\#}$ (p<0.001) and significantly different from Ethanol ***** (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4A.4 DISCUSSION

In the past decades, considerable evidence has established the role of oxidative stress in the pathogenesis of liver complications. Indeed, several studies have reported that ethanol induced liver damage contribute to the accumulation of ROS and antioxidants deficiency (e.g., SOD and GSH) in both experimental animals and patients. This study showed that multi herbal formulation (MHF) increased serum and liver antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) and decreased lipid peroxidation in ethanol induced experimental mice. The current work showed a significant increase in the level of lipid peroxidation (LPO) in serum and liver tissue after 60 days of oral administration of ethanol (50% v/v). LPO is an auto catalytic process leading to oxidative degradation of lipids causing demolition of cell membranes and subsequently cell damage [28]. It is mainly generated by the effect of various reactive oxygen species (ROS) such as hydrogen peroxide, superoxide's and hydroxyl radical [29]. LPO is a chain reaction initiated by the hydrogen abstraction from the side chain of polyunsaturated fatty acids resulting in cell membranes deterioration [30]. Decomposition of latest compounds lead to production of several products particularly malondialdehyde (MDA) [31]. This tending to diminish the fluidity of cell membrane which plays a vital role in cell functioning. The elevated level of MDA observed in the current work, which is an indicator of LPO, denotes cell membrane damage in serum and liver of ethanol treated animals. Treatment with multi herbal formulation (MHF) significantly inhibited increasing of serum and liver lipid peroxidation in comparison with the ethanol treated animals.

Glutathione is a non-enzymatic cellular antioxidant which plays a crucial role in scavenging damaging free radicals. GSH can function as a co-substrate for peroxide detoxification by glutathione peroxidases [32]. GSH also catalyses' the reduction of hydrogen peroxide to water [33]. Depletion of cellular glutathione content may be one of the reasons for the increase in cell vulnerability to oxidative stress [34-36]. In this study decline in GSH level in this investigation could be caused by ethanol intoxication. Treatment with multi herbal formulation (MHF) significantly inhibited decreasing of serum and liver glutathione in comparison with the ethanol treated animals.

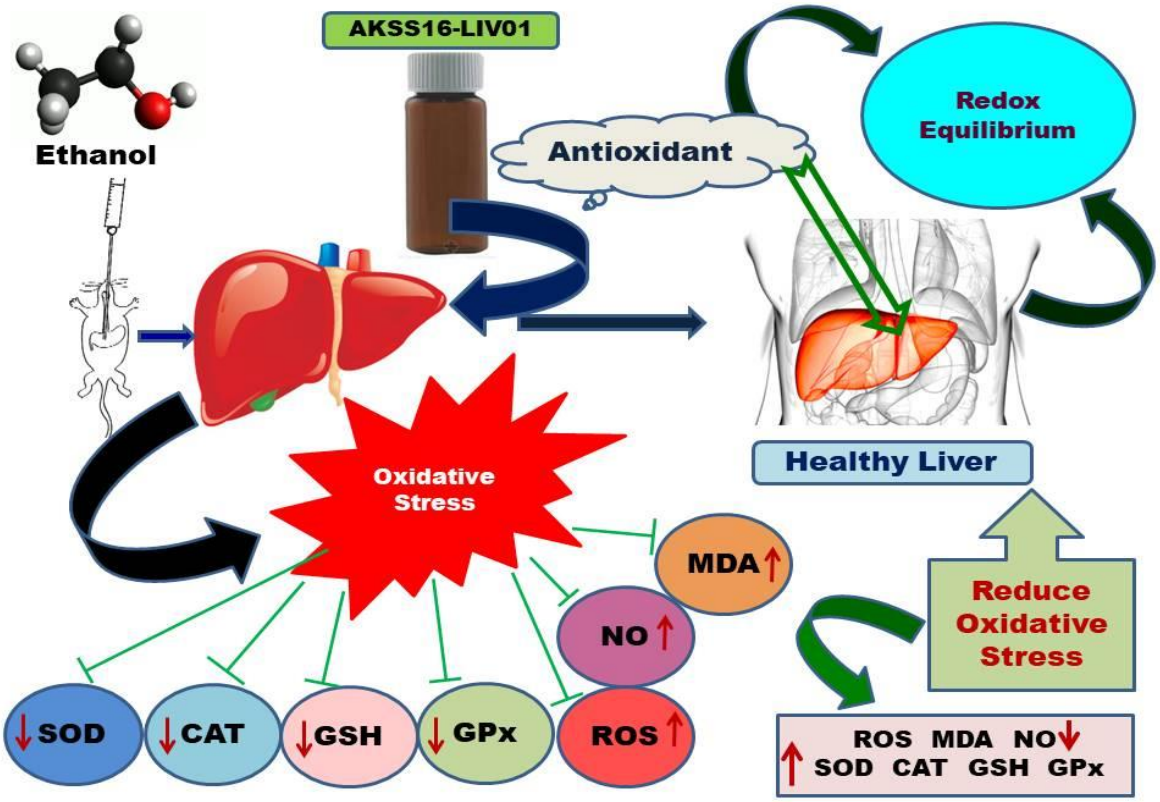
Study showed that there was a significant decrease in SOD and CAT activities in liver and renal tissues following oral administration of ethanol. SOD is an enzyme that repairs cells and decreases their damage through conversion of endogenous cytotoxic superoxide radicals to hydrogen peroxide and ordinary molecular oxygen, which have harmful effects on proteins and polyunsaturated fatty acids [37]. CAT is an important enzyme in protecting the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide to water and oxygen.[32] In the presence of insufficient activity of CAT to decompose hydrogen peroxide, more of it could be converted to toxic hydroxyl radicals that might contribute to oxidative stress after intoxication with ethanol. The significantly suppressed catalase activities after intoxicated with ethanol were recovered by the administration of multi herbal formulation (MHF). The endogenous scavenger, SOD, which removes the superoxide anion radicals by converting them into hydrogen peroxide (H_2O_2) and O_2, was significantly increased in the MHF experimental groups. The observed increase in liver SOD enzyme activity after administration of the multi herbal formulation (MHF) may be a consequence of oxidative activation of enzyme protein or increased of their synthesis. Therefore, the increase in the activity of SOD in liver tissues of treated mice might indicate a reduce accumulation of superoxide anion radical with oxidative stress, contributing decrease liver toxicity [38]. GPx has a role in defending cells against oxidative stress and

this in turn involves GSH as a cofactor. GPx catalyzes the oxidation of GSH to GSSG at the cost of H_2O_2. Decreased GPx activity was observed in the alcohol exposure group. This reduced activity may be involved in either free radical-dependent inactivation of enzyme or depletion of its co-substrate (i.e., GSH) or NADPH on ethanol treatment (35). Administration of multi herbal formulation (MHF) significant increased reduced GPx level activity after alcohol exposure.

CONCLUSION

In summary, the exposure of male Swiss mice to alcohol revealed signs of toxicity that were evidenced by a reduction in antioxidant defense system. Moreover, the activities of SOD, CAT, GPx, and the concentration of MDA and GSH in the liver and kidney clearly indicate that oue developed multi herbal formulation (MHF) is able to inhibit the oxidative stress during the co-exposure with ethanol, but its effect depends on the dose and time of exposure.

SUMMARY



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4B Antioxidant and Immunomodulatory effect of AKSS16-LIV01 – a Multi Herbal Formulation against Alcoholic Liver Dysfunction in Mice

4B.1 INTRODUCTION

Our body continuously exposed with various harmful toxicants as a result liver detoxifying the toxicant and maintains cellular homeostasis [1,2]. People in both developed and underdeveloped countries when consume excesses alcohol or taken alcohol on a continuous basis leading to alcoholic liver disease (ALD). Individuals those are suffering with ALD facing lots of liver complications such as fatty liver disease, hepatic fibrosis, hepatic cirrhosis and hepatic cellular carcinoma even death [3,4]. World health organization (WHO) published a report that stated death due to the liver failure stands the fifth position after cancer, cardiac failure, nervous disorder and respiratory infection [5]. Scientific study stated that consumption of ethanol elevate the ratio of NADH/NAD^+ in the liver cell which create disruption of oxidation of fatty acids in mitochondria developed liver dysfunction [6,7]. Other causes of liver disease deposition of lipid molecule in the liver cells which stop the normal hepatic functions. Alcohol enhances the transportation process of lipids towards the liver from the small intestine which elevate the fatty acids mobilization from adipose tissue, taken up by the liver [8]. This causes damage of the liver cell membrane which releases the transaminases enzymes (AST and ALT) in the blood stream. On the other hand this damaged cell membrane also release alkaline phosphatase (ALP) and gamma glutamyl transferase (GGT) which indicate hepatic damage and inhibit the intercellular homeostasis [9-12].

Superoxide (O_2^-), hydroxyl radical (·OH), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2) are the common reactive oxygen species (ROS) were generated in the liver cell [13] when exposed with certain chemicals, environmental pollutants, xenobiotics etc. Firstly, these free radicals are generated from the oxidation procedure within the cell developed oxidative stress which destabilizes the normal cellular homeostasis [14,15]. Secondly, Chronic intake of alcohol generates ROS via cytochrome P450 2E1 in the liver cell producing DNA damage, loss of membrane integrity, amino acid oxidation and inactivation of specific enzymes through oxidation of their cofactors [16,17]. Important antioxidant enzymes like super oxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) mainly converts these harmful radicals through a series of biochemical reaction into oxygen and water molecule and protect the hepatic cell from the oxidative stress injury. Thus, excess alcohol consumption may accelerate an oxidative mechanism directly or indirectly disrupting cellular antioxidant defense system, which eventually produces cell death (apoptosis) and tissue damage. On the other hand nitric oxide (NO) is an important mediator of many physiological functions responsible for pathogenesis of many diseases. Chronic alcohol consumption increases nitric oxide (NO) levels which may lead to toxicity by peroxynitrite and destroy the membranous integrity. Excessive generation of reactive oxygen species/reactive nitrogen species (ROS/RNS) may occur when its production in the system exceeds the system's ability to neutralize and eliminate them [18].

From the ancient time people from various countries depends on traditional system of medicine for curing the diseases as a safe and symptomatic medications. In the modern world Indian medicinal

herbs and medicinal spices have been extensively used as an alternative medicine because of their promising medicinal property and lesser side effects in comparison to the allopathic drugs [19]. These medicinal herbs and spices are enriched with various constituents likes polyphenols, flavonoids, alkaloids, glycosides, tannins, proteins, amino acids, saphonin etc., which play a key role in cellular protection from the toxicants. Herbal formulations composed of some medicinally sound plants, now have garnered greater interest throughout the world due to its synergistic action. Recently people have a greater interest in herbal medicines because of their lesser side effect in clinical experience, pronounced effectiveness, safe for long term use and relatively low cost [20-22].

Currently throughout the globe fight against various liver dysfunctions such as fatty liver, liver fibrosis, liver cirrhosis etc. through safe and symptomatic medicine is a new challenge. Presently, there is no effective treatment for hepatic dysfunctions. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and hygiene. Our previous reports stated that the formulation have no adverse side effect and no toxicity in mice [23,24]. So, in the present study we formulated a new novel phytomedicine (AKSS16-LIV01) composed of six indigenous medicinal herbs and three medicinal spices those were mentioned in Ayurveda. Commercially available formulations composed of more than twelve medicinal herbs without medicinal spices. On the other hand in composition of the formulation the concentration of the individual herbs is less in comparison to marketed products. So, the developed formulation is an unique one and produced better therapeutic effects on animal models. Therefore, the present study was undertaken to evaluate the oxidative stress induction and tissues injury in liver of adult mice followed exposure to ethanol and its deleterious recovery by the application of unique novel multi herbal formulation (AKSS16-LIV01). The results of the present study are expected to provide a clear picture about the role of our newly formulated AKSS16-LIV01 in ethanol-induced hepatic damage, and may shed light on an achievable ethno-botany driven solution for serious liver problems.

4B.2 MATERIALS AND METHODS

4B.2.1 Chemicals

Trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethanol, TRIS buffers were purchased from SISCO laboratories, India. PBS buffer (pH 7.2) was taken from Sigma-Aldrich, Germany. All the biochemical kits (ALT, AST, GGT, ALP, Cholesterol, Triglyceride, Phospholipid, HDL, LDL etc.) were procured from Span Diagnostic, Surat, India. Hydroxyproline assay kit was procured from Bio Vision, Milpitas, CA. Antioxidant kits (SOD, CAT, GSH and GPx) were obtained from Boehringer, USA. ELISA kit TNF-α and TGF-β_1 were procured from Sigma Aldrich, USA. All others reagents used in this study are laboratory grade.

4B.2.2 Animals

Adult male swiss albino mice weighing 26 g ± 3g were obtained from our CPCSEA registered central animal house facility. The animals were divided into seven experimental groups with 10 animals in each group. For acclimatization mice were kept in the environment controlled animal room for one week before the onset of the experiment. The animals were maintained at 12 h light/dark cycle with

constant temperature (22±2°C) and humidity (54±4%). Standard pellet diets (Procure from Hind liver India Limited, Mumbai) were given to the animal with and water *ad libitum*. The whole experimental procedure were carried out according to the new revised guidelines (2018) of CPCSEA, Ministry of Agricultural and Animal Husbandry, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC), Jadavpur University having approval number (AEC/PHARM/1503/03/2019 dated 30.11.19).

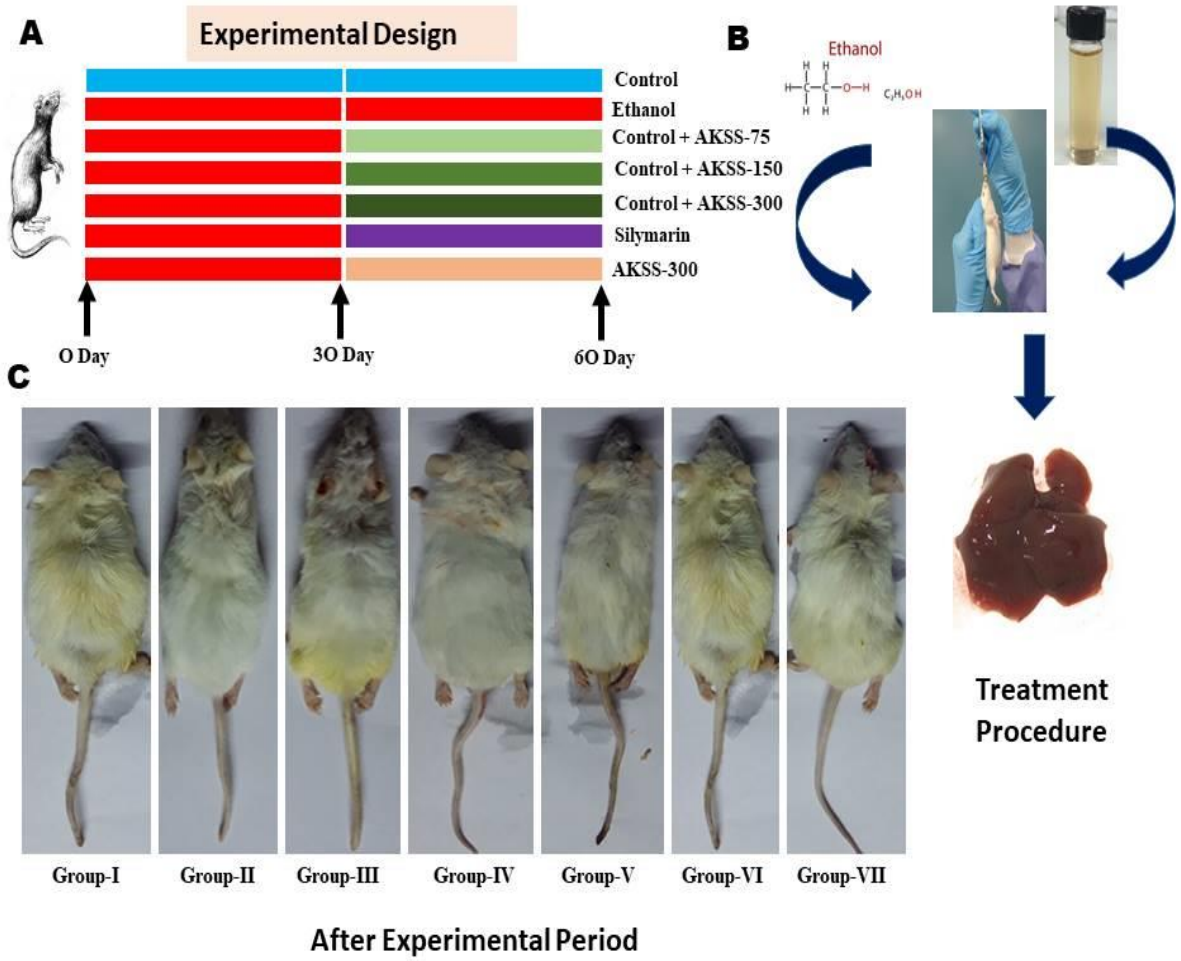


Figure 4B.1: In-vivo experiments upon experimental mice model. A. Experimental design, B. Experimental procedure, C. External views of mice after experimental period.

4B.2.3 Body weight gains and feed efficiency

Routine body weights of the each animal were measured and recorded in every week from the initial day to the final day of experiment and then determined the body weight alteration. Regular food consumption was calculated by measuring food residue on the basis food given at the fix time. Feed conversion was obtained by dividing total feed intake by body weight gain.

4B.2.4 Blood Collection

After the experimental period 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%) from the retro orbital plexus of the mice. Blood collected from animals were placed in slanting position (45°) at room temperature for 2.5 hrs. Then, the blood samples were centrifuged at 4000 g for 10 min. Serum was separated and used for further analyses.

4B.2.5 Hematological Parameters

Blood samples were taken from the retro orbital plexus of experimental mice and collected in heparinized tubes for determination of haematological parameters. Blood parameters were studied in this experiment i.e. haemoglobin (Hb), reticulocyte, hematocrit, total red blood cell, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, total white blood cell and differential count using Sysmax-K1000 automatic Cell Counter.

4B.2.6 Assessment of Liver Function Parameters

All biochemical tests were carried out by using commercial kits with little modification of manufactures instruction. Liver function test (LFT) parameters like aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), globulin, total and direct bilirubin along with total protein (TP) were analysed using biochemical assay kits (Span Diagnostic, Surat, India) following the protocol prescribed by manufacturers. Total protein concentration was determined in the serum by the method of Lowry et al.[28]

4B.2.7 Assessment of serum Lipid profile

Serum lipid profile like Cholesterol, Triglyceride, Phospgolipids, Free fatty acids, LDL-cholesterol and HDL-cholesterol were measured using enzymatic calorimetric kits (ELITech Diagnostic, France) according to manufacture instructions.

4B.2.8 Preparation of Tissue Homogenate

Prior to tissue biochemical analysis, 100 mg/mL of whole liver was homogenized in 50 mM phosphate buffer (pH 7.0). After homogenization the homogenate was centrifuged at 11000 rpm for 12 mins and the supernatant was collected and used for different parameters. Protein concentrations of liver supernatant were determined [28] using commercially available kit (Span Diagnostics Ltd, India) following procedure prescribed by manufacturer.

4B.2.9 Hydroxyproline assay

Hydroxyproline assay to quantify collagen content were performed by established protocol. In brief, 10 mg of liver tissue were excised and homogenized in 100 ml of sterile MQ water followed by hydrolysis in 12N HCl (100 ml) at 120uC. After 3.5 h, 5 ml of tissue lysate was transferred to a 96-well plate and incubated at 37°C for 18 h to evaporate the acid. Samples were incubated with equal

amounts of chloramine T and Ehrlich's reagents for 30 min at 65uC. Absorbance was recorded at 560 nm with an ELISA plate reader (Synergy BioTek, Winooski, VT).

4B.2.10 Assessment of Lipid Peroxidation, NO and iNOS

The level of malonyldialdehyde, as a substance that reacts with thiobarbituric acid (TBARS), was determined in homogenates of the organs and in serum according to the method of Ohkawa [29]. The level of NO and iNOS activity were determined from the liver homogenate through ELISA method using the commercial kit [30].

4B.2.11 Assessments of antioxidant enzymes

Tissue antioxidant enzymes activities were measured according to standard protocol with slight modification. These are as follows:

4B.2.11a Determination of Superoxide Dismutase (SOD)

The activity of superoxide dismutase (SOD) was measured according to a well-established reported method [31]. In brief, 2.5 mL reagent solution (xanthine 0.3 mM, EDTA 0.65mM, 140 µM NBT), sodium carbonate 0.4 M, and bovine albumin (35 mg/30 mL) was added to 0.1 mL sample and 50 µL xanthine oxidase (10 µL in 2 M ammonium sulphate), incubated at 25ºC for 20 min and mixed with,0.1 mL 8 M copper chloride. The developed color was measured at 560 nm and calculates the result with using co-factor.

4B.2.11b Determination Catalase (CAT)

Catalase activity was measured according to the method of Maehly (1955) [32]. Briefly, after addition of 5µL liver homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm was monitored for 1 min to determine catalase activity. The enzyme activity was expressed as U/mg protein.

Determination of Glutathione (GSH) and GPx Content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method [33]. GPx activity was assayed using a modified method of Rotruck et. al. [34]

4B.2.12 Determination TNF-α TGF 1β

Levels of TNF and TGF in the liver were determined using ELISA kits according to the manufacturer's guideline. Protein concentrations were measured according to the manufacturer's guideline.

4B.2.13 Hematoxylin and Eosin staining

Five micron paraffin-embedded liver sections were deparaffinised and washed with water. Hydrated tissue sections were incubated with Mayer's hematoxylin for 5 min. After that the hematoxylin stained tissue section vigorous wash in running tap water. Sections were counter stained with 1% eosin for 2

min. Eosin stained sections were washed with water and dehydrated with alcohol. Dehydrated sections were washed with xylene. Images were taken with a microscope (Olympus BX51 fluorescence microscope).

4B.2.14 Sirius red staining

After the experimental period portion of the liver slices were fixed in 10% neutral buffered formalin for overnight and then transferred to 70% ethanol before imbedding in paraffin blocks. Paraffin embedded liver tissues block were taken and cut into 5 mm thick sections. Deparaffinised sections were incubated for 60 min with Pico-Sirius red solution (Abcam, Cambridge, MA) followed by a brief rinse with acetic acid (0.05%). Sections were dehydrated by washing with absolute alcohol. Sections were observed with a light microscope (Olympus BX51 fluorescence microscope).

4B.2.15 Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

4B.3 RESULTS

4B.3.1 Determination of Body weight, Food consumption, liver weight and liver weight index

Figure 4B.2 showed that chronic administration of ethanol retarded the overall growth and development (Figure 4B.2C) of the mice in compared with control untreated animal. Novel herbal formulation (AKSS16-LIV01) recovered the normal growth. Interestingly ethanol intoxication decreased gross body weight (Fig. 3B), food consumption (Figure 4B.3C) as well as daily water intake. Therapeutic treatment with AKSS16-LIV01 (150 & 300 mg/kg) prevent the toxic effects of ethanol and maintained the normal increasing body weight pattern. Moreover significantly increased liver weight and liver index (Figure 4B.3A) by ethanol intoxication was normalized through natural therapy (Figure 4B.3).

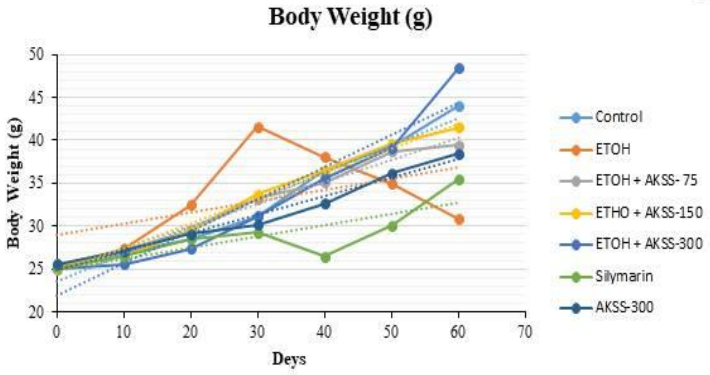
Study of Morphological Parameters

A

Table a: Summary of the body weight, liver weight and liver index of mice

Parameters	Normal	ETHO	ETHO + AKSS-16-75	ETHO + AKSS-16-150	ETHO + AKSS-16-300	Silymarin	AKSS-16-300
Final Body Weight (g)	36.52±1.18	44.94±1.22	34.81±2.15	35.01±1.34	48.01±2.21	38.26±2.17	40.62±1.26
Liver Weight (g)	1.94±0.82	3.50±0.28	2.25±0.47	1.98±0.18	1.86±0.64	2.03±0.64	1.99±0.52
Liver Index	4.02±0.12	7.72±0.15	5.62±0.16	5.65±0.17	4.95±0.17	5.22±0.18	5.51±0.24

B



C

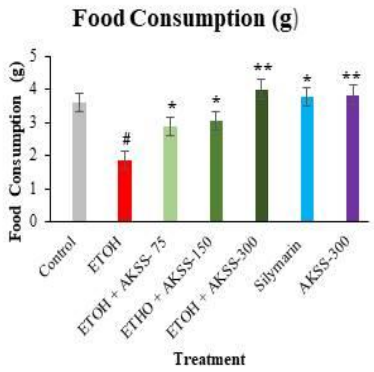


Figure 4B.2: Study of the morphological parameters. A. Table showed summary of the body weight, liver weight and liver index in mice, B. Day wise body weight, C. Mean food consumption. The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p<0.001$ and significantly different from ethanol $^{*}p<0.05$, $^{**}p<0.001$ using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.2 Determination of serum AST, ALT, ALP, GGT and total protein level

Result presented in Figure 4B.3 indicated that levels of serum enzymes such as AST, ALT, ALP and GGT were significantly elevated (P<0.001) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study serum enzymes like AST, ALT, ALP and GGT were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Moreover, Serum total protein level was significantly decreased (P<0.001) in ethanol treated mice compared with normal control mice. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly increased the serum total protein level at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12% better protective effect compared with standard drug

silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

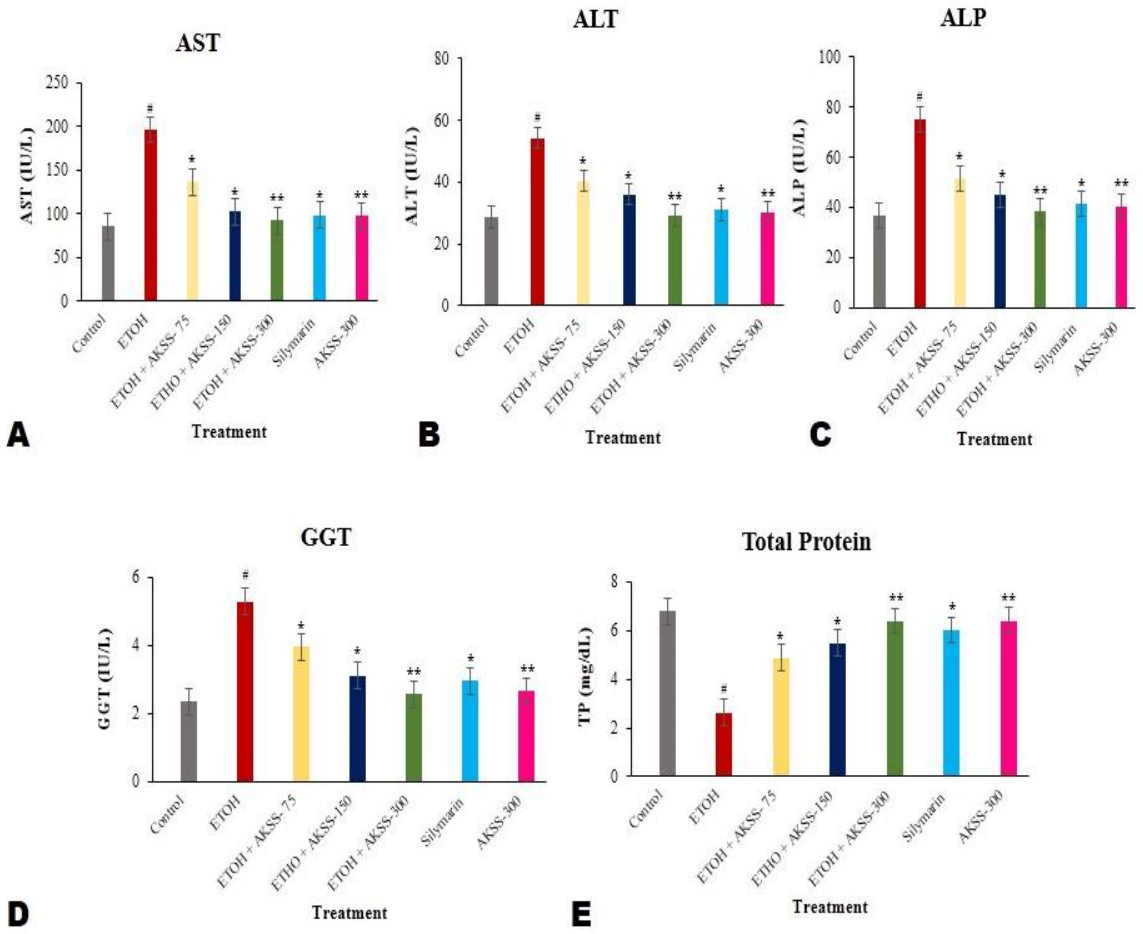


Figure 4B.3: Determination of biochemical parameters (Liver function test).The values are expressed as the mean ± SEM. Significantly difference $^{\#}P< 0.001$ compared to the untreated control group and $^{*}P< 0.001$, $^{**}P< 0.001$ significantly difference compared to the ethanol treated group. Group-I: Control; Group-II: Ethanol treated; Group-III: ASKK-75 mg/kg; Group-IV: AKSS-150 mg/kg; Group-V: AKSS-300 mg/kg; Group-VI: Silymarin – 100 mg/kg; Group-VII: AKSS-300.

4B.3.3 Determination of Haematological parameters

The hematopoietic system is one of the target organs of ethanol toxicity. The results concerning hematologic parameters depicted the Table 4B.1 showed a significant ($p<0.001$) decline in total erythrocyte count, total leukocyte count, hemoglobin concentration, Mean corpuscular haemoglobin concentration, neutrophil content and monocyte content in the ethanol treated animals. On the other hand, reticulocyte content content insignificantly increased in ethanol treated group, when compared

with control animals. Gradually all the above mentioned parameters recover in the dose dependent AKSS16-LIV01 treated groups as compared with standard drug silymarin.

Table 4B.1: Effect of AKSS16-LIV01 on haematological parameters in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol + AKSS16-LIV01 (150)	Ethanol + AKSS16-LIV01 (300)	Ethanol + Silymarin (100)	AKSS16-LIV01 (300)
Hb (g %)	12.1± 1.05	9.03± 0.89$^{\#}$	12.0±1.02*	11.05±0.99*	12.51±0.95**	10.96±0.74	11.21±0.82
RBC (x10^{6} cm^{2})	10.8±0.82	8.1±0.71$^{\#}$	10.5±0.77*	9.44±0.71	10.02±0.85*	9.85±0.79	9.62±0.84
RT (%)	2.7±0.12	4.9±0.26$^{\#}$	2.6±0.14*	3.1±0.14	2.8±0.15*	3.0±0.12*	3.6±0.16
HCT (%)	34.6±0.48	39.4±0.55$^{\#}$	34.1±0.44*	35.8±0.51	34.9±0.56*	34.4±0.51*	35.1±0.77
MCV (µm^{3})	37.8±0.32	31.0±0.68	36.7±0.29*	36.5±0.44	35.9±0.79	36.2±0.43*	35.5±0.36
MCH (pg)	21.2±0.15	22.2±0.14$^{\#}$	22.8±0.23*	21.1±0.12*	21.4±0.11*	21.2±0.14	21.1±0.12
MCHC (%)	41.2±1.06	32.4±0.95$^{\#}$	40.2±1.07	37.1±0.92	39.6±0.87*	38.6±0.99	36.2±0.91
Platelets	6.5±0.02	5.5±0.03	6.5±0.04	5.8±0.05	6.1±0.07	5.5±0.05	5.4±0.06
WBC (x10^{5} cm^{2}))	9.2±0.09	12.4±0.11$^{\#}$	9.1±0.08	10.8±0.12	9.2±0.11**	10.1±0.13	10.7±0.11
Lymphocyte	74±2.98	79±3.04$^{\#}$	72±2.54*	73±3.06*	74±2.58*	72±3.08*	71±3.11
Neutrophil	26±1.12	15±0.49$^{\#}$	24±1.09*	20±0.56*	25±0.69**	24±0.51*	21.52±2.09

Data are expressed as mean ± standard deviation (N=6). Hb: Haemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White Blood corpuscle

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p<0.001$ and significantly different from ethanol $^{*}p<0.05$, $^{**}p<0.001$ using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.4 Determination of serum BUN, total bilirubin, direct bilirubin, albumin level and albumin-globulin ratio

Result presented in Table 4B.2 indicated that levels of serum biochemical hepatotoxic marker such as blood urea nitrogen (BUN), total bilirubin and direct bilirubin were significantly elevated (P<0.001) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study serum enzymes like BUN, total bilirubin and direct bilirubin were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Moreover, Serum total albumin level and albumin-globulin ration was significantly decreased (P<0.001) in ethanol treated mice compared with normal control mice. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly increased the serum total albumin level and albumin-globulin ratio at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 10% better protective effect compared with standard

drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Table 4B.2: Effect of AKSS16-LIV01 on liver function test parameters across the groups in chronic ethanol-induced hepatic damage in mice

Parameters	**Normal**	**Ethanol**	**Ethanol + AKSS16-LIV01 (75)**	**Ethanol + AKSS16-LIV01 (150)**	**Ethanol + AKSS16-LIV01 (300)**	**Ethanol + Silymarin (100)**	**AKSS16-LIV01 (300)**
BUN(mg/dl)	0.41±0.02	0.72±0.04$^{\#}$	0.58±0.04*	0.67±0.02*	0.46±0.02**	0.54±0.03*	0.46±0.03*
Total Bilirubin(mg/dl)	0.12 ± 0.2	0.62 ± 0.11$^{\#}$	0.22±0.09*	0.34±0.08*	0.16±0.09**	0.24±0.08*	0.19±0.11*
Direct Bilirubin (mg/dl)	0.06±0.001	0.33 ± 0.07$^{\#}$	0.19±0.002*	0.09±0.003*	0.09±0.002**	0.11±0.005*	0.07±0.002*
Alb (gr/dL)	3.48±0.186	1.97±0.036$^{\#}$	2.85±0.12*	3.70±0.11*	4.02±0.14**	3.32±0.15*	3.16±0.13*
Alb/globulin	1.18±0.141	0.54±0.013$^{\#}$	0.81±0.091*	0.91±0.096*	1.21±0.95**	1.04±0.091*	0.98±0.135*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p<0.001$ and significantly different from ethanol $^{*}p<0.05$, $^{**}p<0.001$ using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.5 Determination of liver Non enzymatic Markers

The total cholesterol, triglycerides, phospholipids, free fatty acids, LDL level of untreated ethanol control group was significantly higher (P<0.001) and HDL level significantly lower (P<0.001) than the control groups presented in Table 4B.3. In contrast, the levels of cholesterol, triglycerides, phospholipids free fatty acids and LDL of the 75 mg/kg and 150 mg/kg body weight of multi herbal formulation (AKSS16-LIV01) with ethanol groups were significantly lower (P<0.05) and HDL level significantly higher (P<0.05) than the ethanol control group. Administration of multi herbal formulation (AKSS16-LIV01) at a dose of 300mg/kg body, significantly normalise (P<0.001) the deleterious effect (Table 4B.6) caused by ethanol. Multi herbal formulation (AKSS16-LIV01) showed 15% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Table 4B.3: Effect of AKSS16-LIV01 on concentrations of nonenzymatic biochemical parameters in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol + AKSS16-LIV01 (150)	Ethanol + AKSS16-LIV01 (300)	Ethanol + Silymarin (100)	AKSS16-LIV01 (300)
Cholesterol (mg/dL)	80.17 ± 5402	138.99 ± 6.23$^{\#}$	91.25±2.99*	91.47 ± 2.37*	79.36 ± 4.35**	856.11±2.98*	88.05±3.16*
Triglyceride(mg/dL)	42.55 ± 3.56	75.66 ± 4.28$^{\#}$	68.61±3.24*	47.98 ± 1.97*	37.25 ± 1.87**	44.75±3.25*	51.23±3.01*
Phospgolipids (mg/dL)	78.48 ± 5.29	141.51 ± 5.14$^{\#}$	91.72±2.66*	98.51 ± 3.28*	82.69 ± 4.87**	88.03±2.84*	92.37±2.66*
Free fatty acids (mg/dL)	14.07 ± 0.79	34.87 ± 1.87$^{\#}$	24.33±1.91*	21.22 ± 1.69*	14.09 ± 1.22**	19.27±2.01*	20.14±1.96*
LDL-cholesterol (mg/dL)	38.11 ± 1.91	76.94 ± 1.77$^{\#}$	44.61±2.88*	45.78±1.65*	36.85 ± 1.25**	42.52 ± 2.28*	43.61±1.88*
HDL-cholesterol (mg/dL)	19.52 ± 0.88	9.21 ± 0.68$^{\#}$	16.25±0.62*	16.24±0.41*	10.28 ± 0.28**	21.57 ± 0.99*	15.28±0.55*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p<0.001$ and significantly different from ethanol $^{*}p<0.05$, $^{**}p<0.001$ using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.6 Determination of liver hydroxyproline level

Table 4B.4 showed the hydroxyproline level of control and experimental group. In our study, long term ethanol intoxication produced deleterious effects which was clearly indicated when we found that hydroxyprolein level significantly elevated (P<0.001) in experimental mice with compared with normal untreated group. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly reduce at a dose of 75 mg/kg (P<0.05), 150 mg/kg (P<0.05) and 300 mg/kg (P<0.001) respectively compared with ethanol treated mice (Group-II). Multi herbal formulation (AKSS16-LIV01) showed 15% better protective effect compared with standard drug silymarin (Group-VI). Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone (Group-VII) showed no such toxic effect in mice compared with control mice.

4B.3.7 Determination of NO and iNOS

In this study result showed that in Table 4B.4 ethanol intoxication significantly elevated (P<0.001) NO content and iNOS level in mice as compared with normal untreated group. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly reduce at a dose of 75 mg/kg (P<0.05), 150 mg/kg (P<0.05) and 300 mg/kg (P<0.001) respectively compared with ethanol treated mice (Group-II). Multi herbal formulation (AKSS16-LIV01) showed 12% better protective effect compared with standard drug silymarin (Group-VI). Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone (Group-VII) showed no such toxic effect in mice compared with control mice.

4B.3.8 Determination of MDA and ROS level

Result presented in Table 4B.4 and Figure 4B.4 indicated that levels of lipid peroxidation (MDA level) and the tissue ROS levels. ROS content were significantly elevated (P<0.001) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study MDA and ROS content were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12-13% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Table 4B.4: Effect of AKSS16-LIV01 on liver Lipid peroxidation, NO, iNOS levels and Hydroxyproline concentration in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol + AKSS16-LIV01 (150)	Ethanol + AKSS16-LIV01 (300)	Ethanol + Silymarin (100)	AKSS16-LIV01 (300)
Lipid Peroxidation (nm/100g tissue)	68.16±3.52	182.16±3.09	92.35±2.14*	77.92±1.47*	62.58±2.52**	82.57±1.67*	66.57±0.99*
NO(µmol/mg protein)	0.64±0.004	2.29±0.002#	102.77±0.005*	0.84±0.004*	0.69±0.006**	0.88±0.007*	0.65±0.002*
iNOS (U/mg protein)	0.32±0.003	1.11±0.002#	0.62±0.005*	0.46±0.006*	0.38±0.003**	0.45±0.005*	0.39±0.003*
Hydroxyproline (mq/g)	0.42±0.004	0.99±0.004#	0.76±0.006*	0.55±0.003*	0.47±0.002**	0.56±0.004*	0.44±0.005*

The values are expressed as the mean ± SEM. Significantly different from control #p<0.001 and significantly different from ethanol *p<0.05, **p<0.001 using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.9 Determination of SOD, CAT, GSH and GPx level

Result presented in Figure 4B.4 indicated that levels of different antioxidant enzymes such as SOD, CAT, GSH and GPx were significantly reduce (P<0.001) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study the above antioxidant enzymes i.e. SOD, CAT, GSH and GPx were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75mg/kg (P<0.05), 150mg/kg (P<0.05) and 300mg/kg (P<0.001) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12-13% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

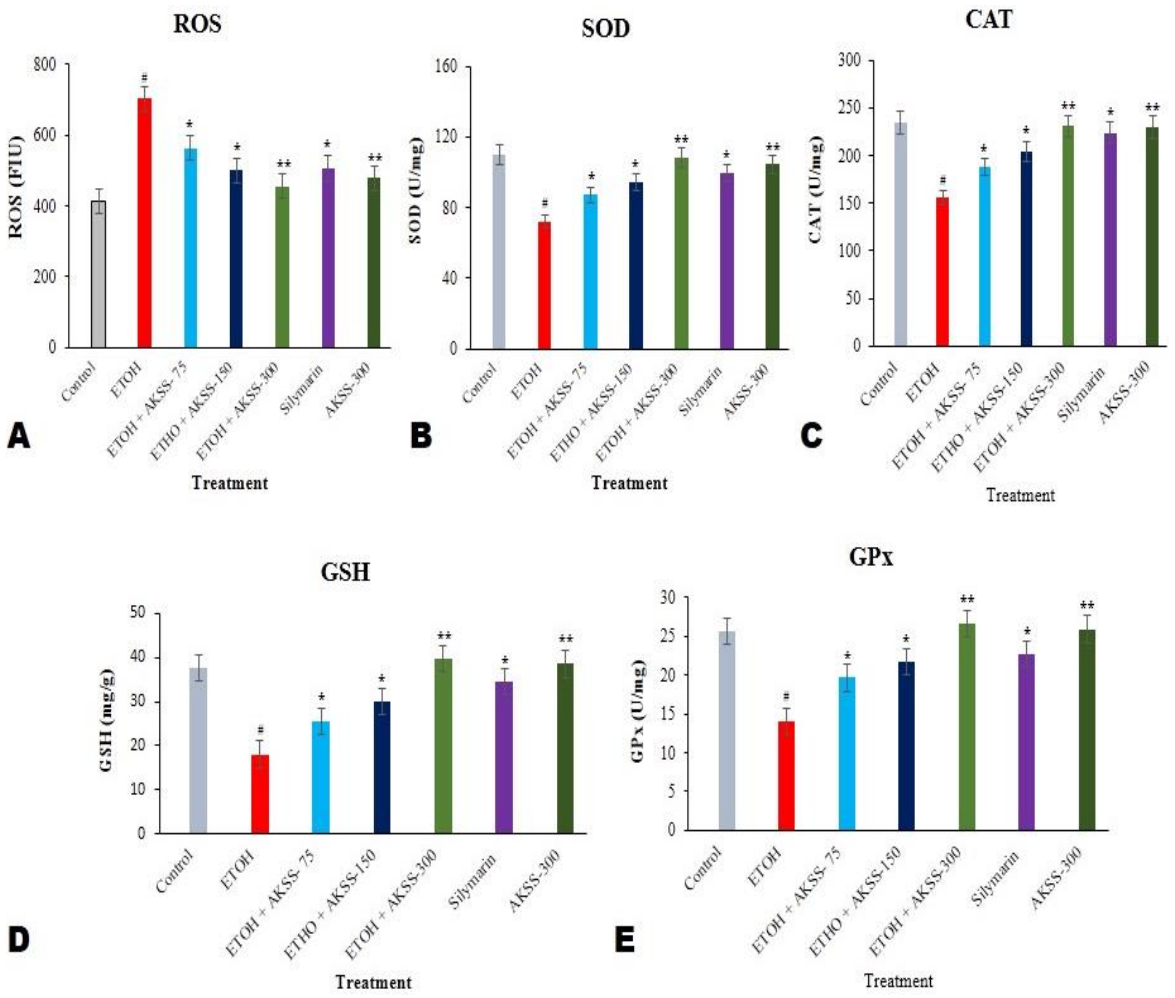


Figure 4B.4: Determination of the total reactive oxygen species (ROS) contents and levels of various antioxidant enzymes. The values are expressed as the mean ± SEM. Significantly difference $^{\#}P< 0.001$ compared to the untreated control group and $^{*}P< 0.001$, $^{**}P< 0.001$ significantly difference compared to the ethanol treated group. Group-I: Control; Group-II: Ethanol treated; Group-III: ASKK-75 mg/kg; Group-IV: AKSS-150 mg/kg; Group-V: AKSS-300 mg/kg; Group-VI: Silymarin – 100 mg/kg; Group-VII: AKSS-300.

4B.3.10 Determination of inflammatory cytokines TNF α and TGF 1β

Inflammation is commonly associated with liver dysfunction and fibrosis during chronic liver injury. The values of proinflammatory cytokines like TNF-α were determined from the hepatic tissue sample. Table 4B.5 shows significant increase ($p<0.001$) of TNF-α in ethanol treated mice when compared to control untreated animals. The elevated level of TNF-α was significantly inhibited ($p<0.05$, $p<0.001$) by the application of multi herbal formulation (AKSS16-LIV01) in dose dependent manner 150 and 300 mg/kg). This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at a dose of 300 mg/kg showed optimum protective potential against

ethanol induce liver intoxication. TGF 1β is the major profibrogenic cytokine. As shown in Table 4B.5 significantly increased level of TGF 1β was observed in ethanol treated mice when compared to control untreated animals. On the other hand, treatment with AKSS16-LIV01 both 150 mg/kg and 300 mg/kg significantly decreased ($p<0.05$, $p<0.001$) the level of TGF 1β when compared with ethanol treated animals. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at a dose of 300 mg/kg alone showed optimum protective potential against ethanol induce liver dysfunction.

Table 4B.5: Effect of AKSS16-LIV01 on TNF α and TGF 1β levels in liver tissue in chronic ethanol-induced hepatic damage in mice

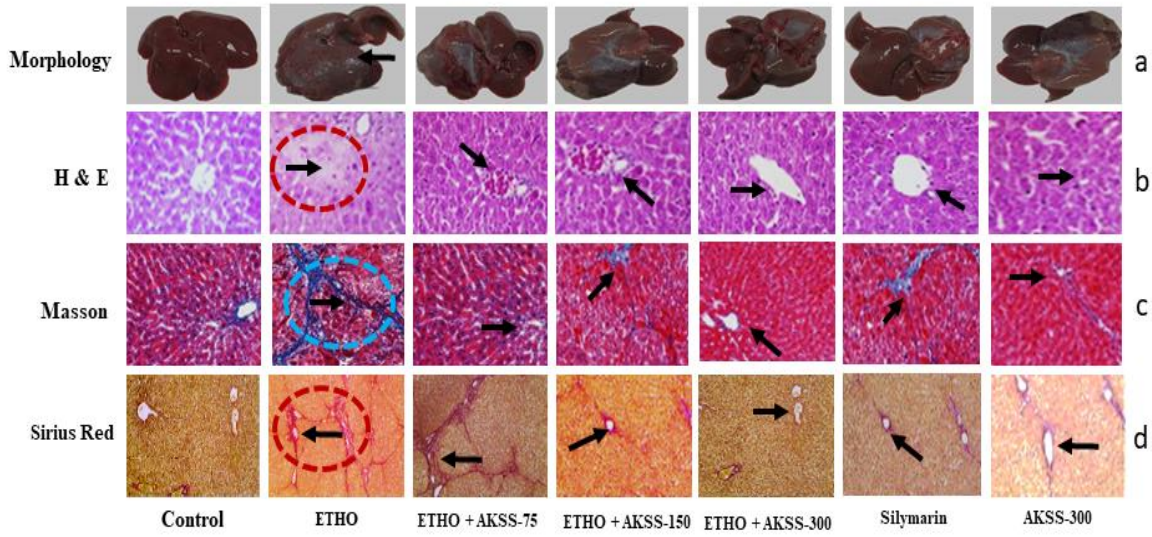
Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol + AKSS16-LIV01 (150)	Ethanol + AKSS16-LIV01 (300)	Ethanol + Silymarin (100)	AKSS16-LIV01 (300)
TNF-α (pg/mg protein)	1411.91±206.53	2344.77±371.98$^{\#}$	1636.77±220.97*	1572.25±236.93*	1437.11±285.83**	1478.26±288.17*	1422.87±198.54*
TGF-1β (pg/mg protein)	485.90±91.34	1237.05±125.78$^{\#}$	702.38±98.77*	598.11±212.45*	474.03±102.76**	552.03±203.84*	492.11±88.16*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p<0.001$ and significantly different from ethanol $^{*}p<0.05$, $^{**}p<0.001$ using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

4B.3.11 Histopathological examination

Histologic examination Figure 4B.6 shows normal morphological architecture under light microscope of H&E in the control group. In the ethanol treated intoxicated groups (50:50 v/v) necrosis, hyperemia, vacuolar degeneration and infiltration of the inflammatory cells were observed which indicated hepatocellular damage (Figure 4B.5). In this regards, administration of multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg showed less damages compared with ethanol intoxicated mice (Figure 4B.1). Mild necrosis vacuolar degeneration and infiltration of the inflammatory cells were observed in the group 4 (150 mg/kg). In the group 5 (300 mg/kg) only mild vacuolar degeneration and infiltration of the inflammatory cells were seen which may indicate that the treatment of multi herbal formulation (AKSS16-LIV01) was very effective (Figure 4B.5). Histopathologic parameters of the liver tissues were graded in Figure 4B.6 (Table 4B.b). Masson trichrom and serius red photographs showed that liver's normal architectures completely massed by ethanol and deleterious effect was completely restored by AKSS16-LIV01 which greatly supported the above results.

Study of Histopathology



e Table: Lesion Scores of Different Liver Alterations among all Experimental Groups

Histopathological lesions	Normal	ETHO	ETHO + AKSS-75	ETHO + AKSS-150	ETHO + AKSS-300	Silymarin	AKSS-300
Fibroblast proliferation in the capsule	–	+++	++	–	–	–	–
Fibroblast proliferation in the portal triad	–	+++	++	+	–	+	–
Focal hepatic necrosis	–	++	+	+	–	–	–
Steatosis of hepatocytes	–	+++	+	–	–	–	–
Cytoplasmic vacuolization of hepatocytes	–	++	–	–	–	–	–
Kupffer cell activation	–	+++	–	–	–	+	–

Figure 4B.5: Representative photomicrographs of livers in different experimental groups. a. liver morphology b. H & E stained liver sections c. Masson trichrom stained liver sections d. Sirius red stained liver sections. e. Table showed histological scoring of the liver section in different groups.

4B.4 DISCUSSION

People all over the globes widely consume alcoholic drinks and consequently suffer from various diseases such as liver cirrhosis, liver fibrosis, fatty liver and, hepatic cell carcinoma (liver cancer). The condition produces hepatic dysfunctions that alter the body's normal homeostasis [35-37]. From ancient times, Indian medicinal herbs and spices are very useful for liver complications by boosting the antioxidant system and make a balance between antioxidants and prooxidants. These herbs and spices are enriched with polyphenols, flavonoids, tannins, proteins, amino acids, saponin, etc. which play a key role in liver protection against various toxicants. It also increases the body's antioxidant ability which further increases the immune power for fighting against disease. In the present study, we

developed a multi herbal formulation (MHF) composed of six Indian medicinal herbs and three Indian medicinal spices (AKSS16-LIV01) have cumulative actions in comparison with the single herbal extract. We try to establish its antioxidant and immune-suppressive effect against ethanol intoxication and find out a new safe and symptomatic medication in liver dysfunction.

The preclinical and clinical studies have already established that ethanol is a potent hepatotoxicant which produces severe liver complications [38]. Liver damage by ethanol is closely associated with the generation of reactive oxygen species (ROS) such as peroxide, singlet oxygen, superoxide anion, hydroxyl ions which elevates MDA (malondialdehyde), NO (nitric oxide) and iNOS (nitric oxide synthase), suppress the cellular integrity. Ethanol intoxication showed the function of the various antioxidant enzymes gets severely reduced causing cell apoptosis [39.40]. The elevation and reduction of enzymatic and non-enzymatic markers of serum were also associated with this condition. Alcoholic liver disease (ALD) was normally found in liver histology, which disrupts the normal liver architecture and reduces regular functions. Our developed formulation AKSS16-LIV01 was enriched with antioxidants that could revert and lower the free radicals level. It had shown that the beneficial effects of this phytomedicine in preventing the ethanol-induced hepatotoxicity caused by free radicals.

In the present study, we evaluated the ameliorative effects of AKSS16-LIV01 against ethanol-induced hepatotoxicity in the animal model. Dose-dependent administration of ethanol increased the gross liver weight and liver-body weight ratio and decreased the cumulative body weight, which caused the changes in the behavior of mice. Administration of AKSS16-LIV01 with three specific doses gradually normalized the changes. Ethanol intoxication elevated the concentrations of key cellular enzymes like AST, ALT, ALP, and GGT present in the liver cells that leak into the serum during liver damage [41-45]. This happens because of a higher concentration of alcohol dehydrogenase in the liver, which catalyzes alcohol to its corresponding aldehyde [46]. Therefore, administration of AKSS16-LIV01 at a dose of 150mg and 300mg body weight could help to normalize the AST, ALT, ALP, and GGT enzyme levels. So, the developed multi herbal formulation AKSS16-LIV01 could reduce the level of these enzyme markers.

These results are also consistent with the protective effects of the developed formulation correlating with its antioxidant ability against alcohol-induced hepatocyte cells of the liver [45, 47– 49]. Our results also showed that administration of ethanol increased the serum essential enzymes blood urea nitrogen (BUN), total and direct bilirubin which was reverted with the treatment of AKSS16-LIV01. On the other hand, it was found that ethanol toxicity reduces the body's different protein concentration and breaks the normal homeostasis. Our results showed that reduced total protein, albumin, and albumin globulin ratio were normalized by AKSS16-LIV01.

Ethanol administration increased serum total cholesterol (TC), total triglyceride (TC), phospholipids, LDL, VLDL, and HDL levels of non-enzymatic markers which caused liver damage. This damage is attributed to the higher concentration of alcohol dehydrogenase enzyme which catalyzes alcohol to aldehyde and accumulation of export type proteins due to inhibition of the secretion of the proteins from the liver of alcoholics [46,50]. Both doses of AKSS16-LIV01 restored the higher level of lipid profile parameters in a dose-dependent manner to normal levels [51-53].

It is reported that in cirrhotic mice caused by ethanol produced significantly higher reactive oxygen species (ROS) and malondialdehyde (MDA) as compared to the control animals [54,55]. In this study our result also supported the above reports. On the other hand the levels of MDA and ROS content were markedly low in the AKSS16-LIV01 treated group (Table4). It is predicted that polyphenol, flavonoids, and other essential constituents rich multi herbal formulation (MHF) inhibit lipid peroxidation and reactive oxygen level in experimental mice [56]. During this study, the antioxidant system of liver fibrotic mice was extensively impaired, causing a high level of MDA and ROS. However, in this study higher levels of liver nitrite/nitrate indicating significantly increased production of hepatocellular NO content in response to chronic alcohol administration via induction of inducible nitric oxide synthase (iNOS) [57, 58]. NO, and its metabolite i.e. peroxynitrite ($ONOO^-$) cross cell membranes through anion channels, which produces nitration of tyrosine and inactivation of biologically important proteins and enzymes [59]. AKSS16-LIV01 administration to ethanolic mice decreased the levels of nitrite/nitrate significantly compared to the ethanolic group. We observed decreased levels of nitrites/nitrates in alcoholic mice receiving AKSS16-LIV01, which might be mainly due to inhibition of the hepatic cytosolic iNOS enzyme activity by the newly developed multi herbal formulation [60].

Oxidative stress (OS) induced by ethanol intoxication causes liver ailment in alcoholism [61,62] and produced various health complications. Indian traditional healthcare system is extensively used for a long time to cure liver dysfunctions and augment body's immune system. Free radical scavenging enzymes such as SOD, CAT, GPx, and GSH are the body's first line of defense against oxidative injury. SOD scavenges superoxide ions and catalase converts H_2O_2 to water. In this study decreased activities of CAT, SOD, and GPx as well GSH in alcohol-treated mice were observed. The decreased SOD activity could be due to the oxidative inactivation of the enzyme. This condition generates massive reactive oxygen species (ROS) and break cellular homeostasis [63]. GSH is a major non-protein thiol and plays a provital role in coordinating the antioxidant defense process. It is involved in the maintenance of normal cell structure & functions through its detoxification process [64]. GSH in association with GPx metabolizes hydrogen peroxide to water, thereby protecting mammalian cells against oxidative damage. Administration of our developed multi herbal formulation against ethanol intoxicated mice normalized these essential antioxidant enzymes concentrations in the liver tissue. The formulation thus protects the liver against ethanol-induced redox healing.

TNF-α is a key factor that initiates a cascade of immune responses involving the induction of cytokines after liver damage. In the damaged liver, predominantly Kuffer cells and infiltrating macrophages and neutrophils will produce TNF-α. TNF-α plays a dichotomous role in the liver, where it not only induces hepatocyte proliferation, apoptosis, and inflammation but also is known to suppress collagen α1 gene expression. Our results showed that the administration of multi herbal formulation (AKSS16-LIV01) with ethanol reduced the level of TNF-α in the liver as compared to ethanol administration alone. This result suggested that AKSS16-LIV01 is capable to suppress TNF-α production against ethanol-induced liver damage.

Hepatic stellate cells (HSC) activation was triggered by TGF-β1, which was released from Kuffer cells as well as oxidative stress caused by ethanol. TGF-β1 regulates the production, degradation, and accumulation of the extracellular matrix (ECM) in liver fibrosis. TGF-β1 leads fibrogenesis through the autocrine and paracrine effects of HSC. Our results showed that the administration of AKSS16-

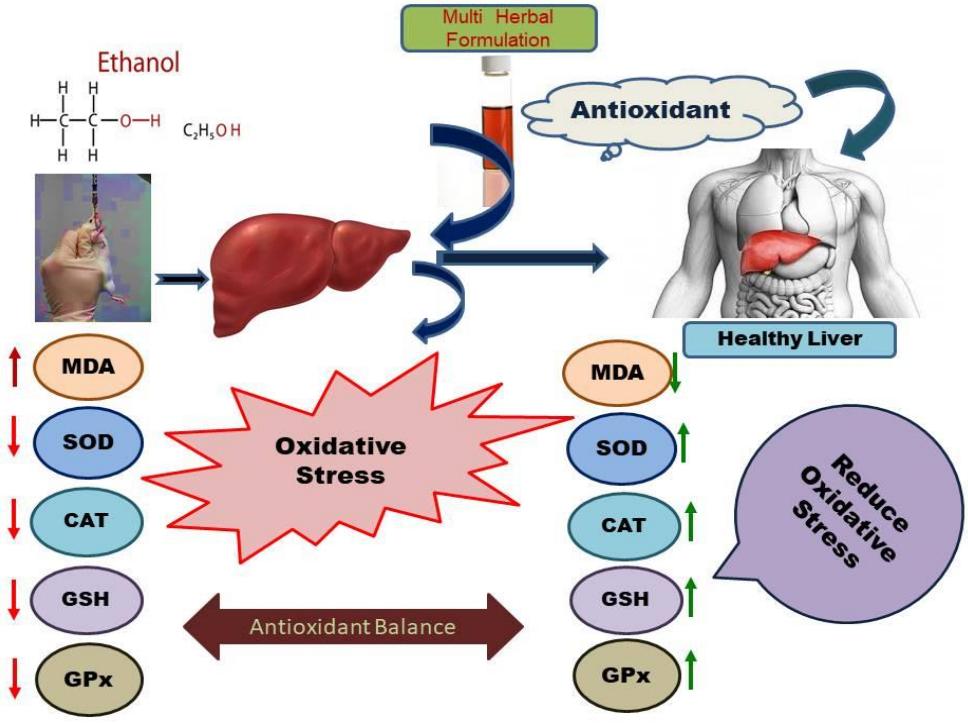
LIV01 with ethanol reduced the level of TGF-β1 in liver tissue as compared with ethanol administration alone. This suggested that the ameliorative effect of AKSS16-LIV01 on ethanol-induced hepatotoxicity was associated with their abilities to inhibit HSC activation by reducing TGF-β1 production.

Histopathological examination was an indication of hepatic damages after administration of ethanol. Chronic administration of ethanol developed cellular necrosis, vacuolar degeneration, hyperemia and infiltration of the inflammatory and lymphocyte cells. The histopathology of the liver confirmed the protective effect of multi herbal formulation (AKSS16-LIV01). Application of the newly developed unique formulation restored the hepatic damage in mice and inhibits the damaging effect of ethanol. The efficacy was shown by the reduction of livers' damages such as necrosis, hyperemia, vacuolar degeneration, and infiltration of the inflammatory cells; the effects were especially evident at the dose of 300 mg/kg. All the above study was compared with standard drug silymarin and it is showed that AKSS16-LIV01 has more potent hepatoprotective effect instead of standard drug

CONCLUSION

In conclusion, our study revealed that multi herbal formulation (AKSS16-LIV01) has shown protective action against alcohol-induced hepatic injury by ameliorating oxidative stress in mice as evidenced by liver histopathological studies, various hepatic enzymes, lowered lipid peroxidation, NO levels, and elevated antioxidant status. Apart from this AKSS16-LIV01 can suppress pro-inflammatory cytokines TNF-α and inhibit HSC activation by reducing profibrogenic cytokines TGF-β1. The presence of various constituents such as tannins, polyphenols, and flavonoids in AKSS16-LIV01 could be contributed to the above mechanism.

SUMMARY



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Chapter - V

Sanative Effect of a Novel Multi Herbal Formulation (AKSS16-LIV01) against Hematological Dysfunctions

5A Ameliorative efficacy of a novel multi herbal formulation (AKSS16-LIV01) upon Haematological modulations induced by fixed dose combination of tramadol hydrochloride/paracetamol (THP)

5A.1 INTRODUCTION

Tramadol hydrochloride/paracetamol is a find dose combination used to treat moderate to moderately severe pain. This fixed dose combination (FDC) contains 37.5 mg of tramadol hydrochloride and 325 mg of paracetamol [1]. immediate release (IR) formulation orally relief pain within an hour. Tramadol has a central acting mechanism via serotonin receptors and acts by binding μ-opioid receptors and neurons, and it is also a serotonin–norepinephrine reuptake inhibitor (SNRI) [2]. Over dose and chronic consumption of this combination produce constipation, itchiness and nausea [3]. Some times more serious adverse effects like insomnia, drug dependency and a high risk of serotonin syndrome may occur [4].

Intake of low dose of tramadol hydrochloride/paracetamol can acts as an effective analgesic but at high dosage and over a prolonged period the combination may cause various complications and disrupt body's homeostasis [5, 6]. Recent study showed that application of tramadol hydrochloride/paracetamol (THP) alters normal value of the various haematological parameters in animals [7]. Apart from this prolonged or chronic administration of THP may cause severe thrombocytopenia, leading to failure of the immune system, anemia and a very low erythrocyte count [8, 9].

Multi herbal formulations mean a dosage form consisting of one or more herbs or processed herbs in specified quantities which have potent therapeutic efficacy without adverse effects [10, 11]. Scientific study revealed that this plant based formulation is very effective to cure anaemia and control the blood [12]. Here we developed a multi herbal formulation (AKSS16-LIV01) based on six Indian medicinal plants and three Indian spices. Our previous study established that the formulation is completely safe in various does upon experimental animals [13]. With view of the above, there is need to developed and safe and symptomatic medication that controls all haematological parameters in the body when system exposed with fixed dose combination.

5A.2 MATERIALS AND METHODS

5A.2.1 Chemicals

Tramadol hydrochloride and paracetamol were obtained from Dey's Medical Stores (Mfg.) Ltd., Kolkata as a gift sample. Ethanol, sodium chloride, sodium hydroxide and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. ALT and AST were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

5A.2.2 Preparation of plant extract

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. Plants parts were cleaned and dry with normal temperature. The dried plant parts were used for preparation of multi herbal formulation as per standard validated protocol [14]. The plants and plant parts used in preparation of the extract are listed in Table 1.

5A.2.3 Animals

Twenty four young, healthy swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

5A.2.4 Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received Multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received Tramadol hydrochloride/paracetamol (THP) daily at dosage of 1.68 g/300 ml of water and Group-IV received THP (1.68 g/300 ml of water) along with AKSS16-LIV01 (400 mg/kg).

5A.2.5 Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

5A.2.6 Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

5A.2.7 Determination of biochemical parameters

Liver function enzymes such as AST and ALT were used as biochemical markers for hepatotoxicity and assayed by the standard.

5A.2.8 Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

5A.3 RESULTS

5A.3.1 Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in Table 5A.1. Administration of Tramadol hydrochloride/paracetamol (THP) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity as compared with Tramadol hydrochloride/paracetamol (THP) treated animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Table 5A.1: Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	Groups			
	Control	**AKSS16-LIV01**	**THP**	**THP + AKSS16-LIV01**
Body weight (g) Initial	25.16±2.57	25.84±2.44	26.01±5.21	25.11±4.85
Body weight (g) Final	36.98±2.55	36.91±2.69	21.22±2.61$^{\#}$	36.67±1.47*
Body weight (g) gain or loss	11.82±0.06	11.07±0.04	4.79±0.006	11.56±0.03
Food consumption (g)	4.81±0.05	4.39±0.07	2.97±0.05$^{\#}$	5.18±0.04*
Water intake (ml)	4.18±0.04	4.27±0.04	2.98±0.02$^{\#}$	4.55±0.04*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (THP) group values at $p<0.001$

5A.3.2 Effect of multi herbal formulation (AKSS16-LIV01) on Haematological parameters

Haematological parameters of control and experimental groups are shown in Figure 5A.1 – 5 and Table 5A.2. Four weeks treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day did not showed significant differences in PCV, haemoglobin (Hb), WBC, RBC, mean corpuscular haemoglobin concentration (MCHC), mean cell volume (MCV), and mean cell hemoglobin (MCH) compared with the control. Significant reduction in Hb ($p<0.001$), PCV

($p<0.001$), MCV ($p = 0.001$), and MCH ($p = 0.001$) was noticed in THP intoxicated mice when compared with the control. The WBC count was significantly ($p = 0.001$) greater in Group C compared with the control. In contrast, no significant differences were observed in RBC and MCHC between the control and Group C. Administration of multi herbal formulation (AKSS16-LIV01) along with THP significantly increased Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p = 0.001$), and MCH ($p = 0.001$) when compared with the THP intoxicated animals. On the other hand WBC count was significantly reduced in Group D THP intoxicated animals. Others haematological parameters (Table 5A.2) like Read Blood corpuscle (RBC); Reticulocyte (RT); Haematocrit (HCT); Mean corpuscular haemoglobin concentration (MCHC) did not show any significant changes upon all the experimental groups.

Table 5A.2: Effect of multi herbal formulation (AKSS16-LIV01) on haematological Parameters

Parameters	**Groups**			
	Control	**AKSS16-LIV01**	**THP**	**THP + AKSS16-LIV01**
RBC ($x10^6$ μL^{-1})	10.8±4.1	10.2±5.3	10.1±4.2	10.6±5.1
RT (%)	2.8±1.1	2.4±1.6	2.8±2.4	2.9±1.6
HCT (%)	34.8±1.3	32.8±2.1	32.8±2.1	35.1±3.1
MCHC (%)	41.4±7.6	41.7±2.4	40.4±1.4	41.4±1.4
Platelets ($x10^3$ μL^{-1})	6.6±2.0	6.9±1.2	6.3±1.2	6.5±2.6

Data are expressed as mean ± standard deviation (N=6)
RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCHC: Mean corpuscular haemoglobin concentration;

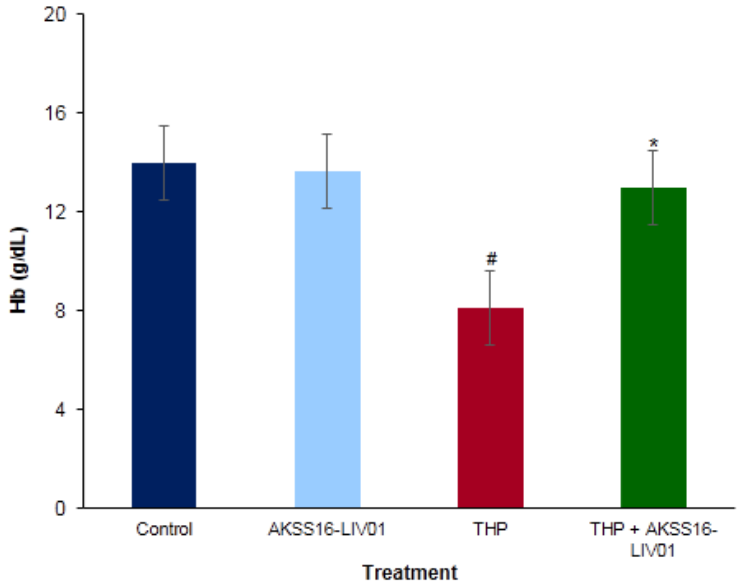

Figure 5A.1. Effect of multi herbal formulation (AKSS16-LIV01) on haemoglobin (Hb) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *Significantly different from (THP) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

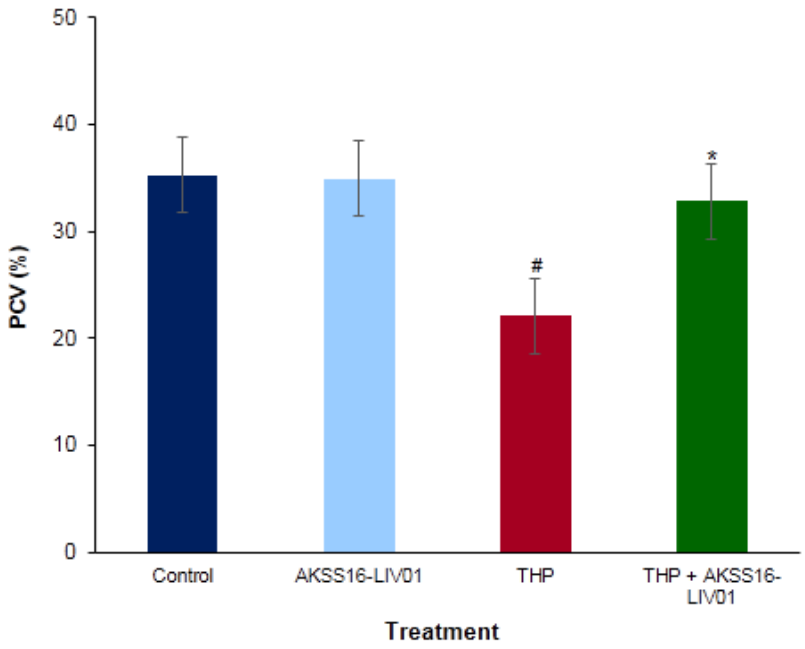

Figure 5A.2. Effect of multi herbal formulation (AKSS16-LIV01) on packed cell volume (PCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

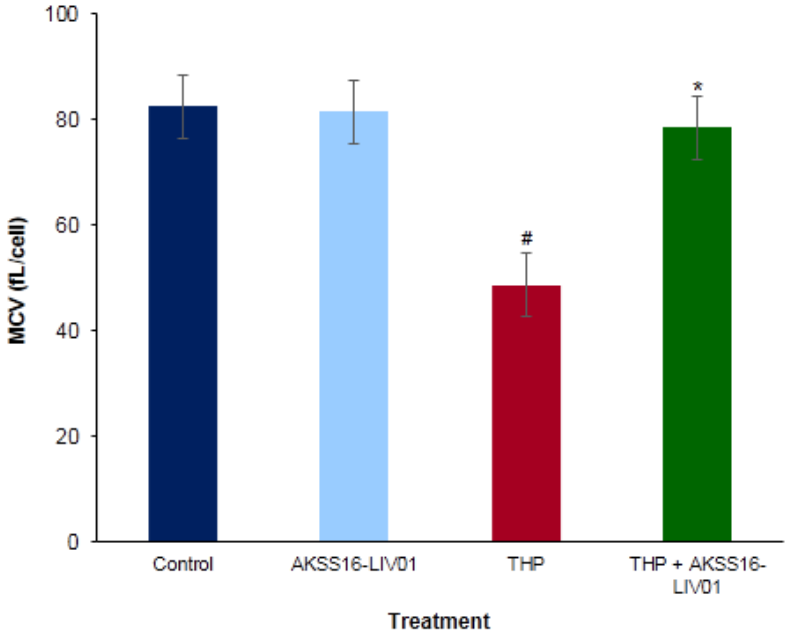

Figure 5A.3. Effect of multi herbal formulation (AKSS16-LIV01) on mean cell volume (MCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

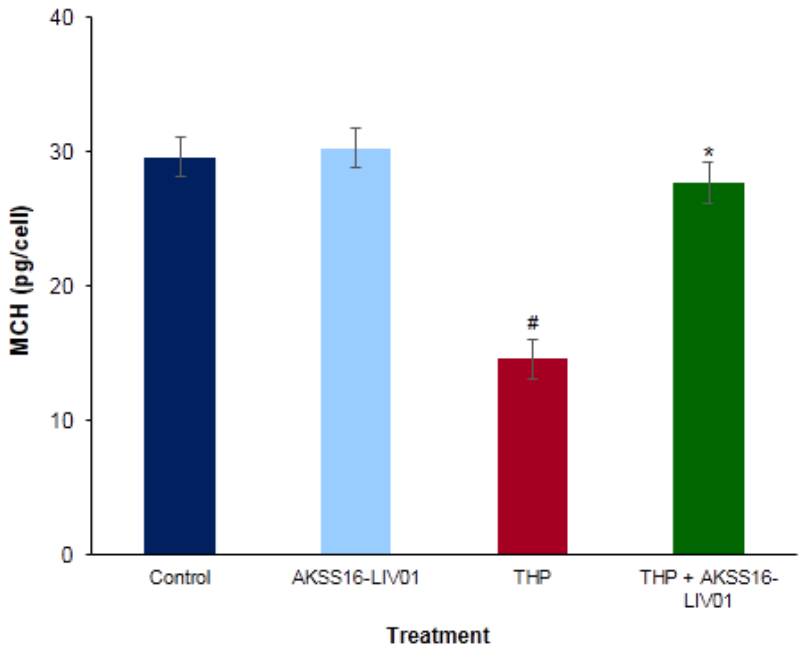

Figure 5A.4. Effect of multi herbal formulation (AKSS16-LIV01) on mean cell hemoglobin (MCH) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

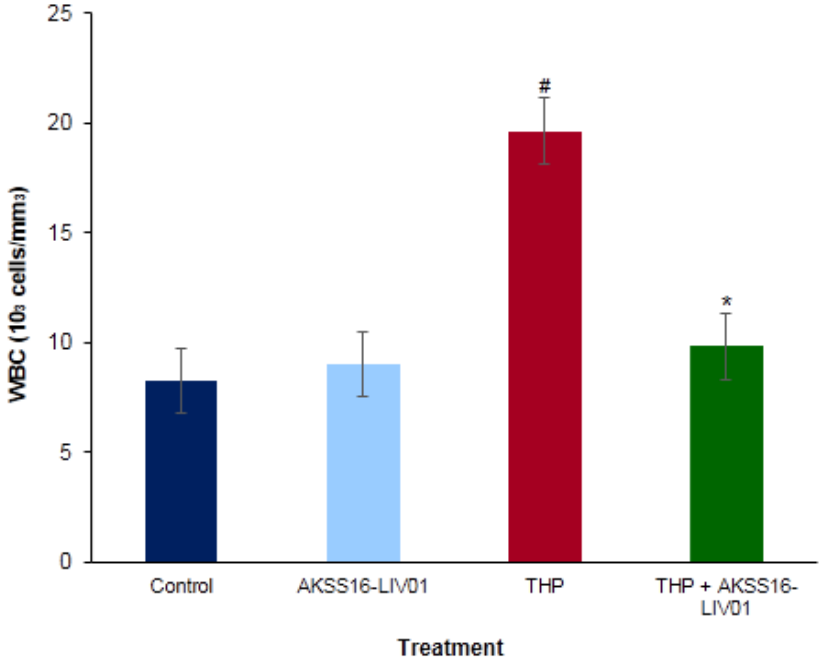

Figure 5A.5. Effect of multi herbal formulation (AKSS16-LIV01) on white blood cell (WBC) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

5A.3.3 Effect of multi herbal formulation (AKSS16-LIV01) on Biochemical parameters

Table 5A.3 shows the mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in control and experimental groups of mice. Data indicate that THP intoxicated mice had significantly greater mean AST and ALT compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day normalized the elevated AST and ALT levels when compared with THP treated mice. Four weeks treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in AST and ALT when compared with control group.

Table 5A.3: Effect of multi herbal formulation (AKSS16-LIV01) on serum biochemical parameters

Groups	AST (Unit/L)	ALT (Unit/L)
Control	55.28±6.34	26.82±4.11
AKSS16-LIV01	54.91±5.81	27.54±4.62
THP	106.28±8.17$^{\#}$	67.59±6.01$^{\#}$
THP + AKSS16-LIV01	62.99±5.44*	31.83±5.18*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (THP) group values at $p<0.001$

5A.4 DISCUSSION

Analgesics as fixed dose combination are very useful for first pain relief. Tramadol hydrochloride/paracetamol (THP) is a fixed dose combination consists of two analgesics tramadol and paracetamol used for treats moderate to severe pain [15]. It is well established that overdose or chronic use for analgesics specially fixed dosses form developed mild to severe adverse effects and sometimes damage various organs like liver, kidney and brain [16]. In very recent study confirm that administration of THP upon animal model severely disturbed hematological and biochemical parameters [17]. To prevent these deleterious effects we simultaneously administered our newly developed multi herbal formulation (AKSS1-LIV01) in mice. It is reported that treated with THP at a dose of 1.68 g/300 ml of water on mice reduced the haemoglobin (Hb), packed cell volume (PCV), and mean corpuscular volume (MCV) values. Another report depict that lower haemoglobin (Hb) value leads to iron deficiency anaemia which is characterized by a microcytic hypochromic blood picture. In the present study our result also confirm that administration of THP (1.68 g/300 ml of water) decline Hb, PCV, MCH and MCV values could be attributed to disturbed hematopoiesis, destruction of erythrocytes. The low PCV and Hb concentration and the abnormally low values of MCV and MCH are indications of microcytic anaemia. Medicinal plants enrich with various compounds capable to control and maintained the various blood parameters. Pre-treatment with newly developed multi herbal formulation (AKSS16-LIV01) along with THP elevate Hb, PCV, MCH and MCV values may indirectly protect the body from the anaemia.

Elevated aspartate transaminase (AST) and alanine transaminase (ALT) levels are strong indicators of inflammatory conditions and injury to the liver, while increased white blood cells (WBC) level is generally recognized as an inflammatory response [18, 19]. Inflammatory conditions may induce malnutrition in the body [20]. It is reported that inflammatory conditions can interfere with the body's ability to use stored iron and absorb iron from the diet[21]. Our result clearly showed that treatment with THP abruptly increased serum aspartate transaminase (AST) and alanine transaminase (ALT) levels as well as elevate white blood cells (WBC) count indicate THP produce inflammatory response and affects liver cell, disturbed homeostasis. On the other hand administration with newly developed multi herbal formulation (AKSS16-LIV01) along with THP decline the AST, ALT value and WBC count protect the liver against THP induced inflammation. Thus our developed multi herbal formulation composed with six medicinal plants and three medicinal spices may be able to protect haematological disturbance caused by THP.

CONCLUSION

This investigation shows that multi herbal formulation (AKSS16-LIV01) has the ability to protect the haematopoietic cells from the damaging effects of exposure to Tramadol hydrochloride/paracetamol (THP) and this protection might be attributed to the anti-oxidative power of multi herbal formulation (AKSS16-LIV01). Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of haematological dysfunction.

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5B Role of newly developed novel multi herbal formulation (AKSS16-LIV01) in ameliorating carbon tetrachloride induced haemato-toxicity in Swiss albino mice

5B.1 INTRODUCTION

Last few decades in various industrial processes carbon tetrachloride (CCl_4) is extensively used as a solution [1]. Due to its solvent property, this hazardous chemical used as refrigerator fluids, as a propellant for aerosol cans, as a dry-cleaning agent in industry, as a household spot remover, as grain fumigant and as intermediate in the synthesis of chlorofluorocarbons. As a result CCl_4 can easily found in the water bodies and contaminant the ground and surface water. Exposure and consumption of excessive CCl_4 disrupt body's homeostasis and make liver and kidney damage [2, 3]. Within the body CCl_4 can generate reactive oxygen species (ROS) like peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen caused oxidative damage. Hepatotoxicity is very common when people exposed with CCl_4 [4].

Adverse effect of carbon tetrachloride (CCl_4) in blood is well established. A recent study depicted that administration of CCl_4 reduced red blood cell (RBC), packed cell volume (PCV) and Haemoglobin (Hb) that disturbed the haematopoiesis [5, 6]. Various ultra-structural abnormalities in the leukocytes in the blood were visible under electron microscopy of mice those treated with CCl_4, clearly demonstrated that this notorious chemical makes the structural deformities in blood [7].

Multi herbal formulations mean a dosage form consisting of one or more herbs or processed herbs in specified quantities which have potent therapeutic efficacy without adverse effects [8, 9]. Scientific study revealed that this plant based formulation is very effective to cure anaemia and control the blood [10, 11]. Here we developed a multi herbal formulation (AKSS16-LIV01) based on six Indian medicinal plants and three Indian spices. Our previous study established that the formulation is completely safe in various doeses upon experimental animals [12, 13]. With view of the above, there is need to developed and safe and symptomatic medication that control all haematological parameters in the body when system exposed with toxicant.

5B.2 MATERIALS AND METHODS

5B.2.1 Chemicals

Carbon tetrachloride (CCl_4) and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. ALT and AST were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

5B.2.2 Preparation of plant extract

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. Plants parts were cleaned and dry with normal temperature. The dried plant parts were used for preparation of multi herbal formulation as per standard validated protocol [12]. The plants and plant parts used in preparation of the extract are listed in Table 5B.1.

Table 5B.1. Details of ingredient(s) present in the newly developed novel multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Family	Part Used	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	Stem	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	Fruit	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	Leaves	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	Leaves & Steam	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	Leaves & Steam	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	Rhizome	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	Seed	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	Seed	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	Seed	10 mg

* Amount required for preparation of 5 ml extract.

5B.2.3 Animals

Twenty four young, healthy swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

5B.2.4 Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg).

5B.2.1 **Body weight, food consumption and water intake**

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed

intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

5B.2.5 Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

5B.2.6 Evaluation of haematological parameters

Complete blood count includes hemoglobin (Hb), packed cell volume (PCV), total red blood corpuscles (TCRBC), total count of white blood cells (TCWBC), differential count (DC), platelets count, RBC indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were analysed by Sysmex KX-21, TRANSASIA, a fully automated 3-part differential hematology analyzer.

5B.2.7 Determination of biochemical parameters

Liver function enzymes such as AST and ALT were used as biochemical markers for hepatotoxicity and assayed by the standard (14).

5B.2.8 Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

5B.3 RESULTS

5B.3.1 Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in Table 5B.2. Administration of carbon tetrachloride (CCl_4) (1 ml/kg-bw) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity and reduced the liver weight as compared with control animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Table 5B.2. Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	Mice			
	Group-I	**Group-II**	**Group-III**	**Group-IV**
Body weight (g) Initial	26.35±1.91	26.51±2.35	26.71±4.2	26.68±5.1
Body weight (g) Final	37.84±2.03	36.94±1.69	21.81±2.41$^{\#}$	36.97±1.67*
Body weight (g) gain or loss	11.49±0.06	10.43±0.04	4.90±0.006	10.29±0.03
Food consumption (g)	4.52±0.05	4.37±0.07	2.94±0.06$^{\#}$	5.11±0.04*
Water intake (ml)	4.01±0.04	4.25±0.04	3.01±0.02$^{\#}$	4.31±0.06*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (CCl_4) group values at $p<0.001$

5B.3.2 Effect of multi herbal formulation (AKSS16-LIV01) on Haematological parameters

Haematological parameters of control and experimental groups are shown in Table 5B.2 as well as Figure 5B.1 to 5. 28days treatment with newly developed novel multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day did not showed significant differences in PCV, haemoglobin (Hb), WBC, mean cell volume (MCV), and mean cell hemoglobin (MCH) compared with the control (Figure 5B.1-5). Significant reduction in Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p = 0.001$), and MCH ($p< 0.001$) was noticed in CCl_4 intoxicated mice when compared with the control. The WBC count was significantly ($p< 0.001$) greater in Group C compared with the control. In contrast, no significant differences were observed in RBC and MCHC between the control and Group C. Administration of multi herbal formulation (AKSS16-LIV01) along with CCl_4 significantly increased Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p< 0.001$), and MCH ($p< 0.001$) when compared with the CCl_4 intoxicated animals. On the other hand WBC count was significantly reduced in Group D CCl_4 intoxicated animals. Others haematological parameters (Table 5B.3) like Read Blood corpuscle (RBC); Reticulocyte (RT); Haematocrit (HCT); Mean corpuscular haemoglobin concentration (MCHC) did not show any significant changes upon all the experimental groups.

Table 5B.3. Effect of novel multi herbal formulation (AKSS16-LIV01) on haematological Parameters

Parameters	Mice			
	Group-I	**Group-II**	**Group-III**	**Group-IV**
RBC ($x10^6$ μL^{-1})	10.8±4.1	10.2±5.3	10.1±4.2	10.6±5.1
RT (%)	2.8±1.1	2.4±1.6	2.8±2.4	2.9±1.6
HCT (%)	34.8±1.3	32.8±2.1	32.8±2.1	35.1±3.1
MCHC (%)	41.4±7.6	41.7±2.4	40.4±1.4	41.4±1.4
Platelets ($x10^3$ μL^{-1})	6.6±2.0	6.9±1.2	6.3±1.2	6.5±2.6
Lymphocyte (%)	76±6.3	72±3.3	73±5.4	73±3.4
Neutrophil (%)	25±6.2	22±4.3	21±5.1	25±6.9
Monocyte (%)	2.3±0.01	2.6±0.01	1.1±0.02	2.4±0.01
Eosinophil (%)	9.6±2.6	9.3±4.1	9.4±2.5	9.2±3.6
Basophil (%)	1.2±0.05	1.5±0.02	1.4±0.02	1.2±0.04

Data are expressed as mean ± standard deviation (N=6)

RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCHC: Mean corpuscular haemoglobin concentration

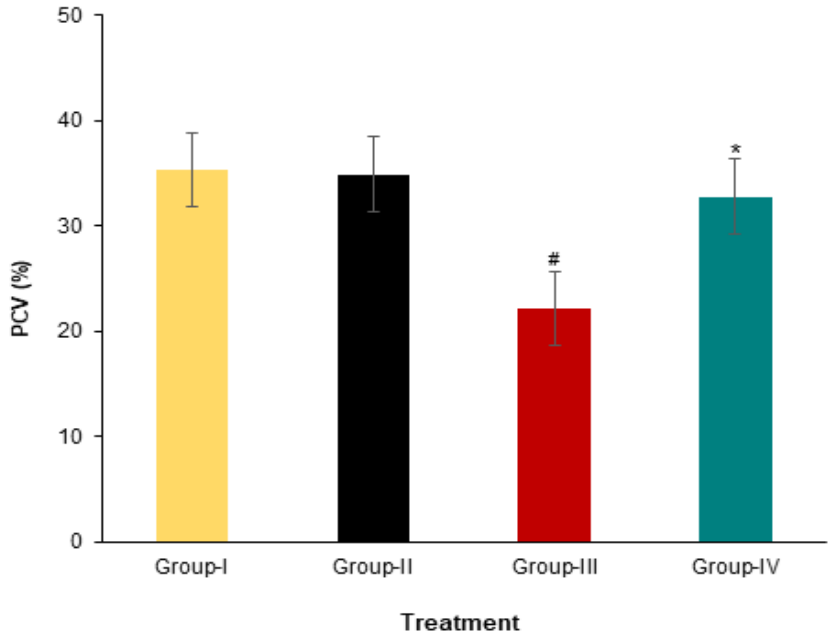


Figure 5B.1: Effect of multi herbal formulation (AKSS16-LIV01) on packed cell volume (PCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *Significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

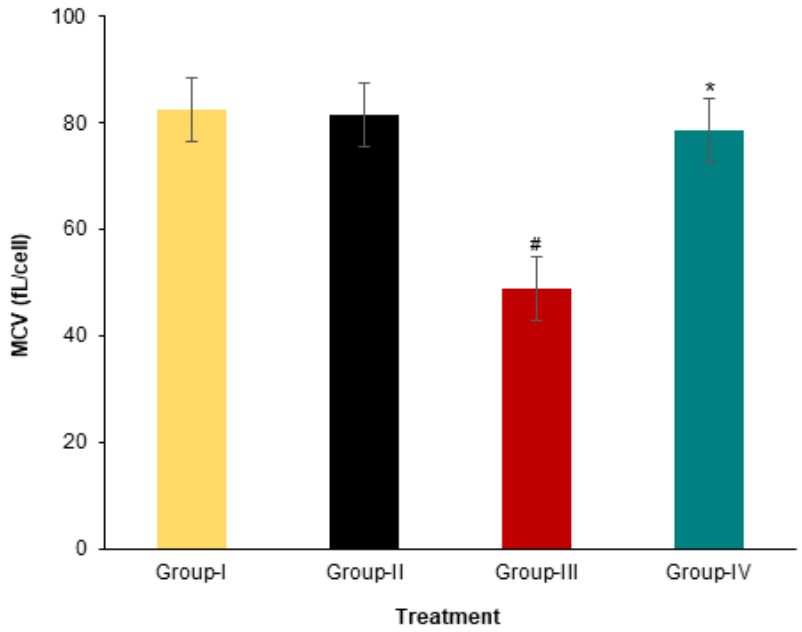


Figure 5B.2: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell volume (MCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

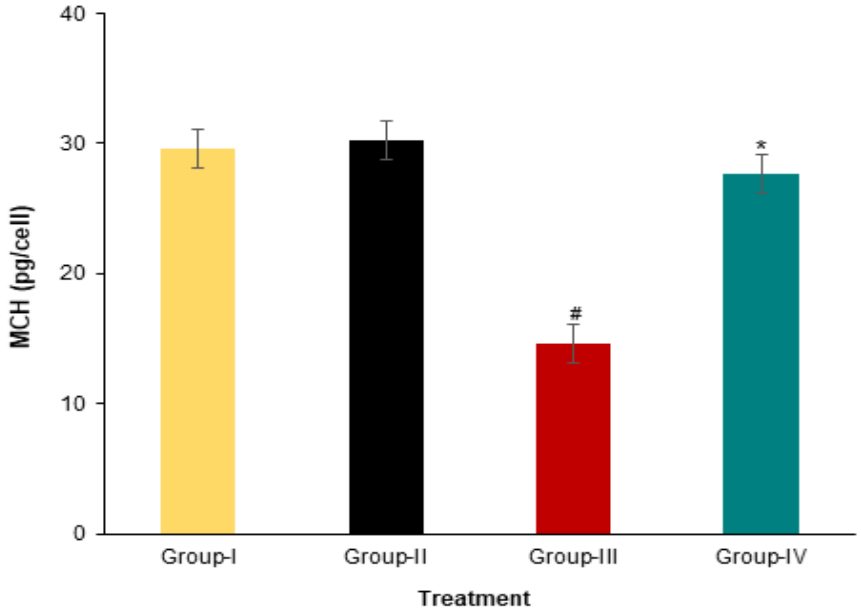


Figure 5B.3: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell hemoglobin (MCH) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

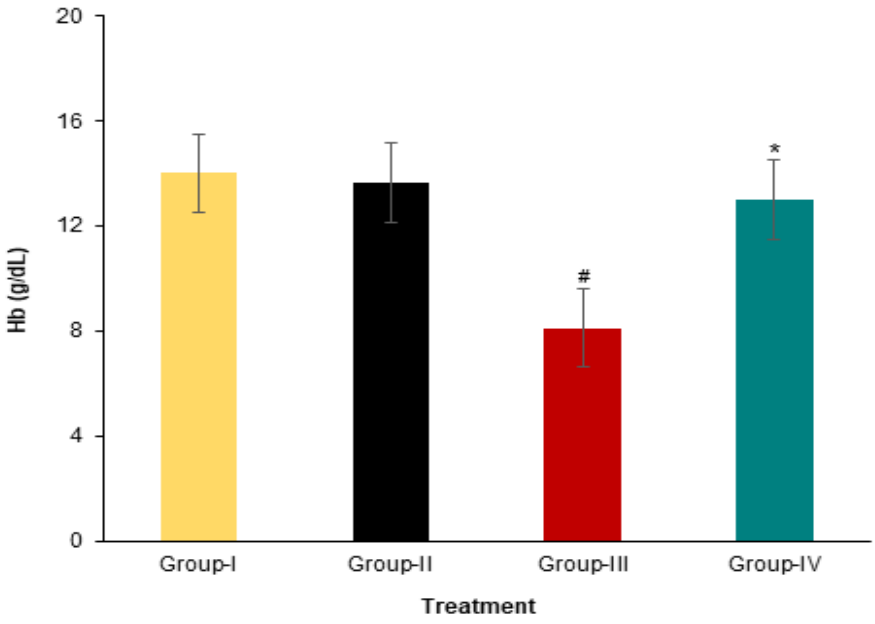


Figure 5B.4: Effect of multi herbal formulation (AKSS16-LIV01) on haemoglobin (Hb) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

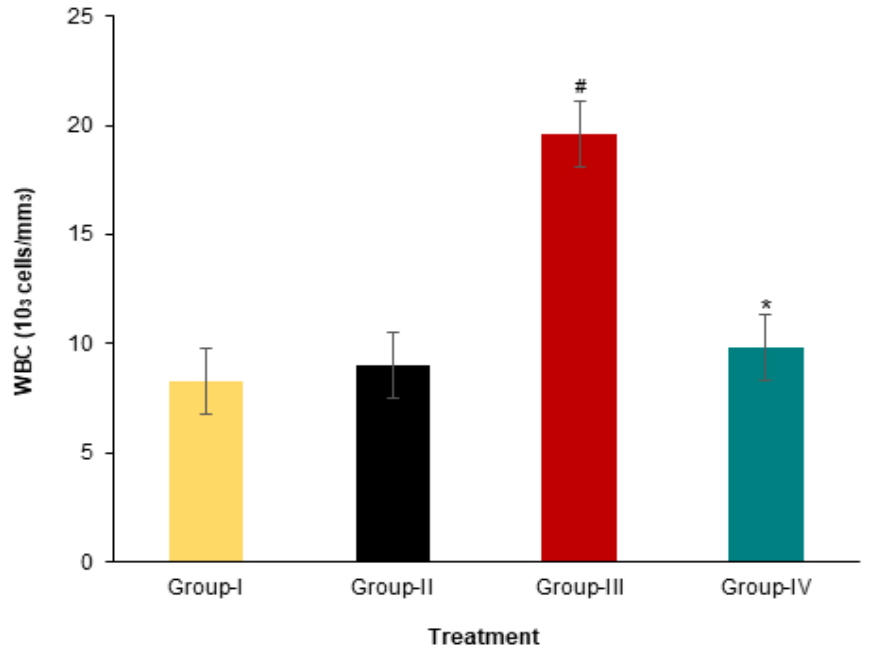


Figure 5B.5: Effect of multi herbal formulation (AKSS16-LIV01) on white blood cell (WBC) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

5B.3.3 Effect of multi herbal formulation (AKSS16-LIV01) on Biochemical parameters

Table 5B.4 shows the mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly greater mean AST and ALT compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day normalized the elevated AST and ALT levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in AST and ALT when compared with control group.

Table 5B.4. Effect of novel multi herbal formulation (AKSS16-LIV01) on serum biochemical parameters

Groups	AST (Unit/L)	ALT (Unit/L)
Group-I	54.25±6.31	27.88±4.58
Group-II	56.92±7.06	29.58±4.64
Group-III	108.95±9.17$^{\#}$	68.57±7.91$^{\#}$
Group-IV	61.28±5.21*	31.09±5.45*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (CCl_4) group values at $p<0.001$

5B.4 DISCUSSION

Hazardous toxicants and chemicals leads to various changes in haematological parameters and develops medical complications. Carbon tetrachloride (CCl_4) being one of the very common solvent is used in various industrial processes traded as an environmental pollutant [14]. It is reported that when treated with CCl_4 at a dose of 0.05 ml on mice it reduced the haemoglobin (Hb), packed cell volume (PCV), and mean corpuscular volume (MCV) values [15]. Another report depict that lower haemoglobin (Hb) value leads to iron deficiency leading to anaemia which is characterized by a microcytic hypochromic blood picture [16]. In the present study our results also confirm that administration of CCl_4 (1 ml per kg body weight) decline Hb, PCV, MCH and MCV values and could be attributed to disturbed hematopoiesis, destruction of erythrocytes. The low PCV and Hb concentration and the abnormally low values of MCV and MCH are indications of microcytic anaemia. Medicinal plants enriched with various compounds are capable to control and maintain the various blood parameters. Pre-treatment with newly developed multi herbal formulation (AKSS16-

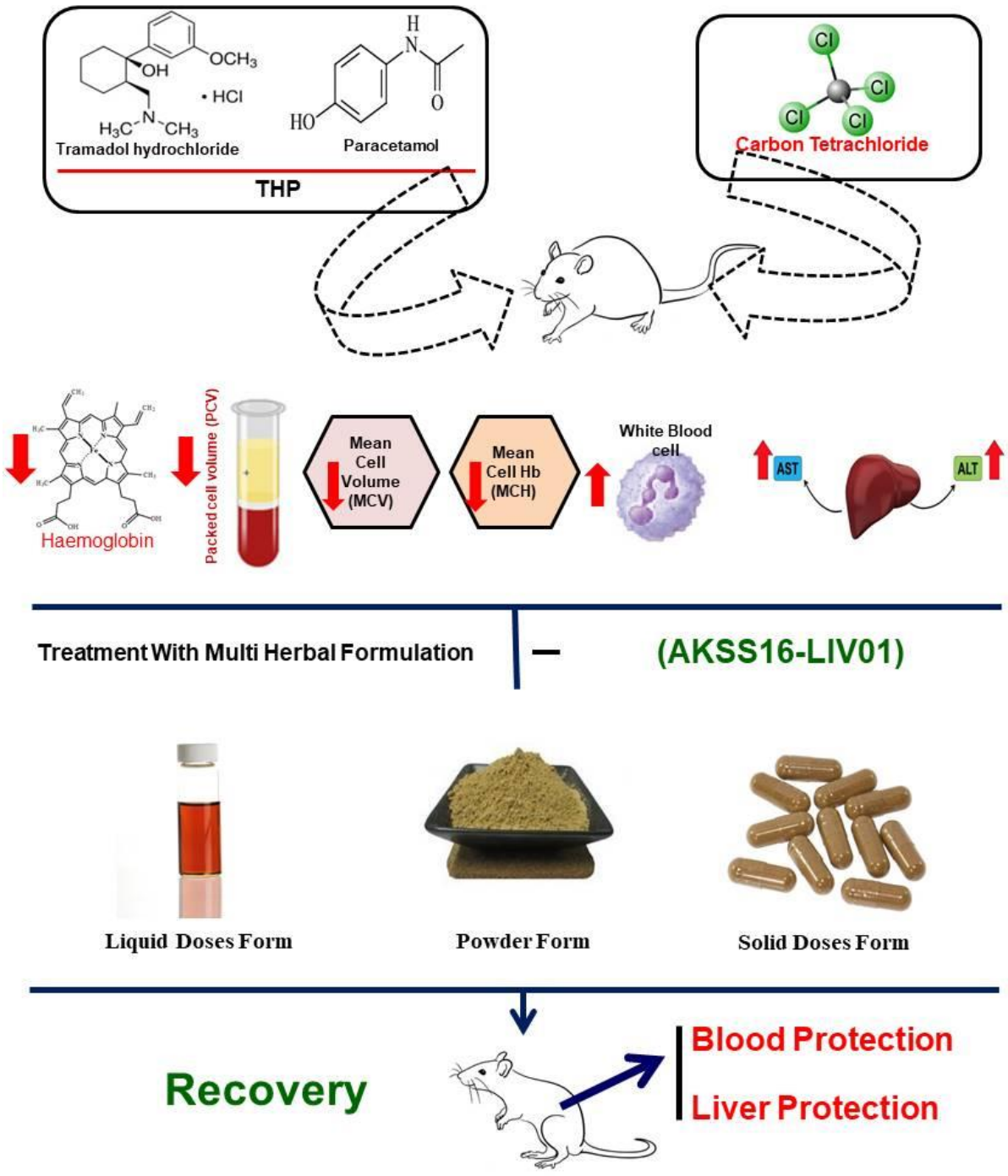
LIV01) along with CCl_4 elevate Hb, PCV, MCH and MCV values may indirectly protect the body from the anaemia.

Elevated aspartate transaminase (AST) and alanine transaminase (ALT) levels are strong indicators of inflammatory conditions and injury to the liver [17], while increased white blood cells (WBC) level is generally recognized as an inflammatory response. Inflammatory conditions may induce malnutrition in the body. It is reported that inflammatory conditions can interfere with the body's ability to use stored iron and absorb iron from the diet [18, 19]. Our result clearly showed that treatment with CCl_4 (1 ml per kg body weight) abruptly increased serum aspartate transaminase (AST) and alanine transaminase (ALT) levels as well as elevate white blood cells (WBC) count indicate CCl_4 produce inflammatory response and affects liver cell, disturbed homeostasis. On the other hand administration with newly developed multi herbal formulation (AKSS16-LIV01) along with CCl_4 decline the AST, ALT value and WBC count protect the liver against CCl_4 induced inflammation. Thus our developed multi herbal formulation composed with six medicinal plants and three medicinal spices may be able to protect haematological disturbance caused by CCl_4.

CONCLUSION

This investigation shows that the developed novel multi herbal formulation (AKSS16-LIV01) has the ability to protect the haematopoietic cells from the damaging effects of exposure to CCl_4 and this protection might be attributed to the anti-oxidative power of multi herbal formulation (AKSS16-LIV01). Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of haematological dysfunction.

SUMMARY



Multi Herbal Formulation (AKSS16-LIV01) ameliorate Hematological Dysfunctions Induced by Tramadol hydrochloride/paracetamol (THP) and Carbon Tetrachloride

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Chapter - VI

A Novel Multi Herbal Formulation (AKSS16-LIV01) Protects Obesity (Non-alcoholic fatty liver disease) and mitigates serum and tissue protein alterations

6A Therapeutic efficacy of a novel multi herbal formulation AKSS16-LIV01 upon HFD induced Obesity in murine model

6A.1 INTRODUCTION

Overweight and Obesity is a serious health problem throughout the world, and is reaching pandemic levels [1] According to World Health Organization's (WHO) report more than one billion people are overweight out of which three hundred million meet the criteria for obesity [2-4] Due to the excessive fat accumulation in the body, people both developed and developing countries are suffering for various overweight related complication such as type 2 diabetes, cardiovascular disease, coronary artery disease, stroke, myocardial infarction, dyslipidemia, anxiety, hypertension, mental stress, hepatic disorder and various type of cancer [5] [6] Another report stated that 26% nonpregnant woman ages between 20 to 39 years are overweight and out of which 29% are obese. World statistics stated that 35% men and 37% woman are overweight and 40% men and 30% women are obese [7, 8].

Abnormal accumulation of free fatty acids (FFA) in the liver is one of the prime causes of obesity which gradually produce fatty liver or hepatic steatosis. Accumulation of >5% triglycerides in hepatocytes is commonly observed in fatty liver related to alcohol consumption, metabolic syndrome and drug use [9].The primary cause of metabolic disorders is insulin resistance which accumulates fat in the adipocyte by suppressing the enzyme hormone-sensitive lipases (HSLs) resulting the released of triglycerides into the blood in the form of fatty acids. Deposition of free fatty acids (FFA) in the liver increases fat accumulation in the hepatic cell developed non-alcoholic fatty liver but the molecular mechanism is not fully understood [10] [11, 12]

Intakes of high fat enrich diet especially junk food and street food developed obesity and increased body fat. Excessive consumption of fatty food generates intracellular reactive oxygen species (ROS) disrupt the balance between oxidant and antioxidant agents. Redox imbalance is the main cause of hepatic mitochondrial dysfunction which produced inflammatory responses, and a breakdown of lipid metabolism [12, 13]. Mitochondria are the major organs involved in ROS production, generates various types of free radicals such as superoxide (O_2^-), peroxyl (RO_2^-), hydroxyl (OH^-), lipid peroxyl (ROO^-) radicals and non-free radicals such as hydrogen peroxide (H_2O_2), singlet oxygen (O_2^{-1}), ozone (O_3) and lipid peroxide (LOOH) those are actively involved for cellular damage. Study revealed that in non-alcoholic fatty liver disease (NAFLD) ROS which can induce damaged mitochondrial membrane potentials and changes in mitochondrial structure causing a breakdown of lipid homeostasis [14]

Medicinal plants are enriched with polyphenols, flavonoids alkaloids, and tannins takes part for prevention of many non-communicable diseases through inhibition of ROS production[15]. Regular consumption of plant polyphenols protects the liver cell by external chemical or foreign injury and save the hepatic cell from fat deposition. During oxidative stress our body required sufficient amount of antioxidant compounds which protects the hepatic cell against the deleterious effects of ROS[16-18] [18-20]. Scientific study revealed that combination therapy produces enormous positive results as herb-herb interaction produces synergism, which could be pharmacokinetic synergism or

pharmacodynamics synergism. Recently scientist and medical practitioners are interested for combination therapy to obtain the better therapeutic efficacy in comparison to single one[16] [21,22].

Prevention of obesity and obesity induced complications is a new challenge throughout the globe. With view of this in the present study we formulated a new novel phytomedicine (AKSS16-LIV01) containing six indigenous medicinal herbs and three medicinal spices which were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defence against free radical damage and are considered to be important in maintaining optimum health and hygiene. Phytochemicals present in these herbs have potent lipid lowering capacity make a barrier against excessive body growth. Our previous reports stated that the formulation have no adverse side effect and no toxicity in mice and medicinally safe for medication [19, 20] [23,24]. Therefore, the present study was undertaken to evaluate the anti-obesity effects and reduction of tissue oxidative stress and its deleterious recovery by the application of multi herbal formulation (AKSS16-LIV01). We therefore used mice with high-fat diet (HFD)-induced obesity to evaluate changes in fat accumulation, liver function enzymes, MDA content and lipid profiles, as well as examined the redox mechanisms.

6A.2 MATERIALS AND METHODS

6A.2.1 Chemicals and reagents

Standard biochemical kits i.e. AST, ALT, GGT, ALP, total protein, albumin etc. were procured from Merck (Germany). Triglyceride, cholesterol, phospholipids, free fatty acids, HDL and LDL were purchased from Thermo Scientific. Antioxidant enzymes determination kits such as SOD, CAT, GSH and GPx were obtained from Boehringer, USA. All other laboratory chemicals and reagents were purchased from Merck (Germany), Sigma (USA) or BDH chemicals (Mumbai, India).

6A.2.2 Collection of Plants and Preparation of Extract

All the medicinal plants and spices were collected from the registered supplier, Kolkata. Plants were authenticated by pharmacognosist of Department of Pharmacy, Jadavpur University for preliminary identification and verification and further identified based on Ayurvedic parameters by a renowned taxonomist of Department of Botany, Uluberia College, University of Calcutta, India and properly kept as voucher specimen. Ingredients used in the formulation are listed in Table 6A.1 and Figure 6A.1. For preparation of extract the plants and spices were cleaned with double distilled water air dried until dry. Then the plants were kept in hot air oven at 75ºC for 10 min and 55ºC for 30 min. then all the plants were grinded by a blade mill to obtained the fine powder. Then 5g of dry plant powder was taken and dissolved into 10 ml methanol followed by sonication for 30 minutes using an ultrasonic bath. Centrifuged the material at 4000 rpm for 15 minutes and collected the supernatants. The procedure was repeated four times and collects all the supernatant. The supernatant were finally evaporated in a rotary evaporator under reduced pressure at 35°C. Finally, the residue was re-constituted in 3 ml of methanol, filtered using Whatman filter papers (GE Healthcare and Life Sciences, MA, USA) and kept at 4ºC for further use[21] [25].

Table 6A.1: Details ingredient(s) present in the newly developed novel multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Family	Part Used	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	Stem	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	Fruit	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	Leaves	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	Leaves & Steam	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	Leaves & Steam	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	Rhizome	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	Seed	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	Seed	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	Seed	10 mg

* Amount required for preparation of 5 ml extract.

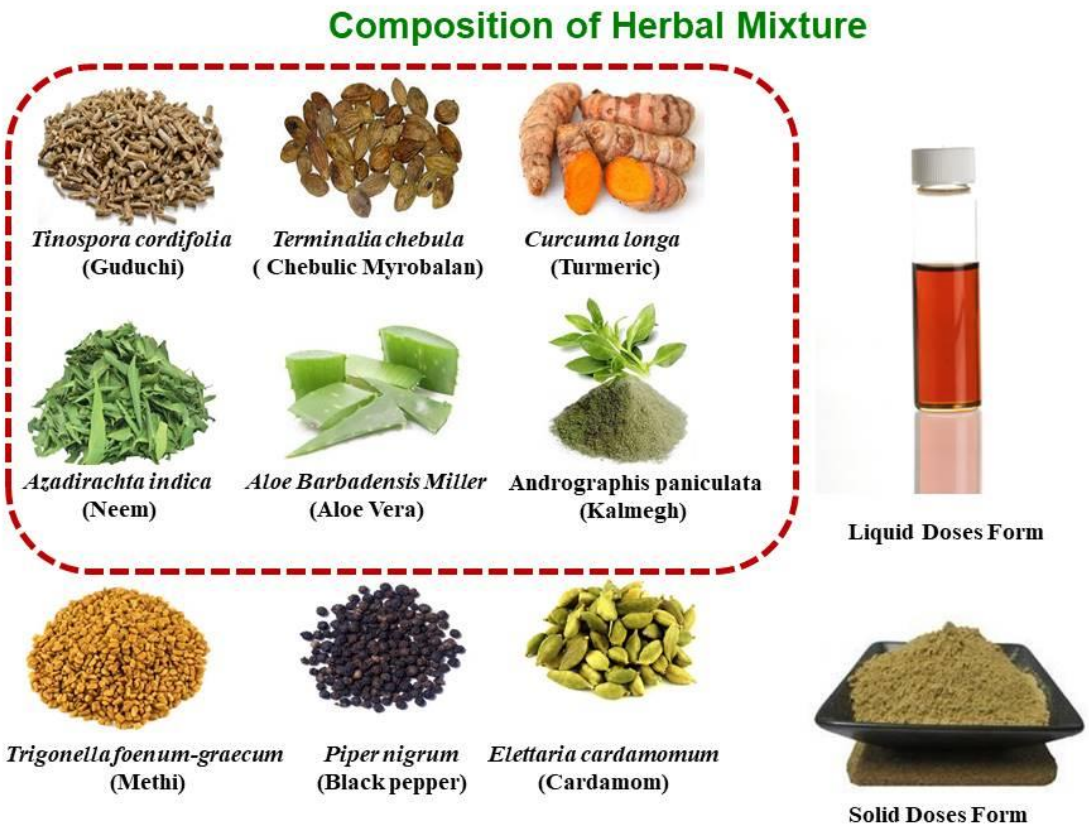


Figure 6A.1: Composition of multi herbal formulation (ASKK16-LIV01) containing six medicinal herbs and three medicinal spices.

6A.2.3 *Animals and treatment*

Experimental animals were taken form registered central animal house of Jadavpur University, Kolkata. Adult C57BL/6J weighing 25 g ± 2g were used to conduct the preclinical study. Before experiment the animals acclimatized for 7 days in an air ventilated room with constant temperature (25±1 °C) and humidity (55±5%). The animals were maintained 12 hours light and dark cycle. Freshly prepared healthy diet was given to the animals with water *ad libitum*. Animal received human care in compliance with the revised guidelines 2018 of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Animal Husbandry, Govt. of India. All the experiments were carrying forward by the prior approval of the Institutional Animal Ethical Committee (IAEC) of Jadavpur University, Kolkata.

6A.2.4 Acute Toxicity Studies

As per the drug development protocol we conduct the acute toxicity study for novel formulation AKSS16-LIV01 followed by the general principles of OECD guideline 423 [22][26]. Overnight fasted healthy twenty four male mice were divided into four (One control and three test) groups and orally given the extract at doses up to 2000 mg/kg body weight (BW). They were observed continuously for 14 days for morbidity and mortality.

6A.2.5 Diets

Healthy standard laboratory diet was used under supervision of the animal care committee of this Institution. Control mice (n=10) were fed standard laboratory diet (7-10% fat, 68-70% CH, 18-20% protein, 1-2% vitamins and minerals; 210 kcal/1000 g/day). High fat (HF) diet was prepared according to the standard method. High fat diet consists of 30% calories from animal fat (30% fat, 50-52% CH, 18-20% protein 1-2% vitamins and minerals; 210 kcal/1000 g/day), the diet was prepared (Table 6A.2) and necessary vitamins and minerals are added.

Table 6A.2: Composition of experimental diets (g/kg diet)

Ingredients	Normal diet	High Fat diet
1. Corn Starch	400	200
2. Sucrose	250	200
3. Cellulose	50	50
4. Protein (Casein)	200	200
5. Fat (ground nut oil and butter)	50	300
6. Salt mixture	35	35
4. Vitamin Mixture	15	15
5. Calories (kcal/100g/day)	210	210

6A.2.6 Treatment Protocol

After acclimatization, animals were randomly divided into four groups (N=10/group) and treated according to the following protocol. All doses were calculated from earlier reports and pilot experiments.

I. Control Group: Received normal food and water on daily basis.
II. HFD Group: High fat diet (30%) given to the animals regularly.
III. AKSS-75 Group: Animal received high fat diet along with multi herbal mixture (AKSS-16-LIV-01) at a dose of 75 mg/kg/day.
IV. AKSS-150 Group: Animal received high fat diet (30%) along with multi herbal mixture (AKSS-16-LIV-01) at a dose of 150 mg/kg/day.
V. AKSS-300 Group: Animal received high fat diet (30 %) along with multi herbal mixture (AKSS-16-LIV-01) at a dose of 300 mg/kg/day.
VI. Std. diet Group: Animal received Lovastatin (50 mg/kg/day) along with high fat diet.
VII. AKSS-300 Group: Animal received only multi herbal mixture (AKSS-16-LIV-01) at a dose of 300 mg/kg/day without high fat diet.

The duration of the study is six weeks.

6A.2.7 Measurement of body weight and food intake

Routine food intake and body weight of each animal was recoded on daily basis using a sensitive digital balance. To obtain the actual food intake subtract the remaining food from the initial food given to the animals. Container of the food were removed at 10.00 a.m. and returned to animals with fresh food at 2:00 p.m. every day.

6A.2.8 Blood Collection

At the end of the experimental period 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%) from the retro orbital plexus of the mice. Blood collected from animals were placed in slanting position (45°) at room temperature for 2.5 hrs. Then, the blood samples were centrifuged at 4000 g for 10 min. Serum was separated and used for further analyses.

6A.2.9 Biochemical estimation

Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) were analysed using standard commercial biochemical kits (Span Diagnostic, Surat, India) following the protocol prescribed by manufacturer. Total protein concentration was determined in the serum by the method of Lowry et al.[23] [28]. Serum lipid profile like Cholesterol, Triglyceride, Phospholipids, LDL-cholesterol, HDL-cholesterol and VLDL-cholesterol (ELITech Diagnostic, France) were measured using enzymatic calorimetric kits according to manufacture instructions.

6A.2.10 Preparation of tissue homogenate

For determination of liver lipid profile the tissue sample was rinsed in ice-cold saline and blotted carefully. The liver tissue was chopped with a scissor and placed in phosphate buffer (at pH 7.4) containing glass tube for homogenization at 6000 g for 10 minutes. Clear supernatant was discarded and collected with a sterilized container for determination of cholesterol, triglyceride, LDL, VLDL and HDL by standard biochemical kit obtained from ELITech Diagnostic, France.

6A.2.11 Measurement of liver malondialdehyde (MDA)

Serum and tissue MDA content were measured spectrophotometrically as Thiobarbituric acid (TBA) reactive substances. In brief TBA reacts with MDA and is formed TBA reactant substances (TBARs) which are basically biomarkers of oxidative damage to polyunsaturated fatty acids. MDA content was measured at 532 nm by spectrophotometer [24][29].

6A.2.12 Determination of Antioxidant Enzymes activities

Serum antioxidant markers such as total ROS content, SOD, CAT, GSH and GPX were determined through Elisa. For determination of liver antioxidant enzymes contents we follow the following standard protocol with slight modification. After the experimental period liver tissue was taken for determination of various antioxidant enzymes activity. Liver tissue was chopped into small pieces and homogenized in ice-cold phosphate buffer (pH 7.2) at a concentration of 15% (weight by volume). Homogenised tissue was centrifuge at 4°C (Hettich Zentrifugen, Germany) at 980 g for 10 minutes. The supernatant was carefully separated and further centrifuged at 7680 g for 30 min at 4°C to obtain the final clear supernatant for evaluation of SOD, CAT, GPx and GSH by standard methods[25-28] [30-33].

6A.2.13 Histopathological examination

A small portion of the liver tissues from all the experimental groups were excised immediately after sacrifice. Tissues were fixed in 10% formalin saline solution (pH 7.0) for 24 hr at room temperature for histopathology. After twenty four hours the tissues were embedded in paraffin wax (melting point 45°C) and sections were cut at 3-5 µm slices and were stained with haematoxylin and eosin (H&E). The mounted stained section was observed under light microscope [34]. Massion's Trichrome and Sirius red staining were carried out according to the manufacture's standard protocol.

6A.2.14 Massion's Trichrome and Sirius red staining

Massion's Trichrome and Sirius red staining were carried out according to the manufacture's standard protocol.

6A.2.15 Oil-Red O Staining

Intracellularly accumulated triglyceride was measured using the Oil-Red O staining assay as in a previous study[29] [35].

6A.2.16 Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

6A.3 RESULTS

6A.3.1 Effect of AKSS-16-LIV-01 on body weight and lipid profile

Anti-obesity effects of the newly developed novel formulation (AKSS-16-LIV-01) were evaluated against high fat diet (HFD) induce obese mice. In this study we analyzed the changes in body weight, food consumption, water intake, body fat, liver weight, and liver body weight ratio (Figure 6A.2) as well as serum lipid profile (Table 6A.3) of HFD-induced hyperlipidemic mice treated with AKSS-16-LIV-01 for 6 weeks. Marked significant differences were detected in the body weight food consumption, water intake, body fat, liver weight, and liver body weight ratio of HFD treated group and HFD + AKSS-16-LIV-01 (150 & 300 mg/kg) treated group as compared with standard lipid lowering drug.

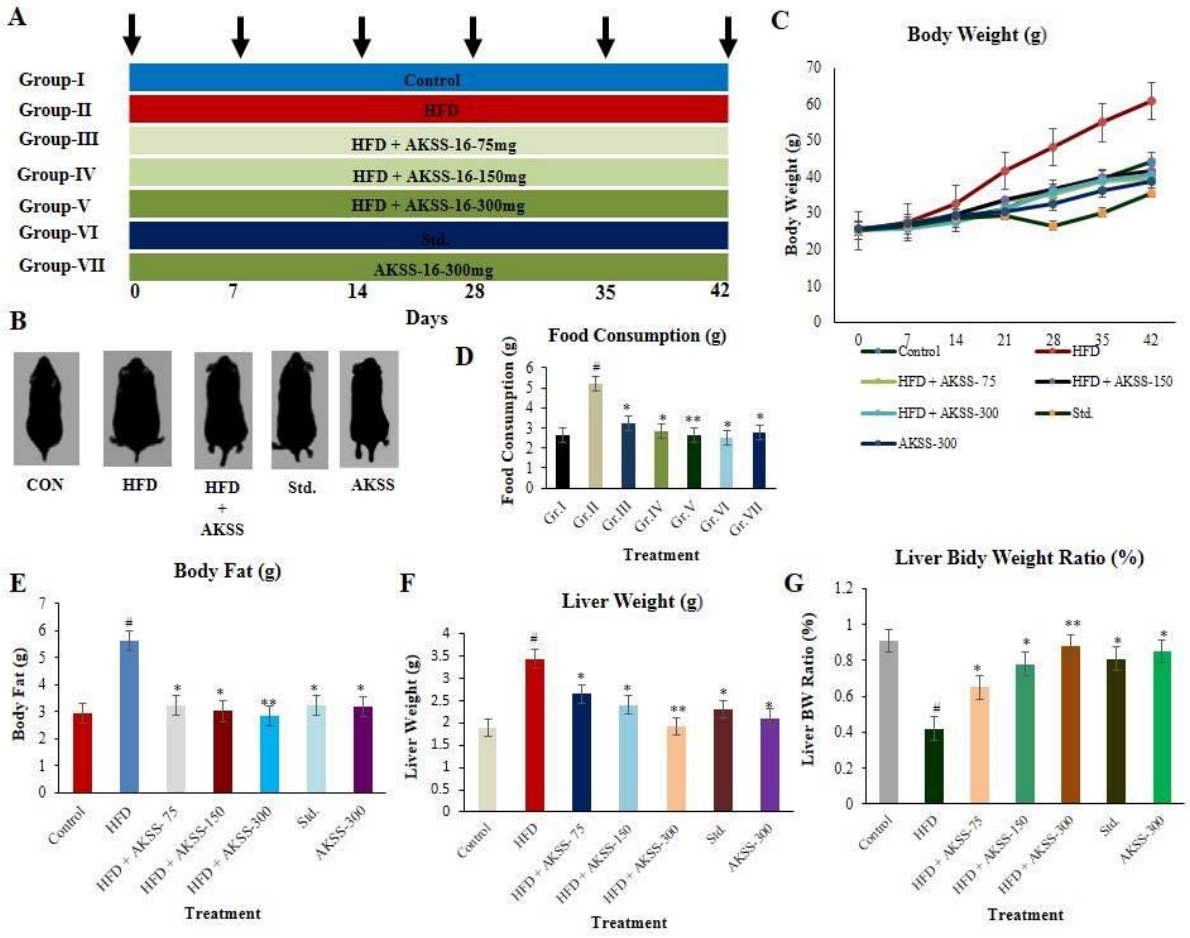


Figure 6A.2: Effect of multi herbal formulation against high fat diet (HFD) induced obesity in mice. A) Experimental design B) Morphological of the mice in different groups C) Measurement of body weight D) Determination of food consumption E) Determination of body fat F) Assessment of liver weight G) Determination of Liver body weight ratio.

Table 6A.3 showed serum lipid profile abruptly elevated in the HFD group and differed after AKSS-16-LIV-01 treatment as compared with standard drug. As shown in Figure 6A.3, all liver tissue lipid profile factors were 80-120% higher in the HFD treated group than in the untreated control group. However, the concentration of LDL, VLDL, TG and TC significantly decreased and HDL concentration was significantly increased in the HFD+ AKSS-16-LIV-01 (150 & 300 mg/kg) treated group as compared to the HFD treated group (P<0.005, P<0.001).These results suggest that AKSS-16-LIV-01 promotes recovery of the serum and tissue lipid profile in HFD-induced obese mice.

Table 6A.3: Effect of AKSS16-LIV01 on serum lipid profile in high fat diet (HFD) induced obesity in mice

Parameters	TC (mg/dL)	TG (mg/dL)	Phospholipids (mg/dL)	VLDL (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
Control	81.03 ± 5.02	40.58 ± 2.05	76.59 ± 6.28	25.61±1.91	39.65 ± 1.96	19.58 ± 0.69
HFD (30% fat of total diet)	135.69 ± 6.15$^{\#}$	72.58 ± 3.28$^{\#}$	142.97 ± 4.69$^{\#}$	36.25±2.15$^{\#}$	76.94 ± 1.77$^{\#}$	10.28 ± 0.28$^{\#}$
HFD + AKSS-16 (75 mg/kg)	86.39±4.29*	60.12±2.15*	88.21±3.24*	31.24±1.98*	44.27±2.01*	16.11±0.62*
HFD + AKSS-16 (150 mg/kg)	88.05±3.16*	51.23±3.01*	92.37±2.66*	28.67±2.36*	43.61±1.88*	15.28±0.55*
HFD + AKSS-16 (300 mg/kg)	79.36 ± 4.35**	37.25 ± 1.87**	77.25 ± 1.87**	24.15±2.47**	36.85 ± 1.25**	10.28 ± 0.28**
Std Drug	91.47 ± 2.37**	41.75±3.25**	88.03±2.84**	28.14±1.95**	44.52 ± 2.28**	22.67 ± 0.99**
AKSS-16 (300 mg/kg)	85.11±2.98	39.64±3.51**	91.51 ± 3.28**	25.11±2.02**	45.78±1.65**	16.24±0.41**

Values are mean of six individual *****(observations in each group ± S.D. Significantly different from control $^{\#}$(p<0.001), and significantly different from HFD group ***** (p<0.05), ****** (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

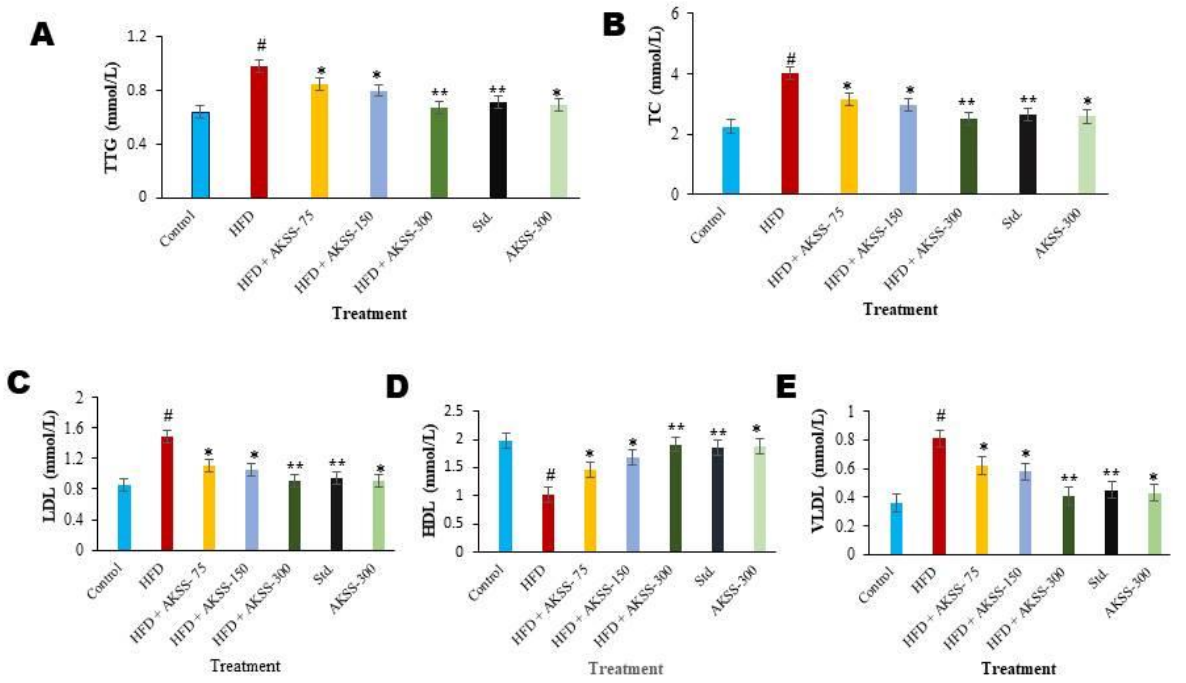


Figure 6A.3: Ameliorative effect of AKSS-16-LIV01 on the activity of Total triglycerides, Total cholesterol, LDL-cholesterol, HDL-cholesterol, VLDL-cholesterol under HFD induced Obesity in mice. Values are expressed as Mean ± SD (n = 6 per group). *Significantly different from control # ($p<0.001$) and significantly different from HFD ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. TTG: Total Triglycerides, TC: Total Cholesterol, LDL: Low-density lipoproteins, HDL: High-density lipoproteins, VLDL: Very-Low-density lipoproteins.

6A.3.2 Effect of AKSS-16-LIV-01 on haematological parameters

Various haematological parameters were presented in Table 6A.2. High fat diet (HFD) significantly reduced ($P<0.05$, $P<0.001$) haemoglobin, Mean corpuscular volume (MCV), Mean corpuscular haemoglobin concentration (MCHC) levels and significantly elevate reticulocyte (RT) and WBC count. Pre-treatment with AKSS-16-LIV-01 150 mg/kg/day and 300 mg/kg/day significantly increased ($P<0.05$, $P<0.001$) haemoglobin, Mean corpuscular volume (MCV), Mean corpuscular haemoglobin concentration (MCHC) levels and significantly decreased reticulocyte (RT) and WBC count. Application of standard drug partially controls the hyperlipidemic effects by the application of high fat diet upon blood parameters. Multi herbal formulation (AKSS-16-LIV-01) at a dose of 300 mg/kg/day provides the optimum therapeutic results as compared with standard modern drug.

Table 6A.4: Effect of AKSS16-LIV01 on haematological parameters in high fat diet (HFD) induced obesity in mice

Parameters	Normal	HFD (30% fat of total diet)	HFD + AKSS16-LIV01 (75)	HFD + AKSS16-LIV01 (150)	HFD + AKSS16-LIV01 (300)	HFD + Std. drug	AKSS16-LIV01 (300)
Hb (g %)	13.6± 2.3	9.03± 0.59$^{\#}$	11.0±1.02**	11.05±0.99*	14.01±1.95*	12.96±0.74*	11.21±0.82*
RBC (x10^{6} cm^{2})	10.8±1.9	9.2±0.96	10.5±0.77	9.44±0.71	10.02±0.85	9.85±0.79	9.62±0.84
RT (%)	2.8±0.15	4.9±0.26$^{\#\#}$	2.6±0.14*	3.1±0.14**	2.8±0.15*	3.0±0.12*	3.6±0.16*
HCT (%)	34.1±2.48	36.4±0.66	34.1±0.44	35.8±1.51	34.9±1.56	34.4±1.51	35.1±0.77
MCV (µm^{3})	37.2±1.3	31.0±1.68$^{\#}$	36.7±0.29**	36.5±0.44*	35.9±0.72*	36.2±0.43*	35.5±0.36*
MCH (pg)	21.4±0.85	22.2±0.14	22.8±0.23	21.1±0.12	21.4±0.11	21.2±0.14	22.1±0.12
MCHC (%)	41.2±1.06	32.4±0.95$^{\#\#}$	40.2±1.07*	37.1±0.92*	39.8±0.87*	38.6±0.99*	36.2±0.91*
Platelets	6.5±0.02	5.5±0.03	6.5±0.04	5.8±0.05	6.1±0.07	5.5±0.05	5.4±0.06
WBC (x10^{5} cm$^{2)}$)	9.2±0.09	12.4±0.15$^{\#}$	9.1±0.08*	10.8±0.12*	9.2±0.11*	10.1±0.13*	10.7±0.11*
Lymphocyte	72±2.98	79±3.04$^{\#}$	72±2.54*	73±3.06*	71±2.58*	72±3.08*	71±3.11*
Neutrophil	26±1.12	16±0.38$^{\#}$	24±1.09*	20±0.56*	25±0.69*	24±0.51*	21.52±2.09*

Data are expressed as mean ± standard deviation (N=6). Hb: Haemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White Blood corpuscle.

Values are mean of six individual *****(observations in each group ± S.D. Significantly different from control $^{\#\#}$(p<0.001), $^{\#}$ (p<0.05) and significantly different from HFD group ****** (p<0.001), *****(p<0.05) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

6A.3.3 *Effect of AKSS-16-LIV-01 on serum biochemical parameters*

Anti-obesity effects of the newly developed novel formulation (AKSS-16-LIV-01) on all the biochemical parameters were depicted in Table 6A.3 and Fig. 6A.4. A significant increase in serum transaminase (ALT and AST), alkaline phosphatase (ALP), γ- glutamyl transferase activity were observed in HFD-intoxicated mice compared to normal control. Treatment with AKSS-16-LIV-01 both 150 mg/kg/day and 300 mg/kg/day for 42 days significantly ($P < 0.05$, $P < 0.001$) reduced ALT, AST, ALP and GGT levels as compared to HFD group. Application of standard drug also showed significant effect but more positive effect observed in AKSS-16-LIV-01 (300 mg/kg/day) group due to presence of medicinal spices in the extracts. Treatment with only AKSS-16-LIV-01 has no significant effect on serum biochemical parameters. On the other hand significantly reduced (P<0.001) serum total protein in HFD group was normalized by the treatment with AKSS-16-LIV-01 (Table 6A.5).

Table 6A.5: Effect of AKSS16-LIV01 on serum biochemical parameters in high fat diet (HFD) induced obesity in mice

Treatment	AST (IU/L)	ALT (IU/L)	GGT (IU/L)	ALP (IU/L)	TP (g/L)
Control	14.26±1.36	27.16±1.36	5.12±0.04	313.02±11.54	6.95±0.91
HFD (30% fat of total diet)	52.19±2.09$^{\#}$	88.02±1.97$^{\#}$	10.16±0.06$^{\#}$	546.74±10.65$^{\#}$	3.25±0.95$^{\#}$
HFD + AKSS-16 (75 mg/kg)	36.59±1.39*	51.22±3.62*	8.01±0.08*	416.91±12.37*	4.12±1.02*
HFD + AKSS-16 (150 mg/kg)	28.91±2.11*	35.11±2.18*	6.11±0.09*	347.84±14.25*	5.78±1.14*
HFD + AKSS-16 (300 mg/kg)	16.06±1.98**	28.35±3.01**	5.29±0.07**	312.05±11.69**	6.58±0.94**
Std Drug	26.02±1.12**	32.06±1.99**	6.01±0.06**	351.74±13.25**	6.01±1.04**
AKSS-16 (300 mg/kg)	22.19±2.06**	30.16±2.15**	5.35±0.04**	330.03±14.05**	6.33±1.11**

Values are mean of six individual *****(observations in each group ± S.D. Significantly different from control $^{\#}$($p<0.001$), and significantly different from HFD group ***** ($p<0.05$), ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

6A.3.4 Effect of AKSS-16-LIV-01 on liver transaminase levels

Figure 6A.4 represented the liver aspartate and alanine transaminase activity. Daily intake of high fat diet (HFD) significantly elevated ($P<0.001$) liver AST and ALT levels as compared with control untreated animals. Treatment with AKSS-16-LIV-01 both 150 mg/kg/day and 300 mg/kg/day for 42 days significantly ($P < 0.05$, $P < 0.001$) reduced tissue ALT and AST levels as compared to HFD group. Administration of standard drug also showed significant effect but optimum effects observed in AKSS-16-LIV-01 (300 mg/kg/day) group due to presence of medicinal spices in the extracts.

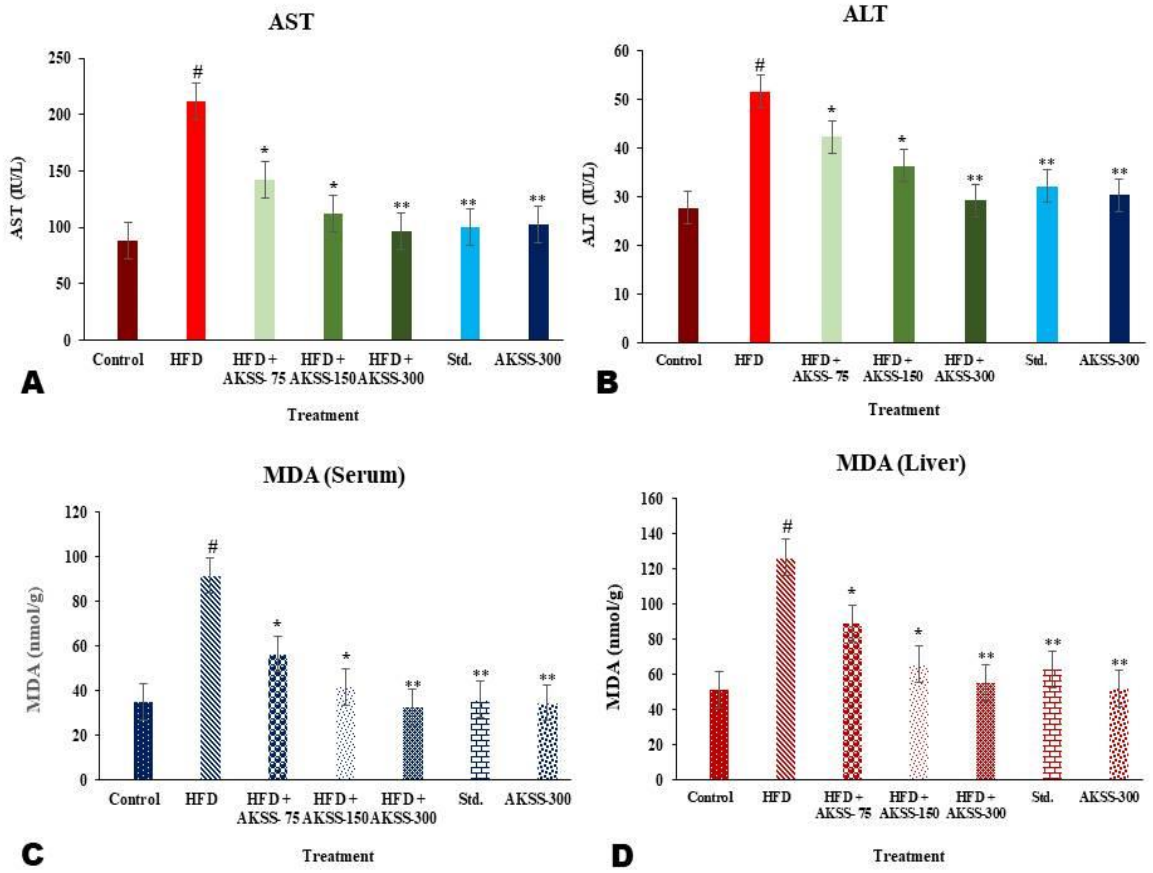


Figure 6A.4: Ameliorative effect of AKSS-16-LIV01 on the activity of Aspartate amino transferase Alanine amino transferase, MDA content in Serum & MDA content in Liver under HFD induced Obesity in mice.

6A.3.5 Effect of AKSS-16-LIV-01 on Lipid peroxidation

Administration of High fat diet (HFD) showed elevated level of MDA both serum and liver when compared to normal control (Figure 6A.4). In comparison with HFD control animals, treatment with AKSS-16-LIV-01 both 150 mg/kg/day and 300 mg/kg/day for 6 weeks has significantly decreased the MDA level in serum and liver. Application of standard drug also showed significant effect but more positive effect observed in AKSS-16-LIV-01 (300 mg/kg/day) group. Treatment with only AKSS-16-LIV-01 has no significant effect on tissue lipid peroxidation.

6A.3.6 Effect of AKSS-16-LIV-01 on serum antioxidant status

The activities of the antioxidant enzymes total ROS content, levels of SOD, CAT, GSH and GPx were altered prominently in serum of HFD group when compared with normal untreated group. Administration of AKSS-16-LIV-01 both 150 mg/kg/day and 300 mg/kg/day for 42 days significantly

increased the serum antioxidant enzyme activities such as SOD, CAT, GSH and GPx in liver tissue (Table 6A.6) . Pre-treatment with multi herbal formulation (AKSS-16-LIV-01) significant reduced the elevated ROS content caused by high fat diet (HFD).

Table 6A.6: Effect of AKSS16-LIV01 on serum antioxidant parameters in high fat diet (HFD) induced obesity in mice

Parameters	ROS (FIU)	SOD (U/mg)	CAT (U/mg)	GSH (mmoles/mg Protein)	Gpx (mmoles/mg Protein)
Control	412.59±14.3	5.53±0.62	14.85±1.02	35.14±3.36	20.09±0.95
HFD (30% fat of total diet)	736.13±17.8$^{\#}$	2.23±0.95$^{\#}$	6.71±1.25$^{\#}$	19.91±4.15$^{\#}$	10.27±2.25$^{\#}$
HFD + AKSS-16 (75 mg/kg)	510.27±14.71*	4.52 ±0.32*	8.02±0.96*	25.47±4.69*	15.62±1.48*
HFD + AKSS-16 (150 mg/kg)	492.81±11.24*	4.96 ±0.75*	10.58±0.94*	29.02±3.48*	18.56±0.98*
HFD + AKSS-16 (300 mg/kg)	434.51±9.65**	5.28±0.85**	14.12±1.12**	37.31±3.78**	22.69±1.48**
Std Drug	478.64±12.69**	4.99 ±0.91**	12.95±1.03**	28.94±5.94**	19.65±0.96**
AKSS-16 (300 mg/kg)	450.27±11.28**	5.21 ±0.54**	13.65±1.67**	35.47±4.85**	20.36±1.25**

Values are mean of six individual *****(observations in each group ± S.D. Significantly different from control $^{\#}$($p<0.001$), and significantly different from HFD group ***** ($p<0.05$), ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

6A.3.7 Effect of AKSS-16-LIV-01 on tissue antioxidant status

The activities of the antioxidant enzymes SOD, CAT, GSH and GPx were decreased prominently in liver tissue of HFD control when compared with normal group. Administration of AKSS-16-LIV-01 both 150 mg/kg/day and 300 mg/kg/day for 42 days significantly increased the antioxidant enzyme activities such as SOD, CAT, GSH and GPx in liver tissue (Figure 6A.5) . Application of standard drug also showed significant effect but more positive effect observed in AKSS-16-LIV-01 (300 mg/kg/day) group. Treatment with only AKSS-16-LIV-01 300 mg/kg/day alone has no significant effect on tissue lipid peroxidation.

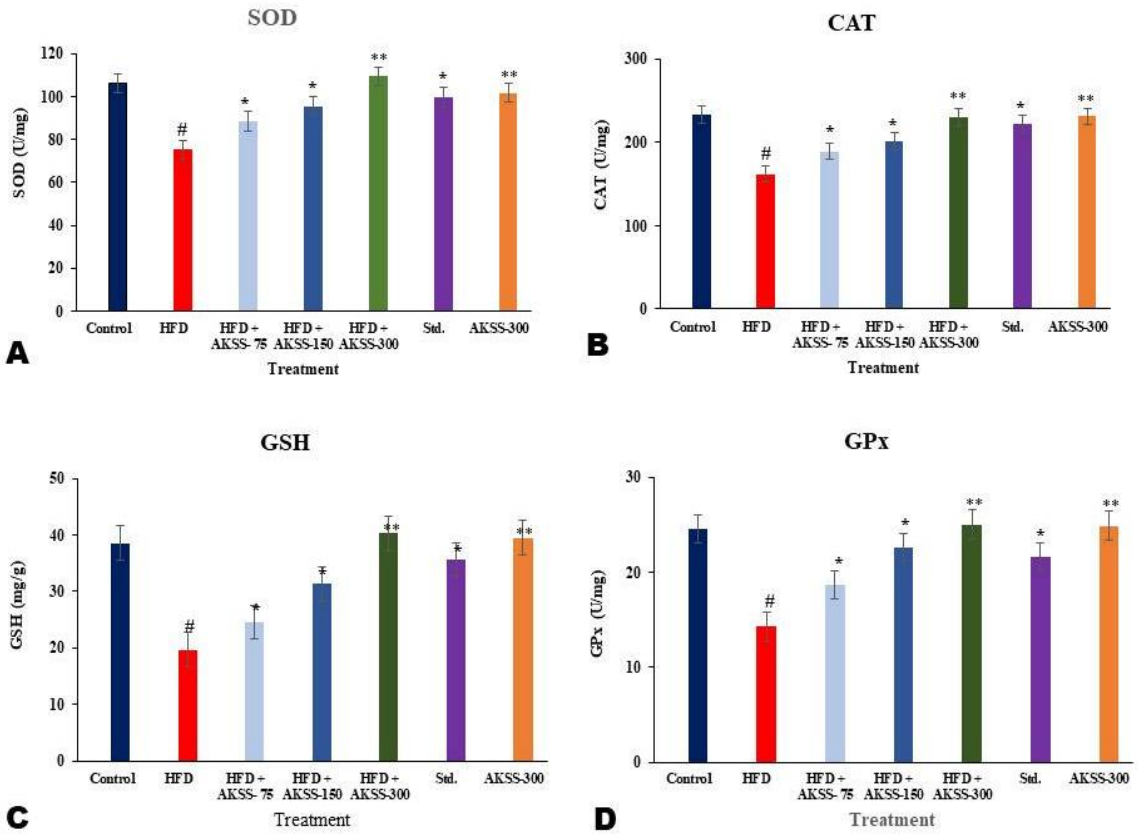


Figure 6A.5: Free radical scavenging activity (SOD, CAT, and GSH & Gpx). Values are expressed as Mean ± SD (n = 6 per group). *Significantly different from control $^{\#}$ ($p<0.001$) and significantly different from HFD ***** ($p<0.05$) ****** ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test. SOD: Super oxide dismutase, CAT: Catalase, GSH: Glutathione, GPx: Glutathione Peroxidase.

6A.3.8 Effect of AKSS-16-LIV-01 on Histopathology of liver

The liver of the High fat diet group was significantly enlarged and the colour of liver becomes pale. The physical appearance, i.e. colour, size and smoothness of the liver of AKSS-16-LIV-01 supplemented with HFD group remain unaltered comparing those of the normal control group. Figure 6, shows the microscopic appearance of the liver tissues. Steatosis (fatty change) clearly existed in the liver tissue of the HFD group as shown in Fig. 6A.6. Lipid accumulation in the hepatocytes as vacuoles, and large droplets were observed in most hepatocytes, in particular in the periportal regions. In contrast administration of AKSS-16-LIV-01, protected the liver tissue from high fat diet, little or no fatty droplets were observed in the AKSS-16-LIV-01 treated group, and decreased the number of vacuoles appeared (Figure 6A.6).

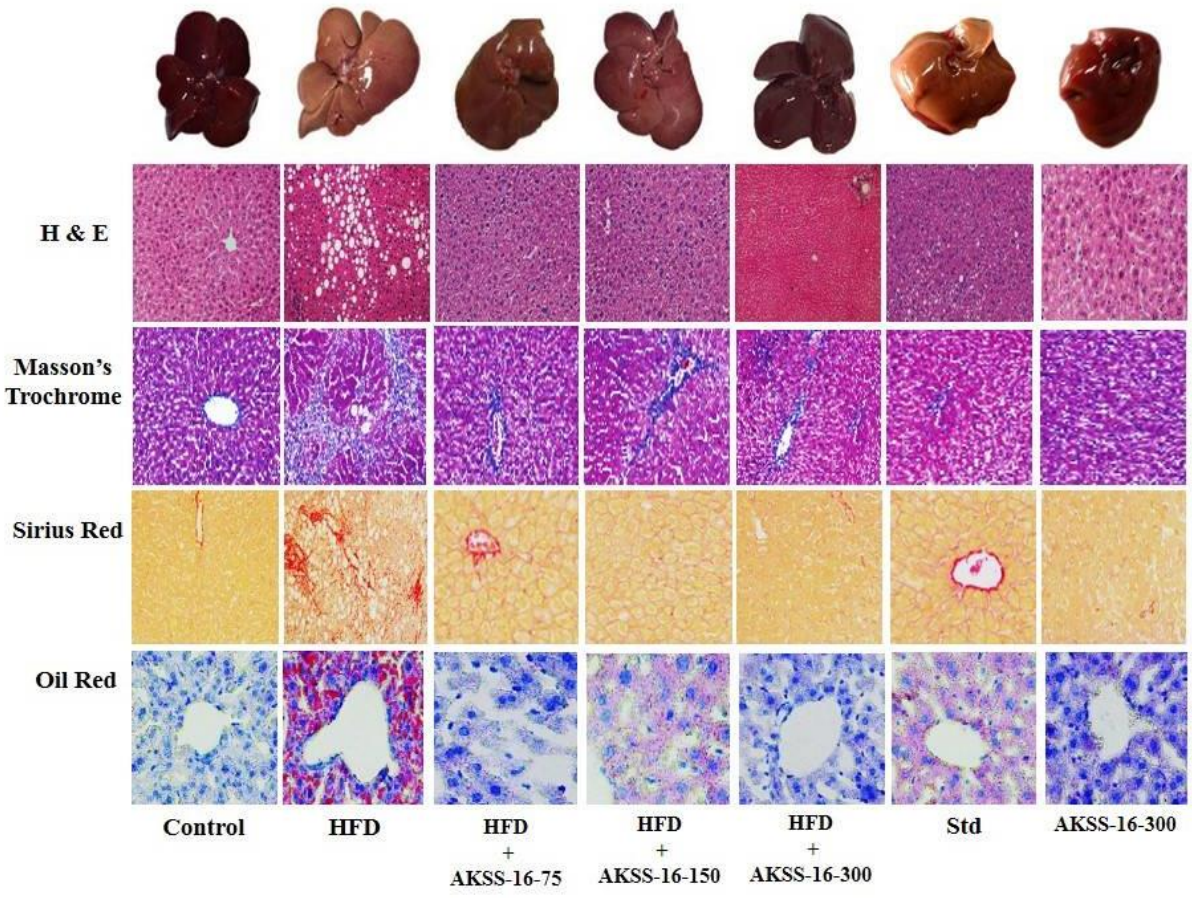


Figure 6A.6: Ameliorative effect of AKSS-16-LIV01 on different morphology & Histology under HFD induced Obesity in mice.

No fatty infiltration was observed in the Normal group. Mice in the HFD group developed macrovesicular steatosis, steatohepatitis changes, inflammation, and massive infiltration of inflammatory cells. Multi herbal formulation ameliorated these morphological features in the HFD+ AKSS-16-LIV-01 groups. Moreover, compared with the HFD group, hepatocyte lipid accumulation, especially in the HFD+ AKSS-16-LIV-01 groups, was significantly decreased Figure 6A.6.

Tables 6A.7: Effect of AKSS16-LIV01 on Histopathological scoring of liver section both HFD and treatment groups.

Groups	**Injury of scores**				
Treatment	**Fatty degeneration**	**Necrosis**	**Cell swelling**	**Inflammation**	**Total score**
Group-I	0	0	0	0	0
Group-II	4	4	3	3	14
Group-III	1	0	1	1	3
Group-IV	1	1	0	0	2
Group-V	0	0	0	0	0
Group-VI	1	0	0	1	2
Group-VII	0	0	0	0	0

6A.4 DISCUSSION

Obesity is closely associated with liver abnormalities, includings liver fibrosis, steatohepatitis, fatty liver, non-alcoholic fatty liver disease (NAFLD) [30, 31][36,37]. People all over the globe are really worried about that. To overcome this situation presently scientists and researchers are immersed in research to find out a permanent safe and symptomatic anti-obesity solution. Unfortunately, different indirect strategies like diet, exercise & yoga, behavioural adaptation modifications not only make a solution to prevent obesity but also showed several health complications. Therefore, world need a novel, effective, and safe antiobesity interventions which not only prevent obesity but also minimise the related complications. High-fat diet (HFD) is a major cause of obesity which is closely linked to a variety of health issues, including coronary heart disease, high blood[32] pressure, stroke, liver diseases, diabetes and cancer[33] [38]. Reactive Oxygen Species (ROS) are closely associated with obesity leads to numerous degenerative diseases such as atherosclerosis, ischemic heart disease and aging, etc. In the obese condition ROS generally suppressed the antioxidant enzymes activity which disrupt the antioxidant defence mechanism produces cellular imbalance and ultimately cell apoptosis [32, 34, 35] [39-41]. So, oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of linked diseases[36, 37] [42,43]. In the present study, administration of high fat diet (HFD) showed an increase in TC, TG, LDL-C and VLDL-C level and decrease HDL-C level in mice. Furthermore, administration of AKSS-16-LIV-01 at a dose of 300mg /kg/day showed a significant ($p<0.001$) reduction in TC, TG, LDL-C and VLDL-C levels and increase in HDL-C level in HFD treated mice. Deviations from cholesterol transport increases LDL levels or decreases HDL cholesterol flux, which may result in accumulation of cholesterol in extra hepatic tissues. Therefore, from the present findings it is presumed that the prevention of hyperlipidemia may be due to the inhibition of biosynthesis of cholesterol and triglycerides by the developed novel formulation.

Scientific study revealed that oxidative stress is a causal factor that associates hyperlipidemia with the pathogenesis of obesity. HFD disrupt body's homeostasis and generate free radicals which produces oxidative stress [38-41] [44-47]. In the present study appears to validate the view that the HFD group mice have shown a marked increase in MDA levels in both serum and liver tissues. Our study showed

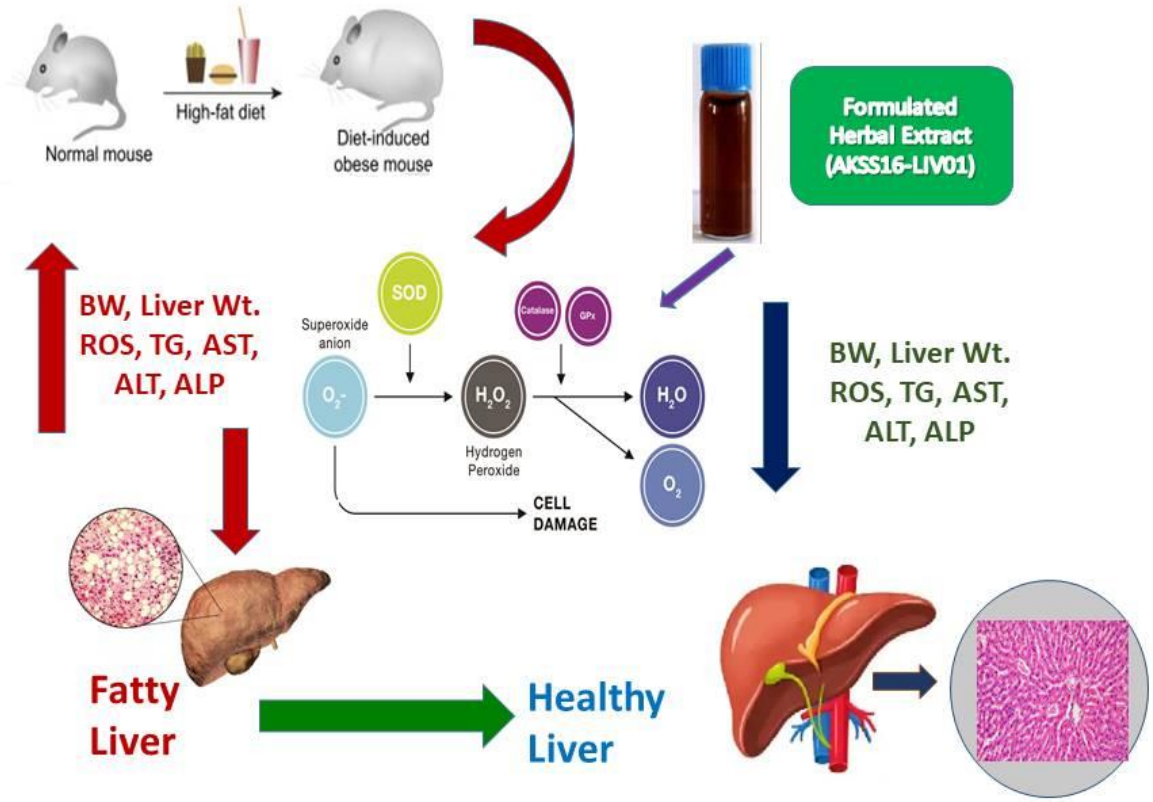
that treatment with the novel multi herbal formulation AKSS-16-LIV-01 at a dose of 300mg /kg/day prevented the lipid peroxidation in the serum and liver tissues of hyperlipidemic obese mice. Our study also indicate that obese mice showed marked decline in the SOD, catalase, GSH and GPx essential first and second order antioxidant enzymes activities when compared with the normal control, and the treatment with AKSS-16-LIV-01 at a dose of 300mg /kg/day has significantly ($p<0.001$) restored these enzymes in a dose-dependent manner when compared with the HFD group animals. Our study also established that AKSS-16-LIV-01 with the therapeutic optimum dose control the obesity as compared to standard marketed drug.

Microscopic and histologic examinations revealed reduction in the mass of adipose tissue and adipocyte cell sizes of the HFD + AKSS-16-LIV-01 treated groups as compared to the HFD group. The liver weight and number of lipid drops also decreased in the HFD+ AKSS-16-LIV-01 treated organ. Scientific study established that adipose tissue is widely distributed in the body and acts as a key energy reservoir [45]. The microscopic images have shown marked reduction of lipid accumulation in adipocyte of visceral adipose tissue of HFD group animals on the administration of AKSS-16-LIV-01 at a dose of 300mg /kg/day. Moreover histological scoring of the liver tissue clearly showed that fatty degeneration, necrosis, cell swelling and inflammation were completely in control as compared with HFD group.

CONCLUSION

Our developed novel multi herbal formulation (AKSS-16-LIV-01) composed of six Indian medicinal herbs and three Indian medicinal spices have a potent anti-obesity activity in experimental animals. AKSS-16-LIV-01showed optimum hypolipidemic and antioxidant properties at a dose of 300mg /kg/day against HFD induced obese mice. In addition, the AKSS-16-LIV-01 significantly protected the liver tissues against oxidative stress triggered by HFD, as a new therapeutic medicine. The present study also successfully demonstrated a modified method for lipid staining using Oil Red O dye which helps observing the lipid droplets in the cells drawn from in vivo system. Further research is required to validate the other possible mechanism of action of AKSS-16-LIV-01 responsible for hypolipidemic activity and effects on hepatic dysfunctions and to understand the molecular pathway.

SUMMARY



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6B Carbon Tetrachloride (CCl_4) induced serum and tissue protein alterations and its mitigation by novel multi herbal formulation (AKSS16LIV01) in experimental murine model

6B.1 INTRODUCTION

The body needs *protein* to function and survive. Alteration of serum total protein leads to various complications and sometimes damage vital organs [1]. The major components of the serum protein are albumin and globulin, represents the nutritional status of the body which maintain the colloidal osmotic pressure in blood [2-4]. These two proteins also maintain body's immune function, prevent infection [5]. Scientific literature revelled that albumin-to-globulin ratio (AGR) is a prognostic factor of various diseases and medical complications [6,7]. Clinical study indicate that decline albumin levels showed poor nutrition status, sometimes very fatal to survive [8,9].

Carbon tetrachloride (CCl_4) is a major industrial pollutant associated with production of free radicals which creates various organ dysfunction like liver and kidney [10]. It is established that metabolic activation of CCl_4 by cytochrome P450 produced trichloromethyl radical ($\cdot CCl_3$) and peroxy trichloromethyl radical ($\cdot OOCCl_3$) which initiates lipid peroxidation, responsible for membrane disruption leads to liver and kidney injury [11]. Long-time exposure of CCl_4 alter the normal protein level in the body which creates various type of organ dysfunctions [12]. Animal study showed that administration of CCl_4 decrease normal food and water intake produces nutrition deficiency syndrome [13].

Long term safe and symptomatic medication without side effects is one of the main approach of alternative alternative system of medicine comprising herbal products [14]. The plant based formulation is enrich with various essential phytochemicals and enormous antioxidants, serrates to prevent diseases [15,16]. We developed a novel, low cost herbal formulation composed of medicinal plants and spices. Our previous study upon animals showed that this formulation does not produces any toxic effects and safe for therapeutic medication [17,18]. Here we try to apply this traditional medicine for maintained the essential protein levels caused by CCl_4.

6B.2 METHODOLOGY

6B.2.1 Chemicals

Carbon tetrachloride (CCl_4) and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. total protein, albumin and globulin were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

6B.2.2 Animals

Twenty four young, healthy swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

6B.2.3 Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received Multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg).

6B.2.4 Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

6B.2.5 Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

6B.2.6 Preparation of tissue homogenate

A small portion of the liver and kidney tissues was homogenized in ice-cold 0.9% w/v saline using a homogenizer to obtain 20% homogenate. Aliquots of the liver homogenate were stored at 4°C prior to biochemical analysis.

6B.2.7 Determination of serum, liver and kidney protein

Serum and tissue homogenate were used for the determination of total protein, albumin and globulin. Total protein, albumin and globulin were determined according to the standard biochemical protocol with slight modification using colorimetric kit obtained from Thermo Scientific, USA.

6B.2.8 Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

6B.3 RESULTS

6B.3.1 Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in Table 6B.1. Administration of carbon tetrachloride (CCl_4) (1 ml/kg-bw) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity and reduced the liver weight as compared with control animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Table-6B.1: Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	**Mice**			
	Group-I	**Group-II**	**Group-III**	**Group-IV**
Body weight (g) Initial	26.35±1.91	26.51±2.35	26.71±4.2	26.68±5.1
Body weight (g) Final	37.84±2.03	36.94±1.69	21.81±2.41$^{\#}$	36.97±1.67*
Body weight (g) gain or loss	11.49±0.06	10.43±0.04	4.90±0.006	10.29±0.03
Food consumption (g)	4.52±0.05	4.37±0.07	2.94±0.06$^{\#}$	5.11±0.04*
Water intake (ml)	4.01±0.04	4.25±0.04	3.01±0.02$^{\#}$	4.31±0.06*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from (CCl_4) group values at $p<0.001$

6B.3.2 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney total protein

Figure 6B.1 shows the mean serum, liver and kidney total protein (TP) levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney total protein compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline total protein levels when compared with CCl_4 treated mice. 28days treatment with newly developed

multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney protein levels when compared with control group.

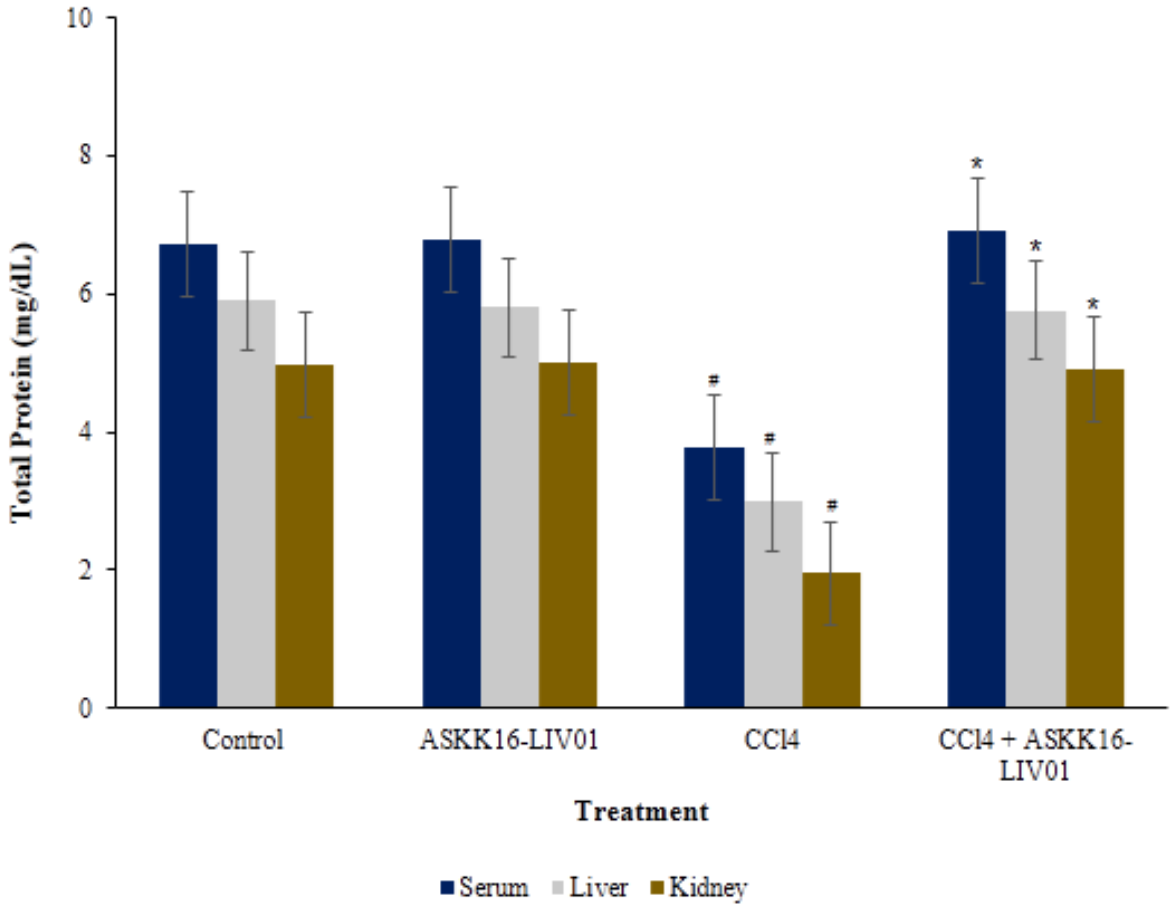

Figure 6B.1: Effect of multi herbal formulation (AKSS16-LIV01) on Total protein levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

6B.3.3 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin

Figure 6B.2 shows the mean serum, liver and kidney albumin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney albumin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline albumin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney albumin levels when compared with control group.

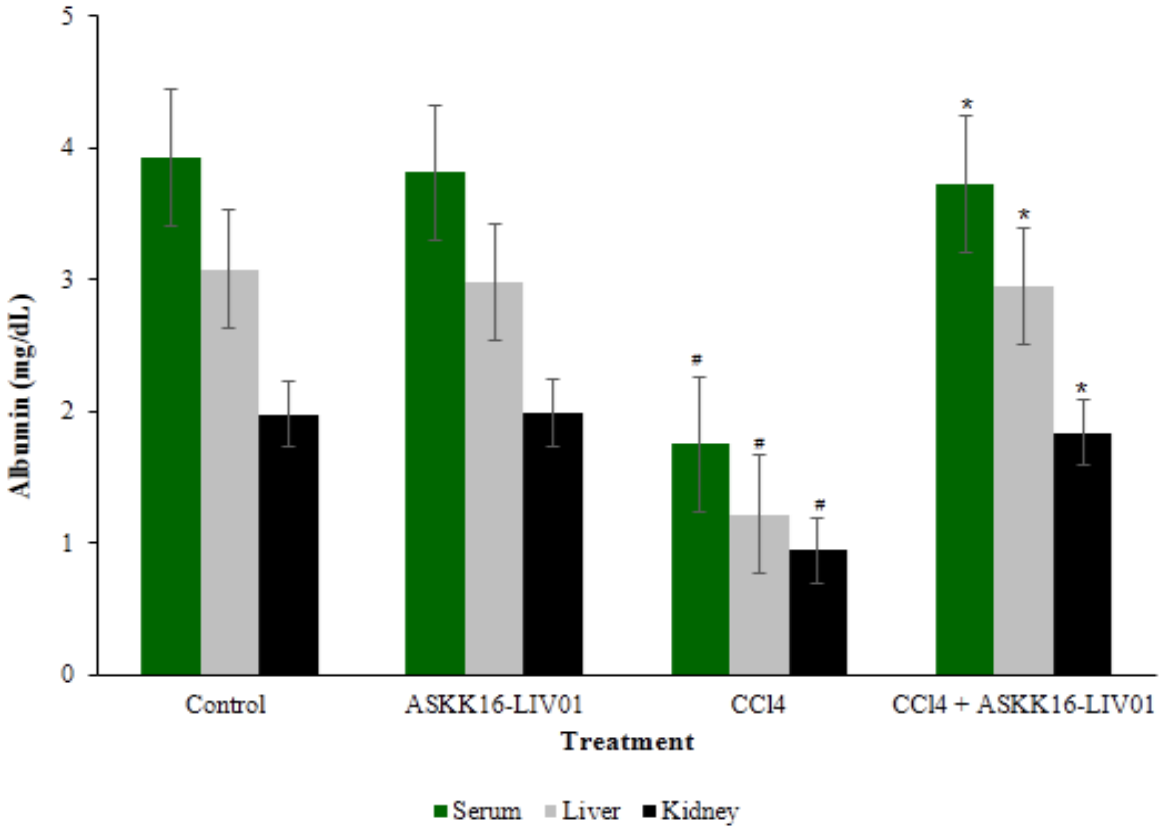

Figure 6B.2: Effect of multi herbal formulation (AKSS16-LIV01) on albumin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

6B.3.4 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney globulin

Figure 6B.3 shows the mean serum, liver and kidney globulin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney globulin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline globulin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney globulin levels when compared with control group.

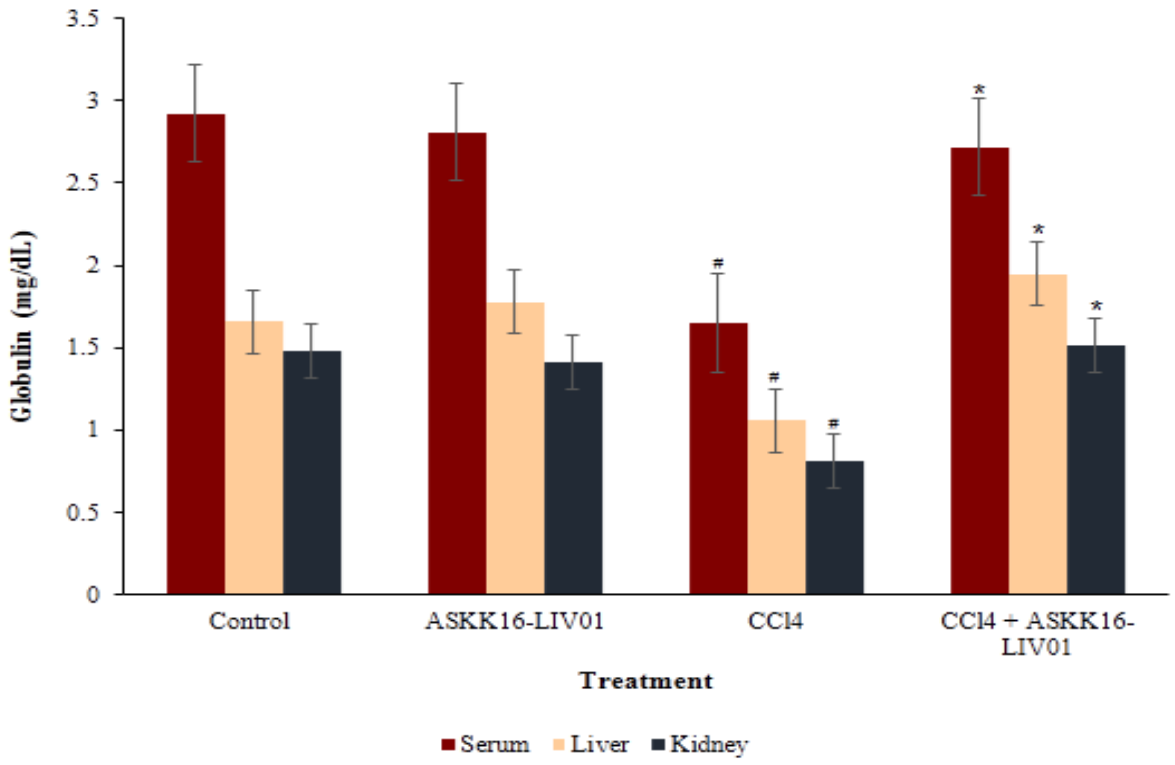

Figure 6B.3: Effect of multi herbal formulation (AKSS16-LIV01) on globulin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (CCl_4) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

6B.3.5 Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin/globulin ratio

Table 6B.2 shows the mean serum, liver and kidney albumin/globulin ratio in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney albumin/globulin ratio compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline albumin/globulin ratio levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney albumin/globulin ratio levels when compared with control group.

Table 6B.2: Effect of AKSS16-LIV01 on serum, liver and kidney albumin/globulin in CCl_4 induced toxicity

Groups	Albumin/Globulin ratio		
	Serum	Liver	Kidney
Control	1.32±0.12	1.45±0.14	1.48±0.11
AKSS16-LIV01	1.31±0.11	1.37±0.16	1.41±0.12
CCl_4	1.76±0.16	1.58±0.14	1.57±0.15
CCl_4 + AKSS16-LIV01	1.26±0.13	1.43±0.19	1.50±0.18

6B.4 DISCUSSION

Various secondary metabolites of the medicinal plants are mainly responsible for therapeutic effects [21]. Poly herbal drug are very useful for treatment of various diseases due to their synergistic effects of different plants [22]. Phenolic compounds and flavonoids present in the aromatic plants are mainly responsible for pharmacological functions and prevent oxidative stress [23]. Our study showed that administration of carbon tetra chloride (CCl_4) inhibit normal body growth, food consumption and water intake. Co administration of our developed formulation retained the body weight, food consumption and water intake.

Protein is responsible for normal body growth and development. Abnormal protein level inhibit the body growth which may be occur when subject exposed with environmental toxin [24,25]. Total serum protein is an indicator in liver and kidney damage [26]. In the present study we observed that carbon tetra chloride (CCl_4) significantly decreased the serum, liver and kidney protein levels. Co administration with AKSS16-LIV01 maintained the normal serum, liver and kidney protein levels.

Albumin play a crucial role to maintain physiological activities of human body [27,28]. It is one of the liver biomarker as it generates from the liver cells. Low level of albumin is responsible for poor nutrition [29-32]. In this study we observed that chronic administration of CCl_4 decline normal albumin levels in serum, liver and kidney which was recovered when animals pre-treated with novel multi herbal formulation (AKSS16-LIV01). The result clearly indicate that AKSS16-LIV01 capable to maintain the normal albumin level against the environmental toxicant like CCl_4. On the other hand scientific study revealed that serum globulin is involved in chronic inflammation. Recent study showed that carbon tetra chloride (CCl_4) alter the serum, liver and kidney globulin and disrupt normal homeostasis. Our study also confirms that application of CCl_4 decreased normal globulin levels in serum, liver and kidney. Treatment with the developed formulation (AKSS16-LIV01) normalized the globulin level in experimental animals. Albumin/globulin ratio also confirms the protein alteration.

CONCLUSION

Chronic administration of carbon tetrachloride (CCl_4) suppressed the normal body growth and reduced normal food and water intake capacity in mice. This environmental toxin reduced the total protein, albumin and globulin levels both in serum and tissues. Our developed novel multi herbal formulation might be able to maintain the normal essential protein values and prevent the CCl_4 induced deleterious effects in mice. Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future.

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Chapter - VII

A Novel Phytomedicine, AKSS16-LIV01 ameliorates chronic kidney disease (CKD) through redox modulation in experimental animal

7A A Novel Phytomedicine ameliorates Chronic Kidney Disease (CKD) through redox modulation in mice

7A.1 INTRODUCTION

Chronic kidney disease (CKD) the gradually decreases normal kidney function and if persists kidney failure occurs. CKD is one of the serious health complications all over the globe [1]. Various factors like continuous exposure of toxins, drugs, environmental toxic substances, junk food habit etc. generate reactive oxygen species (ROS) developed chronic kidney disease [2]. Unwanted but inevitable by-product of aerobic oxygen metabolism generates ROS which might be fatal for cell. Enormous amount of ROS production in renal system leads to developed various undesirable physiological complications which affects normal kidney function [3,4]. Renal oxidative stress through ROS also developed renal inflammation which indirectly correlated with diabetes, cancer, and atherosclerosis. Imbalance between production of ROS and cellular antioxidant defence might be dangerous for normal cellular function and disrupt normal renal homeostasis [5]. Excessive production of ROS loses cellular normal physiological condition and for continuation developed cell apoptosis [6]. Function of kidney when gradually decreased it directly hamper the filtration process of the body and also produced urinary abnormality. The understanding that proper cell functioning critically requires a dynamic balance between oxidative stress and distress (i.e., cellular redox homeostasis) forms the conceptual framework of redox medicine, a novel therapeutics that reduces the oxidative distress while maintaining the normal redox circuitry [7-9].

Cisplatin (CP) is a well-established popular chemotherapeutic agent which is extensively used for the treatment of various solid tumors, including ovarian, cervical, testicular, and non-small-cell lung cancer throughout the world. Clinical literature reported that one third of the patients who received treatment with cisplatin (CP) drug through prescription developed clinical nephrotoxicity, as indicated by reduced glomerular filtration rate, increased serum BUN, urea, creatinine, and deregulated serum electrolyte levels [10-12]. Medical practitioners restrict prescribed this drug as well as controlled its doses for the therapy for cancer treatment due to its excessive adverse drug reaction (ADR). Administration of high-dose and over dose of cisplatin generates different serious side effects, particularly nephrotoxicity. Proximal convoluted tubules (PCT) is the targeted site of drug deposition initially slower down the glomerular filtration and finally close down the process [13,14]. Scientific publication stated that chronic used of cisplatin developed chronic kidney disease (CKD) through ROS generation [15]. But till today exact mechanism of cisplatin-induced nephrotoxicity remains incompletely understood. Very limited research has been suggested that renal tubular cell apoptosis and inflammatory responses play an important role in the pathogenesis of cisplatin-induced nephrotoxicity [16,17].

Researcher and scientific community all over the globe engaged to invent an effective medication for chronic kidney disease (CKD) but unfortunately till today we have no effective and fruitful medication which can eradicate this serious complication permanently [18-21]. Now a day's natural therapies have a great interest for prevention of many diseases because of their lesser side effects and low cost. Moreover, the therapeutic efficacy of the natural products is very high due to presences of various

phytochemicals [22-26]. With this notion in mind we have focused to develop a new medication composed of Indian medicinal plants and spices, which are potentially sound for various diseases due to their synergistic action. In this study, our major aim was to develop novel multi herbal formulation (AKSS16-LIV01) as a redox medicine against CKD,a serious global public health problem (with 8–16% worldwide prevalence) that originates from redox imbalance due to mitochondrial dysfunction.

7A.2 MATERIAL AND METHODS

7A.2.1 Chemicals and reagents

Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), NaCl, NaOH, ethanol, TRIS buffers were purchased from SISCO laboratories, India. PBS buffer (pH 7.2) was purchase from Sigma-Aldrich, Germany. All the biochemical kits (BUN, albumin, urea, creatinine etc.) were procured from Autospan Liquid Gold, Span Diagnostic Ltd., India. GFR disagnistic kit was purchased from FITC–inulin, Sigma, USA. Hydroxyproline assay kit was procured from Bio Vision, Milpitas, CA. Antioxidant kits (SOD, CAT, GSH and GPx) were obtained from Boehringer, USA. ELISA kit IL-6, IL-10 and TNF-α were procured from Merck Millipore, Billerica, MA, USA. All others reagents utilized in this study are laboratory grade.

7A.2.2 Collection of herbs and Preparation of herbal formulation

All the required medicinal plants and species were procured from registered suppliers and authenticated by renounced taxonomist, department of Pharmaceutical Technology, Jadavpur University, Kolkata, India. After washing and dyeing the formulation was prepared according to standard protocol. Details of the protocol were given in the previous chapter.

7A.2.3 Animals and treatment

Adult healthy swiss albino mice (8–10 weeks old, weighing 27 ± 2.3 g) were used for the experiment. All animals were kept in clean polypropylene cages (temperature 22 ± 2 °C; relative humidity 45–55%; 1:1 light and dark cycle). Pathogen free clean water *ad libitum* and standard laboratory pellet diet (Saha Enterprise, Kolkata, India) were given to the animals throughout the experimental period. They were allowed to acclimatize for 14 days before the treatment. The guideline of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India, was followed and the study was approved by the Institutional Animal Ethics Committee (Ethical Clearance No. - IAEC/PHARM/1503/03/ 2019 dated 30.11.19).

The mice were randomly divided into four groups (n = 10 for each group): (1) control; (2) cisplatin; (3) cisplatin + AKSS16-LIV01 and (4) only AKSS16-LIV01 (without cisplatin). The experimental model of CKD was established according to the established method. In brief, for induction of CKD, we used 8 mg kg^{-1} BW cisplatin (i.p.) in each alternative day for 30 days. After induction, we treated AKSS16-LIV01 at 200 mg kg^{-1} BW (orally) for another 30 days. There was an overlap of 7 days between induction and treatment. All the doses were finalized based on reported literature and pilot experimentation.

7A.2.4 Body weight gains, water intake and feed efficiency

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Water intake was monitored on daily basis and after collection of all data then finalized the mean result. Feed conversion was obtained by dividing total feed intake by body weight gain.

7A.2.5 Biochemical evaluations.

Whole blood samples from treated mice were collected from retro-orbital sinus plexus and centrifuged at 2500 × g for 20 min to separate the serum. Urine samples were collected in metabolic cages during 24-h fasting conditions. Biochemical parameters [27,28] such as blood urea nitrogen (BUN), serum albumin, urea and creatinine were performed using commercially available kits (Autospan Liquid Gold, Span Diagnostic Ltd., India) following the protocol described by respective manufacturers. GFR was estimated by the determination of urinary excretion of fluorescein-labeled inulin (FITC–inulin, Sigma, USA).

7A.2.6 Renal hydroxyproline measurement

For the measurement of renal hydroxyproline content, a previously described method was used[32]. In brief, snap-frozen kidney specimens (200 mg) were weighed hydrolyzed in HCl (6 M; Merck, USA) for 12 h at 100 °C. Next, they were oxidized with Chloramine-T (SRL, India). Next, Ehrlich reagent (Sigma, USA) was added which resulted in the formation of a chromophore [29]. Absorbance was measured at 550 nm. Data were normalized to kidney wet weight.

7A.2.7 Renal homogenate preparation

Freshly collected kidney tissue were homogenized in cold phosphate buffer [(0.1 M; pH 7.4), and centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatants were collected for further experimentation.

7A.2.8 Levels of Malonedialdehyde (MDA)

Lipid peroxidation was estimated in the serum and tissue samples by measuring the malondialdehyde (MDA) formation using the thiobarbituric acid method [30]. Briefly, 100 µL of animals was mixed with 500 µL of 150 mM Tris-HCl and 1.5 mL of 0.375% TBA and vortexed for 10 sec. Then the action mixture was incubated at 100°C for 45 min in a water bath. At the end of incubation, the samples were centrifuged at 1000 ×g for 10 min. The MDA content was calculated from the absorbance measurement at 532 nm and using a Shimadzu spectrophotometer (Tokyo, Japan) an absorption coefficient = $1.56 \times 105\ cm^{-1}\ M^{-1}$.

7A.2.8 Determination of NO and iNOS activity

The level of NO and iNOS activity were determined from the liver homogenate through ELISA method using the commercial kit [31].

7A.2.10 Determination of ROS activity

Amount of ROS in blood was measured [32] using 2', 7'-dichlrofluorescein dictate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci *et al*. 20 Fluorescence was determined at 488 nm excitation and 525 nm[25] emission using a fluorescence plate reader (Tecan Spectra Fluor Plus).

7A.2.11 Assessments of antioxidant enzymes

First and second order antioxidant enzymes were measured according to standard protocol with slight modification. These are as follows:

7A.2.12 Determination of superoxide dismutase (SOD)

The activity of superoxide dismutase (SOD) was measured according to a well-established reported method [33]. In brief, 2.5 mL reagent solution (xanthine 0.3 mM, 581 EDTA 0.65 mM, 140 μM NBT), sodium carbonate 0.4 M, and bovine albumin (35 mg/30 mL) was added to 0.1 mL sample and 50 μL xanthine oxidase (10 μL in 2 M ammonium sulphate), incubated at 25 °C for 20 min and mixed with, 0.1 mL 8 M copper chloride. The developed color was measured at nm and calculates the result with using co-factor.

7A.2.13 Determination catalase (CAT)

Catalase activity was measured according to the established method [34]. Briefly, on the aftermath of the addition of 5 μL kidney homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 593 7.0), change in absorbance at 240 nm up to 1 min to determine catalase activity. The enzymatic activity was expressed as U/mg protein.

7A.2.14 Determination of glutathione (GSH) and GPx content

Reduced glutathione (GSH) was determined from kidney homogenate spectrophotometrically according to Ellman's method [35]. GPx activity was assayed using a modified method of Hafeman. *et. al* [36].

7A.2.15 Cytokines Assays

Plasma IL-6 and IL-10 samples were analysed by the standard established method through ELISA [37]. In addition, plasma levels of tumor necrosis factor-alpha (TNF-α) were measured using ELISA kits (Merck Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Protein concentrations were measured according to the manufacturer's guideline [38].

7A.2.16 Histological examination

After incision, tissues from randomly selected mice were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into a 5 μm thick section. After de-waxing and gradual hydration with ethanol (Merck, USA), the sections were stained with hematoxylin and eosin (SRL, India). Another set of

sections were stained with periodic acid–Schiff (PAS) [39. The sections were then observed under an optical microscope (Olympus, Tokyo, Japan). It is noteworthy to mention here that the histopathologist was impartial to the treatment groups while scoring and evaluating the samples.

7A.2.17 Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p < 0.05 was considered significant.

7A.3 RESULTS

7A.3.1 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption, Water Intake and Kidney weight*

Table 7A.1 shows the changes in gross body weights and its changes, food consumption and water intake in the four experimental groups of animals. The gross body weight, food consumption and water intake capacity of the animals administered with Cisplatin (8 mg kg^{-1} BW) significantly reduced ($p<0.001$) as compared to the control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 200mg/kg/day normalized the body weight, daily food intake and water intake capacity. Administration of AKSS16-LIV01 alone did not show any abnormal changes as compared with control animals. Gross kidney weight of the mice treated with Cisplatin (8 mg kg^{-1} BW) significantly increased ($p<0.001$) as compared to the control animals (Figure 7A.1). After treatment with multi herbal formulation (AKSS16-LIV01) 200mg/kg/day normalized the kidney weight as compared with cisplatin treated animals. Treatment with only AKSS16-LIV01did not produced any changes.

Table 7A.1: Effect of herbal composition (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	**Groups**			
	Control	Cisplatin	Cisplatin + AKSS16-LIV01	AKSS16-LIV01
Body weight (g) Initial	25.28±2.56	26.07±5.46	25.19±4.81	25.55±2.45
Body weight (g) Final	39.64±2.25	19.27±2.31$^{\#}$	37.67±1.57*	40.91±2.69
Body weight (g) gain or loss	14.36±0.06 (+)	6.8±0.006 (-)	12.48±0.03 (+)	15.36±0.04 (+)
Food consumption (g)	4.61±0.05	2.89±0.05$^{\#}$	5.18±0.04*	4.29±0.07
Water intake (ml)	4.22±0.04	2.71±0.04$^{\#}$	4.51±0.06*	4.08±0.05
Kidney weight (g)	0.41±0.02	0.56±0.04$^{\#}$	0.40±0.03*	0.44±0.03

All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. $^{\#}$Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

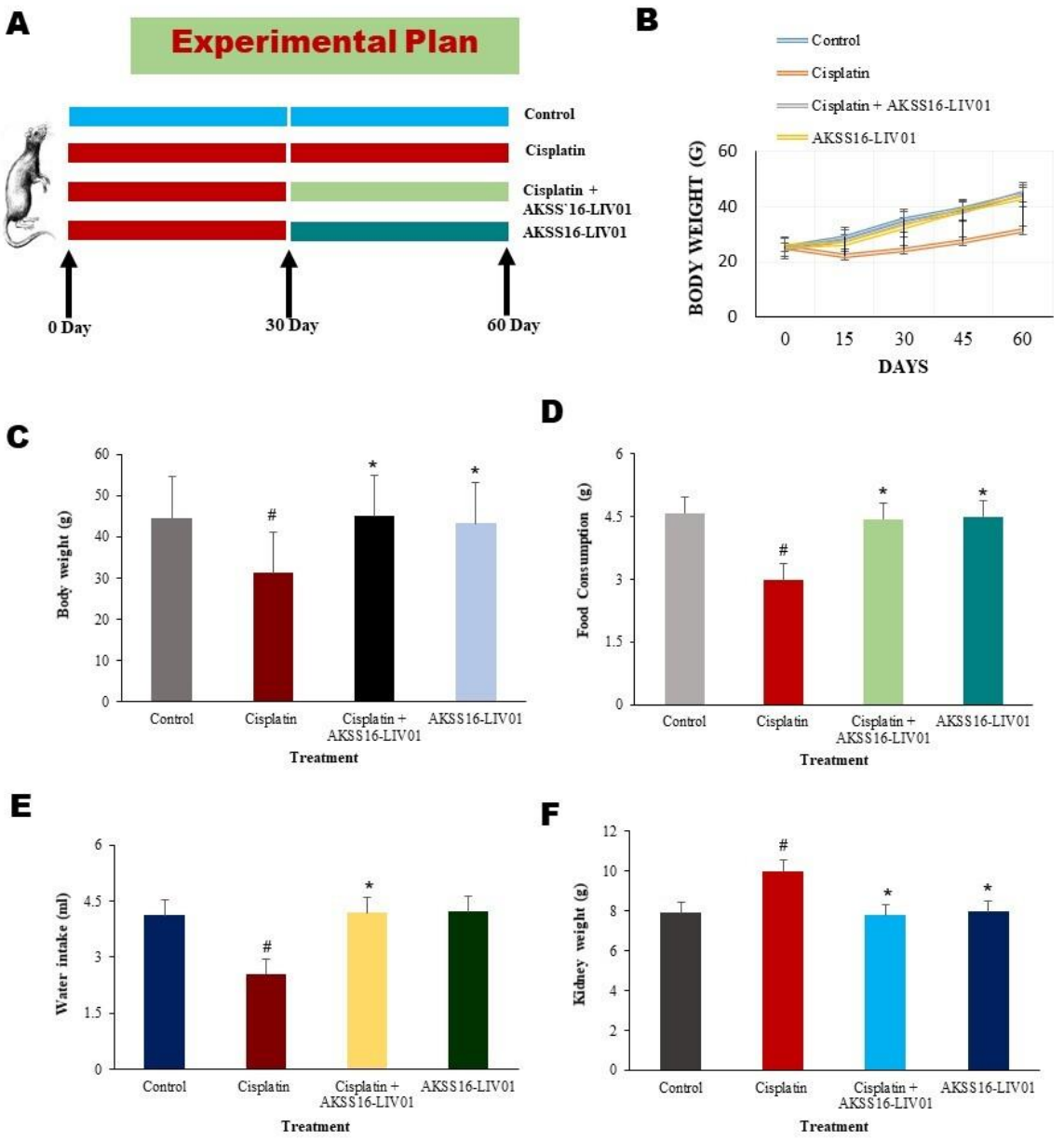


Figure 7A.1. Therapeutic effect of multi herbal formulation (AKSS16- LIV01) against Cisplatin induced chronic kidney disease. (A) Experimental design, (B) Gross body weight (C) Changes of body weight (D) Food consumption (E) Water intake and (F) Kidney weight. All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

7A.3.2 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on GFR, Urine ACR, Creatinine clearance, uric acid and Hydroxypoline level in cisplatin toxicity*

Table 7A.2 shows the changes in GFR, Urine ACR, Creatinine clearance, uric acid and Hydroxypoline level upon experimental animals. GFR, Urine ACR, uric acid and Hydroxypoline level of the animals administered with Cisplatin (8 mg kg^{-1} BW) significantly increased ($p<0.001$) whereas creatinine clearance significantly decreased ($p<0.001$) as compared to the control animals. Treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 200mg/kg/day normalized the in GFR, Urine ACR, Creatinine clearance, uric acid and Hydroxypoline level in mice. Administration of AKSS16-LIV01 without Cisplatin did not show any abnormal changes as compared with control animals. Administration of Cisplatin (8 mg kg^{-1} BW) directly effects GFR, Urine ACR, Creatinine clearance hydroxypoline and uric acid levels and completely restored the changes (Table 7A.2) after administration of AKSS16-LIV01.

Table 7A.2: Effect of Novel Multi Herbal Formulation (AKSS16-LIV01) on nephrotoxic biomarkers.

Group	GFR (µL min−1 g−1 BW)	Urine ACR	Creatinine clearance (µmol min−1)	Uric acid (mg dL−1)	Hydroxypoline (mg g^{-1} tissue)
Control	11.26±0.95	0.35±0.06	1.62±0.07	1.29±0.12	0.56±0.04
Cisplatin	32.54±3.65$^{\#}$	4.98±0.09$^{\#}$	0.47±0.03$^{\#}$	3.11±0.54$^{\#}$	1.61±0.07$^{\#}$
Cisplatin + AKSS16-LIV01	14.25±1.02*	0.84±0.04*	1.48±0.04**	1.38±0.24*	0.68±0.05*
AKSS16-LIV01	10.51 ±1.11*	0.51±0.05*	1.56±0.04*	1.24±0.18*	0.51±0.03*

Data expressed as Mean ± SD (N = 10/group). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed for comparison among the groups. GFR glomerular filtration rate, ACR albumin to creatine ratio. #$p < 0.001$ compared to Cisplatin treated animals. *$p < 0.001$ compared Control animals.**$p < 0.05$ compared Control animals.

7A.3.3 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on serum and kidney MDA content, iNOS concentration and* Na-K-ATPase activity *in cisplatin toxicity*

Serum and tissue (kidney) lipid peroxidation (MDA levels) are depicted in Table 7A.3. Serum MDA and tissue content in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly higher than that of the controls (101.52±1.87 vs 45.14 ±1.64 nmol/g). Treatment with herbal combination (AKSS16-LIV01) significantly reduced serum MDA content compared with Cisplatin intoxicated mice (49.43±0.91 vs 101.52±1.87 nmol/g). Renal MDA content in the Cisplatin intoxicated mice was significantly high than that of controls (61.85±0.96 vs 32,98±2.08 nmol/g). Treatment with multi herbal formulation (AKSS16-LIV01) alone significantly decreased (Table 7A.3) kidney MDA content compared with Cisplatin intoxicated mice (34.77±0.87 vs. 61.85±0.96 nmol/g). Administration of

AKSS16-LIV01 alone did not show any abnormal changes of the said parameters as compared with control animals.

Nitric oxide synthase (iNOS) concentration in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly increased (1.18±0.08 vs 0.36±0.06 µmol/mg protein) than that of the controls animals. Application of multi herbal formulation (AKSS16-LIV01) significantly reduced iNOS concentration compared with Cisplatin intoxicated mice (0.41±0.05 vs 1.18±0.08 µmol/mg protein). Application of multi herbal formulation (AKSS16-LIV01) alone did not show any abnormal changes of the said parameters as compared with control animals.

On the other hand in Cisplatin (8 mg kg^{-1} BW) intoxicated mice Na-K-ATPase activity was significantly decreased (4.18±0.91 vs 9.32±1.26 nmol Pi/min/mg) than that of the controls animals. Treatment with multi herbal formulation (AKSS16-LIV01) significantly elevated Na-K-ATPase activity (Table 7A.3) compared with Cisplatin intoxicated mice (11.48±1.02 vs 4.18±0.91 nmol Pi/min/mg). Multi herbal formulation (AKSS16-LIV01) did not produce any abnormal changes upon experimental animals.

Table 7A.3: Effect of Novel Multi Herbal Formulation (AKSS16-LIV01) on nephrotoxic biomarkers.

Group	Serum MDA (nmol/g)	Kidney MDA (nmol/g)	iNOS (µmol/mg protein	Na-K-ATPase activity (nmol Pi/min/mg)
Control	45.14 ±1.64	32.98±2.08	0.36±0.06	9.32±1.26
Cisplatin	101.52±1.87$^{\#}$	61.85±0.96$^{\#}$	1.18±0.08$^{\#}$	4.18±0.91$^{\#}$
Cisplatin + AKSS16-LIV01	49.43±0.91*	34.77±0.87*	0.41±0.05*	11.48±1.02**
AKSS16-LIV01	42.11±1.02*	35.12±1.09*	0.35±0.05*	9.58±0.87*

Data expressed as Mean ± SD (N = 10/group). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed for comparison among the groups. GFR glomerular filtration rate, ACR albumin to creatine ratio. #p < 0.001 compared to Cisplatin treated animals. *p < 0.001 compared Control animals.**p < 0.05 compared Control animals.

7A.3.4 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on blood urea nitrogen (BUN), albumin, Urea and Creatinine in cisplatin toxicity*

Table 7A.4 shows the changes in blood urea nitrogen (BUN), serum albumin, Urea and Creatinine levels Upon experimental animals. Blood urea nitrogen (BUN), Urea and Creatinine of the animals administered with Cisplatin (8 mg kg^{-1} BW) significantly increased (p<0.001) whereas serum albumin level significantly decreased (p<0.001) as compared to the control animals. Treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 200mg/kg/day normalized the in blood urea nitrogen (BUN), Urea, Creatinine and serum albumin levels in mice. Administration of AKSS16-LIV01 without Cisplatin did not show any abnormal changes as compared with control animals. Administration of Cisplatin (8 mg kg^{-1} BW) directly effects blood urea nitrogen (BUN), Urea,

Creatinine and serum albumin levels and completely restored the changes (Table 7A.4) after administration of AKSS16-LIV01.

Table 7A.4: Effect of Novel Multi Herbal Formulation (AKSS16-LIV01) on nephrotoxic biomarkers.

Group	BUN (mg/dL^{-1}))	Albumin (g/ dL^{-1})	Urea (mg/dL^{-1}))	Creatinine (mg/dL^{-1})
Control	9.58±1.95	6.35±0.26	52.52±4.26	0.79±0.11
Cisplatin	22.59±3.85$^{\#}$	2.98±0.49$^{\#}$	224.58±8.91$^{\#}$	3.81±0.34$^{\#}$
Cisplatin + AKSS16-LIV01	12.25±1.45*	5.84±0.24*	61.48±5.02**	0.98±0.21*
AKSS16-LIV01	10.88 ±1.27*	5.51±0.25*	51.56±3.27*	0.84±0.17*

Data expressed as Mean ± SD (N = 10/group). One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was performed for comparison among the groups. GFR glomerular filtration rate, ACR albumin to creatine ratio. #p < 0.001 compared to Cisplatin treated animals. *p < 0.001 compared Control animals. **p < 0.05 compared Control animals.

7A.3.5 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on serum and renal antioxidant enzymes activity in cisplatin toxicity*

Super oxide dismutase (SOD) activities of serum and kidney are depicted in Figure 7A.2&3**.** Serum and tissue activity of SOD in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was decreased significantly (p<0.001) than that of the controls. Treatment with multi herbal formulation (AKSS16-LIV01) significantly increased (p<0.001) serum and tissue SOD activity compared with cisplatin intoxicated mice.

Serum and kidney catalase (CAT) activities are depicted in Figure 7A.2&3**.** Serum and tissue CAT activity in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly less (p<0.001) than that of the normal untreated animals. Treatment with multi herbal formulation (AKSS16-LIV01) significantly increased (p<0.001) serum and tissue CAT activity compared with cisplatin intoxicated mice.

Serum and kidney reduced glutathione (GSH) activities are depicted in Figure 7A.2&3**.** Serum GSH activity in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly reduced (p<0.001) than that of the controls. Treatment with multi herbal formulation (AKSS16-LIV01) significantly increased (p<0.001) kidney and serum GSH activity compared with cisplatin intoxicated mice.

Serum and renal Glutathione –S Transferase (GST) activities are depicted in Figure 7A.2&3**.** Serum GSH activity in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly declined (p<0.001) than that of the controls. Treatment with multi herbal formulation (AKSS16-LIV01) significantly elevated (p<0.001) serum and tissue GST activity compared with cisplatin intoxicated mice.

Serum and renal Glutathione peroxidase (GPx) activities are depicted in Figure 7A.2&3**.** Serum GPx activity in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly less (p<0.001) than that of

the controls. Treatment with multi herbal formulation (AKSS16-LIV01) significantly increased ($p<0.001$) Serum GPx activity compared with cisplatin intoxicated mice.

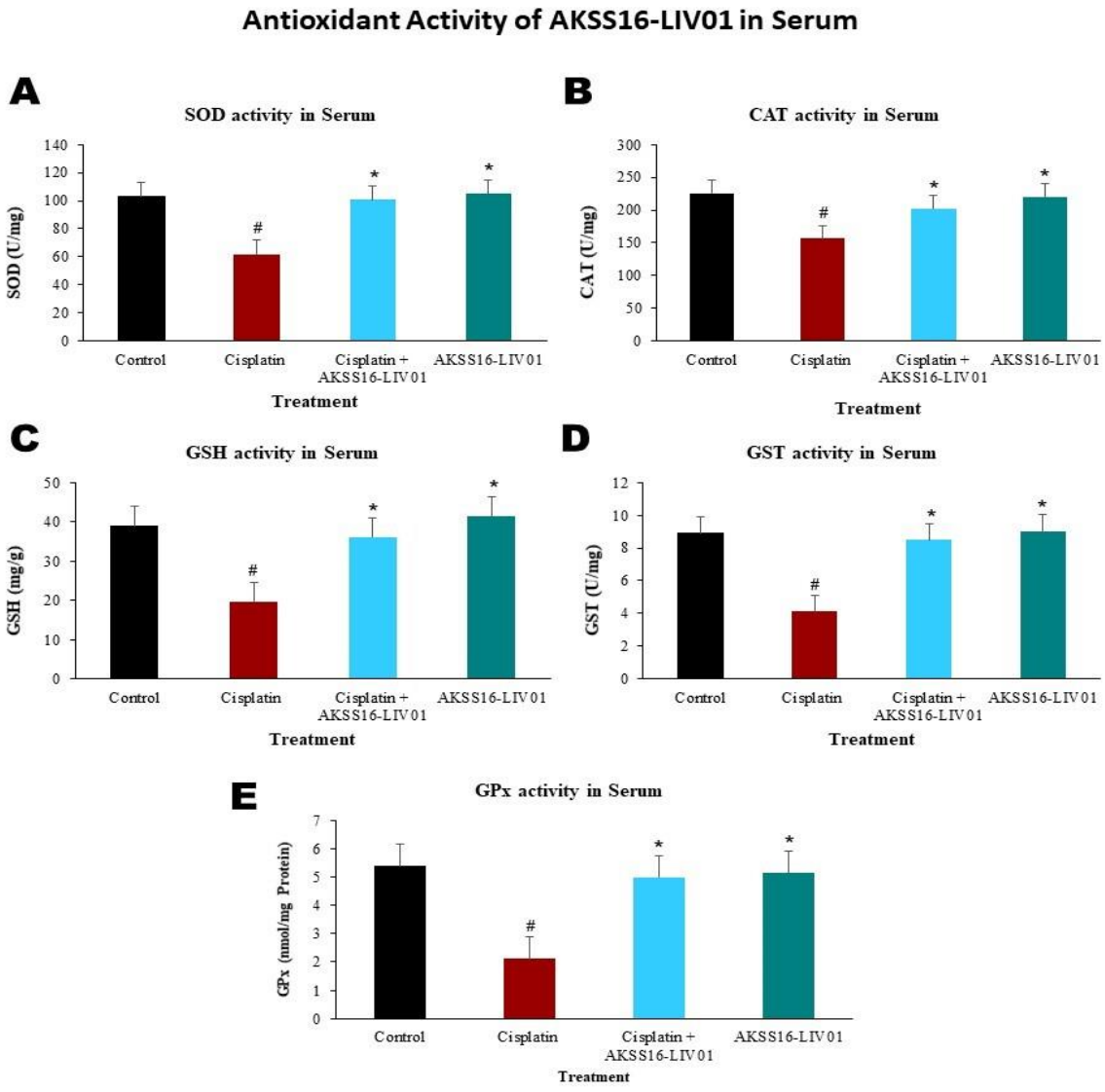


Figure 7A.2. Therapeutic effect of multi herbal formulation (AKSS16- LIV01) against Cisplatin induced chronic kidney disease. (A) Serum SOD activity, (B) Serum CAT activity (C) Serum GSH activity (D) Serum GST activity and (E) Serum GPx activity. All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

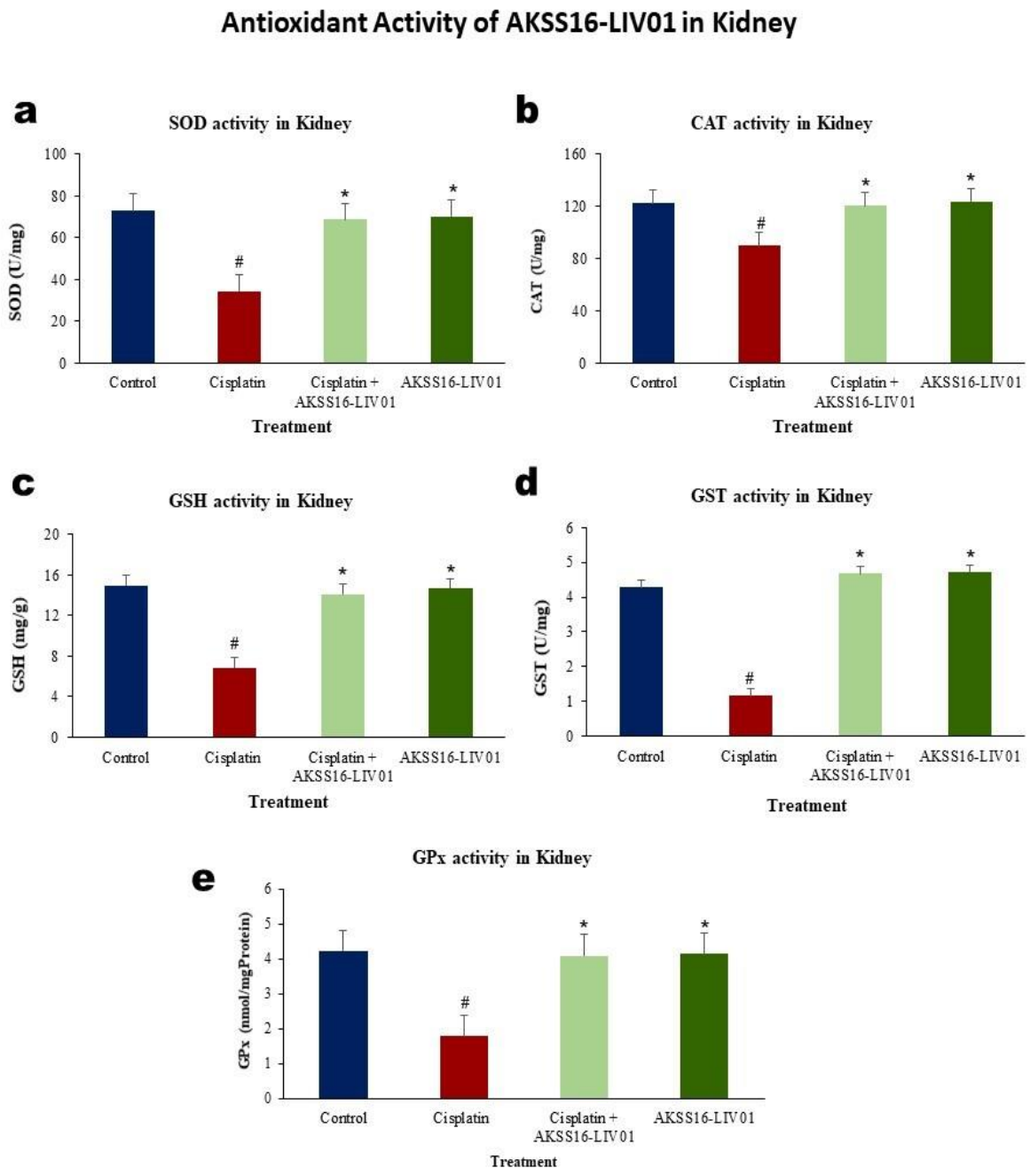


Figure 7A.3. Therapeutic effect of multi herbal formulation (AKSS16- LIV01) against Cisplatin induced chronic kidney disease. (A) Kidney SOD activity, (B) Kidney CAT activity (C) Kidney GSH activity (D) Kidney GST activity and (E) Kidney GPx activity. All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

7A.3.6 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on total ROS, NO level, activity of IL-6, TNF-α and IL-10 in cisplatin induced toxicity*

Total ROS and NO activities are depicted in Figure 7A.4**.** ROS and NO level in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly higher ($P<0.001$) than that of the controls animals. Treatment with multi herbal formulation (AKSS16-LIV01) significantly decreased ($P<0.001$) ROS concentration and NO level compared with Cisplatin (8 mg kg^{-1} BW) intoxicated mice.

Cytokines like IL-6, TNF-α and IL-10 are depicted in Figure 4**.** IL-6 and TNF-α level in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly higher than that of the controls ($P<0.001$). Treatment with multi herbal formulation (AKSS16-LIV01) significantly decreased IL-6 and TNF-α level compared with cisplatin intoxicated mice ($P<0.001$). Level of IL-10 in the Cisplatin (8 mg kg^{-1} BW) intoxicated mice was significantly lower than that of the controls ($P<0.001$). Treatment with multi herbal formulation (AKSS16-LIV01) significantly increased IL-10 level compared with cisplatin intoxicated mice ($P<0.001$).

Chronic administration of Cisplatin (8 mg $kg^{-1\ BW}$) on experimental mice excessively increased total ROS content (612.34 ± 11.05) in comparison to normal untreated animals ((388.78 ± 10.55). Normal health condition of the mice were altered and so many behavioral changes noted. On the other hand application of newly developed multi herbal formulation (AKSS16-LIV01) significantly reduced the elevated ROS level (432.24 ± 14.19) in compared with Cisplatin (8 mg $kg^{-1\ BW}$) induced experimental mice (612.34 ± 11.05). The positive protection made due to synergistic effects of medicinal herbs and medicinal spices, which is an unique blend. Application of multi herbal formulation (AKSS16-LIV01) alone without Cisplatin did not produced any changes of ROS level indicate the newly developed formulation have no toxic and adverse effects on animals.

Intoxication with Cisplatin (8 mg $kg^{-1\ BW}$) on mice model significantly increased signaling cytokines IL-6 (118.75 ± 9.54) and TNF-α level (232.41 ± 8.92) in comparison to normal untreated animals (52.18 ± 6.56 & 78.54 ± 6.28). On the other hand application of newly developed multi herbal formulation (AKSS16-LIV01) significantly reduced the elevated IL-6 (68.75 ± 5.12) and TNF-α level (97.02 ± 6.11) in compared with Cisplatin (8 mg $kg^{-1\ BW}$) induced experimental mice (118.75 ± 9.54 & 232.41 ± 8.92). The therapeutic recovery was made due to synergistic effects of medicinal herbs and medicinal spices, which is an unique blend. Application of multi herbal formulation (AKSS16-LIV01) along without Cisplatin did not produced any changes of IL-6 (56.99 ± 3.58) and TNF-α level (86.22 ± 8.07) level indicate the newly developed formulation have no toxic and adverse effects on animals. Level of IL-10 was significantly reduced (88.25 ± 4.85) after application of Cisplatin (8 mg $kg^{-1\ BW}$) as compared with normal untreated animals (162.58 ± 6.91). Therapeutic application of newly developed multi herbal formulation (AKSS16-LIV01) significantly increased IL-10 level (151.36 ± 4.91) in compared with Cisplatin (8 mg $kg^{-1\ BW}$) induced experimental mice (162.58 ± 6.91). Administration of AKSS16-LIV01 did not produced any effects on experimental mice.

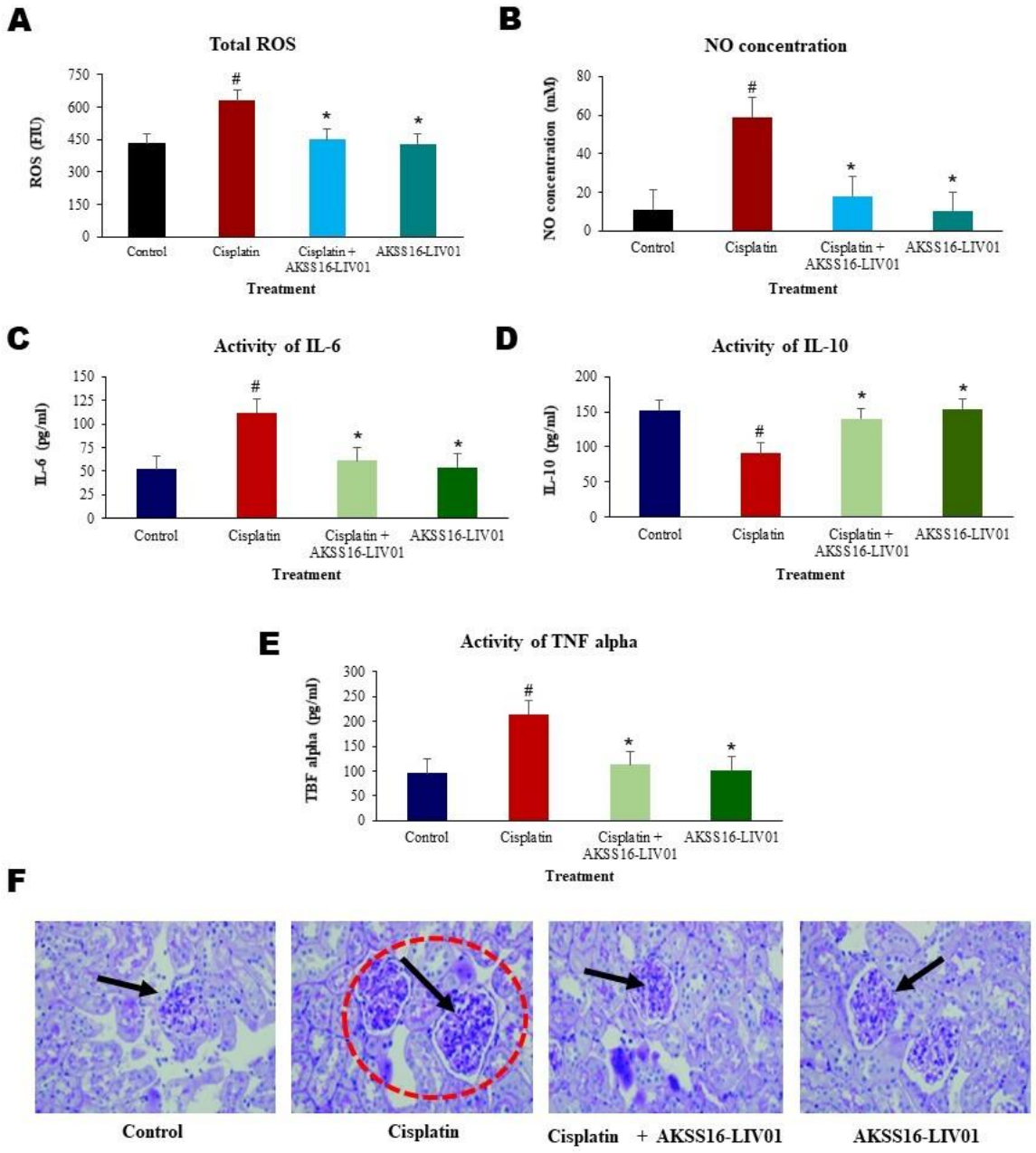


Figure 7A.4. Therapeutic effect of multi herbal formulation (AKSS16- LIV01) against Cisplatin induced chronic kidney disease. (A) RO activity, (B) NO activity (C) IL-6 level (D) IL-10 level (E) TNF-α level and (F) PAS staining sections of kidney. All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

7A.3.7 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on kidney index and morphology*

Figure 7A.5 shows the changes in gross morphology of kidney and kidney index in the four groups of experimental animals. The kidney weight and index of the animals administered with Cisplatin (8 mg kg^{-1} BW) significantly increased ($p<0.001$) as compared to the control animals. Treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 200mg/kg/day significantly ($p<0.001$) reduced the kidney weight and index. Administration of AKSS16-LIV01alone did not show any abnormal changes as compared with control animals. In gross morphology of the colour of the kidney in cisplatin treated animals was completely change (Pale brown colour) whereas it is restored by the administration of AKSS16-LIV01 (Figure 7A.5).

Dark reddish brown colour of kidney which is marker of normal and healthy kidney compleately converted into yellowish pale brown colour after long term administration of Cisplatin (8 mg $kg^{-1\ BW}$), a popular drug commonly used in cancer therapy. To overcome the drug induced kidney injury (DIKI) we administered our newly developed multi herbal formulation (AKSS16-LIV01) consisting of six Indian medicinal plants and three Indian common medicinal spices at a dose of 200mg/kg/day throughout the recovery period. The morphology of the kidney as well as texture and colour showed darkish brown that clearly indicate the therapeutic efficacy of the formulation. On the other hand chronic administration of Cisplatin (8 mg $kg^{-1\ BW}$) significantly increased gross kidney weight and kidney index which indicate the deleterious effect of Cisplatin on renal system. Therapeutic oral application of newly developed unique multi herbal formulation (AKSS16-LIV01) significantly reduced the elevated gross kidney weight and kidney index as compared with Cisplatin induced experimental animals. The effect caused due to the synergistic effects of multi herbal formulation (AKSS16-LIV01) as the presences of various active ingredients within it. AKSS16-LIV01 administration alone without Cisplatin did not produced any changes of gross kidney weight and kidney index indicate the newly developed formulation have no toxic and adverse effects on animals. The formulation successfully protects the kidney against drug induced renal complication.

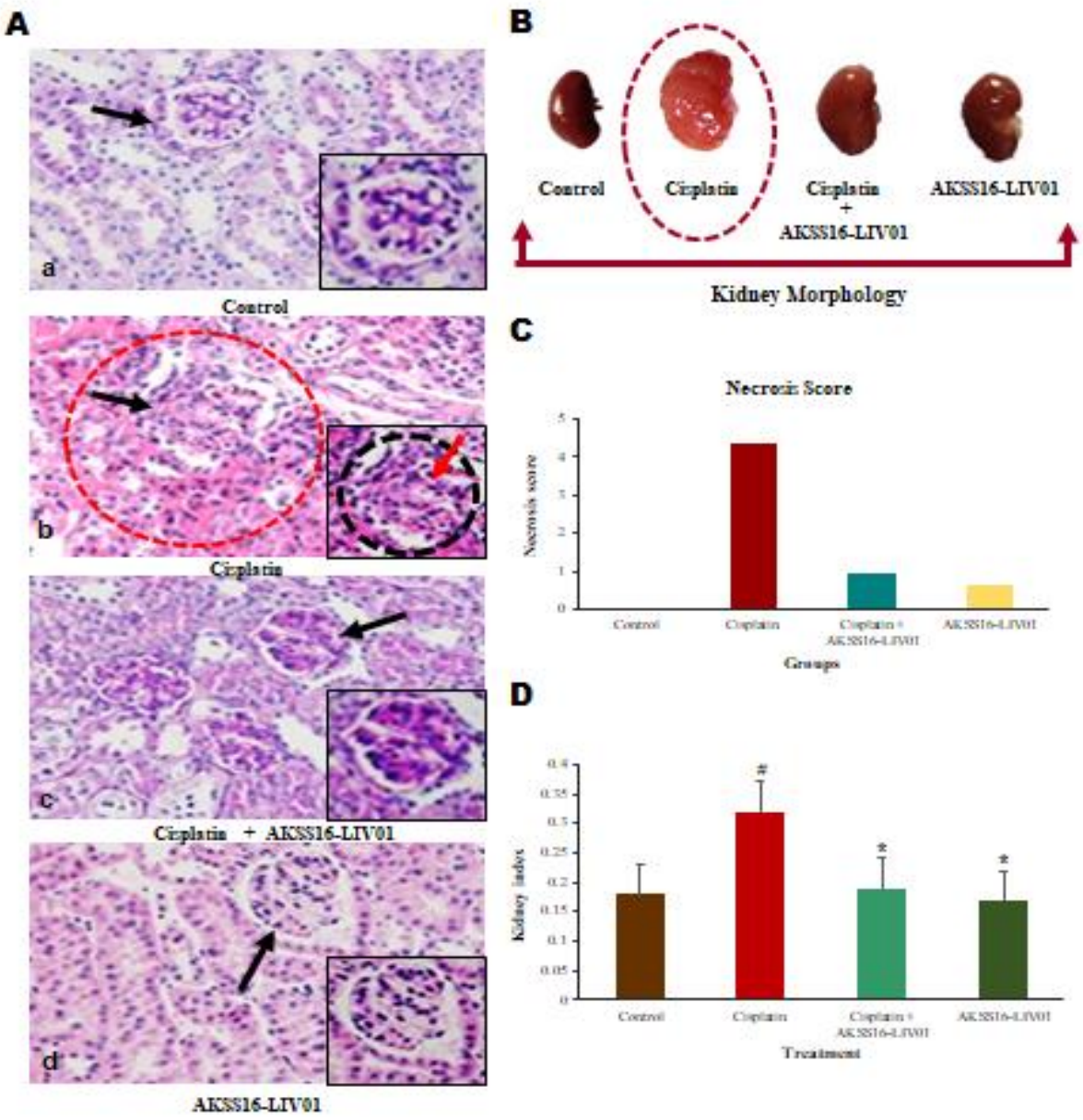


Figure 7A.5. Therapeutic effect of multi herbal formulation (AKSS16- LIV01) against Cisplatin induced chronic kidney disease. (A) Histology of kidney sections, (B) Morphology of kidney (C) Kidney necrosis score and (D) Kidney index. All data were expressed as means± SE (n=10/group). Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from Cisplatin group values at $p<0.001$

7A.3.8 *Therapeutic effect of multi herbal formulation (AKSS16-LIV01) on kidney histology*

Renal histopathological changes of mice should be taken into consideration to assess the efficacy of AKSS16-LIV01 in protecting kidney from Cisplatin (8 mg kg^{-1} BW)-induced renal damage (Figure 7A.4). Light microscopy examination of renal tissues in normal mice revealed normal glomerulus structure and renal tubular interstitial with no evidence of cell necrosis and inflammatory infiltration (Figure 7A.5a). The mice treated with cisplatin showed typical damage characteristics, for example, necrosis and shedding of renal tubular epithelial cells, vacuolization of the renal cortex and inflammatory infiltrations (Figure 7A.5b). In comparison, treatment with AKSS16-LIV01 reduced the number of apoptotic and infiltration of inflammatory cells (Figure 7A.5c). PAS staining of the kidney section also directly established that application of novel multi herbal formulation suppresses the cisplatin induced kidney damage by its synergistic action. The renal tubular necrosis score was significantly reduced compared with that in the cisplatin group, suggesting that AKSS16-LIV01 exerted potential renal protection on cisplatin-induced nephrotoxicity (Figure 7A.4F).

7A.4 DISCUSSION

Long term consumption of cisplatin accumulated in the renal parenchymal cells which decreased the blood level concentration of cisplatin and increased the kidney concentration. This condition increased the blood creatinine level which leads to extensive renal damage by failure of creatinine clearance [40-44]. Here our results also showed that chronic administration of cisplatin elevated blood urea nitrogen (BUN), urea and creatinine clearance. Treatment with AKSS16-LIV01 inhibit the cisplatin related renal damage and normalized all the said parameters.

Nephrotoxicity is frequently noticed in about 25–35% of patients through cisplatin application. The drug significantly reduced renal function when applied even on a single dose. Till now the molecular mechanism of cisplatin induced nephrotoxicity is not fully known [45,46].

Different scientific studies have established that generation of excessive ROS developed oxidative stress involved for cellular apoptotic changes through signalling pathways. Action of ROS on renal cells are involved in the pathogenesis of acute kidney injury [47,48]. Our study demonstrated that cisplatin significantly elevated total ROS concentration as well as declined levels of various antioxidant enzymes as super oxide dismutase (SOD), catalase (CAT), glutathione –S transferase (GST), reduced glutathione (GSH) and glutathione peroxidase (GPx). The deleterious action of cisplatin was restored by our developed multiherbal formulation (AKSS16-LIV01). The study also confirmed that the developed formulation did not produce any adverse effects on experimental animals.

TNF-α is a typical infammatory mediator, which promotes cytokines generation and infammation reaction by stimulating neutrophils and macrophages, ultimately resulting in cellular necrosis or apoptosis [49]. Similarly, IL-6 and IL-10 also involved inflammatory reactions as stimulates the development and differentiation of the immune system [50]. In this work, multi herbal formulation (AKSS16-LIV01) was reported to inhibit the overproduction of TNF-α and IL-6 in cisplatin-induced kidney injury in swiss albino mice. On the other hand decline the levels of IL-10 induced by cisplatin

were normalized by AKSS16-LIV01, depicting the suppression the inflammation associated with excessive cisplatin-induced loss of renal function.

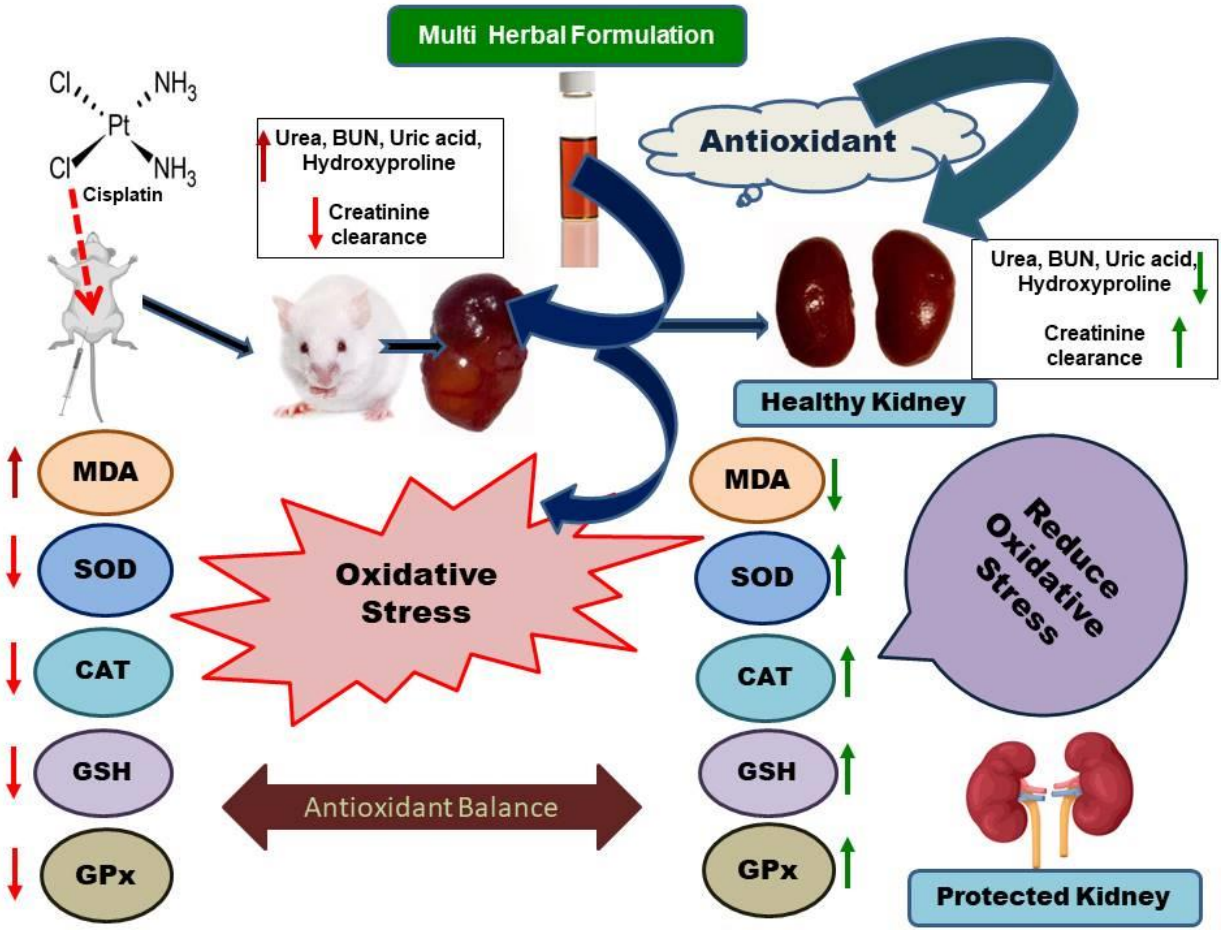
Production and generation of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) and its regulation under physiological and pathophysiological conditions is well established [51]. Overproductions of NO in the mammalian system are very harmful for cell because it is an important biological mediator in the living organism.

On the summary, we can say this study provides a new foresight into the potential molecular mechanisms through the newly developed novel multi herbal formulation (AKSS16-LIV01) and protects cells against cisplatin-induced injury and maintains normal cellular physiological activity and function. Treatment with AKSS16-LIV01 suppressed cisplatin-induced apoptosis, oxidative stress and immunological alteration via the activation of antioxidant enzymes systems. Administration of AKSS16-LIV01 may be considered as a therapeutic strategy to prevent cisplatin-induced chronic renal injury. Thus chronic kidney disease may be treated in future through this natural medicine.

CONCLUSION

Treatment of chronic kidney disease (CKD) is a scientific challenge. There is no fruitful drug still now that prevents CKD permanently. Little literature have been published that focuses on oxidative stress being one. Our study showed that multi herbal formulation of the prime causes that pursuit kidney damage (AKSS16-LIV01) could be an efficient newly developed herbal medicine that attenuate renal injury by preventing oxidative stress and improve normal renal function. The formulation also controls all the altered biochemical parameters and protect kidney against oxidative injury. It also maintained Na/K balance of kidney and maintained kidney homeostasis. So, we could suggest that this newly developed multi herbal formulation (AKSS16-LIV01) may an effective therapy in future against chronic kidney disease.

SUMMARY



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Chapter - VIII

Therapeutic effect of Andrographis paniculata, A component of AKSS16-LIV01 against Phenylhydrazine hydrochloride (PTZ) induced Hyperbilirubinemia: A Safe and Symptomatic Medication

8A Therapeutic and symptomatic medicinal use of *Andrographis paniculata* for hyperbilirubinemia treatment in mice

8A.1 INTRODUCTION

Hyperbilirubinemia is the most prevalent clinical conditions in newborn [1]. Neonatal hyperbilirubinemia is a common clinical problem encountered during the neonatal period, especially in the first week of life [2]. Nearly 8% to 11% of neonates develop hyperbilirubinemia. When the total serum bilirubin (TSB) rises above the 95th percentile for age (high-risk zone) during the first week of life, it will be considered as hyperbilirubinemia [3]. Between 60%–80% of healthy infants are expected to present with idiopathic neonatal jaundice [4]. Neonatal jaundice is the discoloration of skin and sclera color to yellowish in a newborn by bilirubinTherefore it can create concern in the physician and anxiety in the parents.

The pathophysiology of jaundice and the metabolism of bilirubin take place in three phases: viz. pre-hepatic phase, the intra-hepatic phase, and the post-hepatic phase. A problem in any of these phases can lead to jaundice. Bilirubin is the metabolic (or breakdown) product of hemoglobin in erythrocytes. The heme metabolism has a central role for bilirubin production [5]. The unconjugated bilirubin is largely insoluble in water, but can be reversibly conjugated to albumin. It is transported to the liver, escaping the filtration in kidneys. Generally 90–95% of the bilirubin circulating in the blood is unconjugated. In the case of hypoalbuminemia (a type of hypoproteinemia), bilirubin displacement from the albumin molecules may cause diffusion of bilirubin across the BBB. This is by means of an assortment of drugs and/or increase in the blood unconjugated bilirubin levels. If the higher amount of bilirubin crosses the BBB and the level of unconjugated bilirubin in the blood reaches 15–20 mg/dL, it causes bilirubin encephalopathy, or kernicterus (a bilirubin induced brain dysfunction) [6]. The conjugation of bilirubin takes place across the sinusoidal membrane within the hepatocytes after the hepatic uptake, followed by the action of microsomal uridine diphosphoglucuronyl transferase (UDPGT), converting bilirubin to water-soluble form and facilitating its excretion into bile and by the urine [6]. The metabolism of heme results in about 4 mg/kg/day bilirubin production. Maximum amount (about 80%) of heme moiety is utilized by the catabolism of erythrocytes and the rest 20% resulting from the ineffective erythropoiesis and breakdown of muscle myoglobin and cytochromes [7].

Bilirubin is not merely a nuisance molecule that has dire consequences, but bilirubin such as uric acid is an important antioxidant circulating in biologic system of neonate [8-10]. The main causes of increased bilirubin mostly are: race, genetic polymorphisms; inherited and acquired defects e.g. spherocytosis, Gilbert's syndrome, Najjar 1 and 2 Molecular genetics studies have shown the correlations between neonates hyperbilirubinemia and different genetic variations which can change in enzyme activity. Thus our aim and object was to evaluate the developed a potential therapeutic safe and symptomatic medication to prevent hiperbilirubenimia.

8A.2 MATERIAL AND METHODS

8A.2.1 Chemicals and reagents

Cholesterol, triglyceride, phospholipids, free fatty acids, HDL and LDL kits were obtained from Merck, Germany. AST, ALT kits were procured from Merck (Germany). All other laboratory chemicals and reagents were purchased from Merck (Germany), Sigma (USA) or BDH chemicals (Mumbai, India). Triple distilled water prepared by Millipore (New Delhi, India) was used throughout the experiment.

8A.2.2 Preparation of Extract

Plant parts were air dried after cleaning with double distilled water and kept in oven at 80ºC for 10 min and 60ºC for 30 min. Then they were ground by a blade mill to fine powder. Subsequently, the extraction of the polar fraction was performed according to the method of Taamalli et al. (2015) with some modification. 5 gm of dry plant parts were dissolved using 10 ml of methanol, sonicated at room temperature for 30 min using an ultrasonic bath, centrifuged at 3000 rpm for 15 min, and finally the supernatant was removed. This procedure was repeated four times, collecting all the supernatants, which were finally evaporated in a rotary evaporator under reduced pressure at 35°C. Finally, the residue was re-constituted in 3 ml of methanol, filtered using Whatman filter papers (GE Healthcare and Life Sciences, MA, USA) and kept at 4ºC for further use.

8A.2.3 Animals and treatment

Swiss albino male mice weighing approximately 25–30 g were obtained from the animal house facility of the Jadavpur University, Kolkata, West Bengal. All animals received humane care in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The institutional Animal Ethical Committee (IAEC) of Jadavpur University, Kolkata, West Bengal, India approved the protocols for the experiments. Prior to dosing, they were acclimatized for 7 days to light from 06:00 to 18:00 h, alternating with 12 h darkness. The animals were housed in stainless steel cages in an air-conditioned room with temperature maintained at 25 ± 2°C. Mice were allowed standard chow diet (Amrut feeds, Pranav Agro, New Delhi, India) throughout the experiment and water ad libitum.

8A.2.4 Treatment protocol: Phase I

Forty eight mice of either sex were randomly divided into five groups of eight mice each. Group I served as the vehicle control and was given olive oil daily (0.5 ml/kg BW) for a period of 2 weeks. For inducing hyperbilirubinemia and hepatotoxicity (in vivo), animals of groups II, III and IV were administered with PTZ – intoxication for a period of 2 weeks. PTZ is a well-known hepatotoxic agent frequently used to study hepatoprotective activity of new drugs in in vivo experimental model. After PTZ intoxication, group II served as the PTZ control and was left untreated. Group III was administered with citrate-capped AP (100 mg/kg BW) for 7 days. Group IV was administered with citrate-capped AP (200 mg/kg BW) for 7 days. Group VI served as the positive control and was administered silymarin (100 mg/kg BW) daily for a period of 1 week. All the treatments were

executed by intraperitoneal injection. At the end of the experiment, the animals were kept in fasting condition overnight and sacrificed by cervical dislocation.

8A.2.5 Treatment protocol: PhaseII

For studying the efficiency of BR degradation by citrate-capped *Andrographis paniculata* (AP) extract over standard drug silymarin, 48 mice were divided into four groups (n = 12 per group). Group I served as vehicle control and received olive oil daily (0.5 ml/kg BW) for a period of 3 weeks. Other four groups were administered with PTZ – intoxicated Control for a period of 3 weeks to induce hyperbilirubinemia. After induction, group II left untreated, group III was administered with AP (100 mg/kg BW) twice daily, group IV was administered with AP (200 mg/kg BW) and group V received silymarin (100 mg/kg BW) twice daily. Serum biochemical tests were performed from blood collected at 2, 6, 12 and 24 h.

8A.2.5a Measurement of serum biochemical parameters

For biochemical studies, blood samples were collected just before sacrifice in sterile tubes (nonheparinized) from retro-orbital plexus and allowed to clot for 45 min. Serum was separated by centrifugation at 3000 rpm for 15 min. All serum samples were sterile, hemolysis-free, and were kept at 4°C before determination of the biochemical parameters. Serum BR concentration (total and direct) was measured using commercially available test kits and results were expressed as mg/dl. Liver damage was assessed by the estimation of serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), γ-glutamyltranferase (GGT) using commercially available test kits. The results were expressed as units/liter (IU/l). Total protein concentration was estimated and expressed as gm/dL. All the kits for measurement of serum biochemical parameters were purchased from Autospan Liquid Gold, Span Diagnostics Ltd (Surat, India). All hematological tests were performed spectrophotometrically following the protocols described by the corresponding manufacturers.

8A.2.5b Hematological study

For hematological studies, the blood was collected in heparinized tubes. Blood-cell count was done using blood smears in Sysmax-K1000 Cell Counter. Parameters studied were hemoglobin, total red blood cell, reticulocyte, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, total white blood cell.

8A.2.5c Histopathological examination

The liver was excised immediately after collection of blood, washed with ice-cold phosphate buffer and dried with tissue paper. It was weighed and fixed in neutral formalin solution (10%), dehydrated in graduated ethanol (50–100%), cleared in xylene and embedded in paraffin. Sections 4–5 μm thick were prepared using microtome and then stained with hematoxylin and eosin (H&E) dye and examined for histopathological changes under the microscope.

8A.2.5d Statistical analysis

All quantitative data are expressed as mean ± standard deviation. Comparison of different parameters between the groups was performed using one-way analysis of variance followed by Tukey's multiple comparison test using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA [38]. $p < 0.05$ was considered significant

8A.3 RESULTS

8A.3.1 Phase-I study

In the phase I study, the total serum BR (Tsb) level of PTZ treated group (group II) increased to 0.91 ± 0.08 mg/dl which is far higher than the control group (group I 0.31 ± 0.06 mg/dl), indicative of hyperbilirubinemia. This group (group II) was left untreated for a week to see the auto recovery. The serum biochemical tests were done after treatment of seven days and represented in Table 8A.1. The results show that treatment with the A. paniculata extract (100 and 200 mg/kg) significantly decreased BR level (total and direct) even lower than the sham control (Figure 8A.1). But for group II, we have seen very mild decrease in serum BR concentration even after removal of the hepatoxin, indicating slow recovery process. However, in case of direct BR level, the reduction is not significantly different from the clinical standard silymarin (Table 8A.1). The *A. paniculata* extract directly interacts with the BR and degrade the same. In the case of direct BR (soluble in aqueous environments and complex with glucuronic acid), hindrance of the interaction with A. paniculata extract could be a possible reason of lower efficacy of the A. paniculata extract in reducing direct BR.

Table 8A.1: Effect of *Andrographis paniculata* (AP) extract on serum Bilirubin level in PTZ-intoxicated mice

Gr.	Design of treatment	Total Bilirubin (mg/dL)		Direct Bilirubin (mg/dL)	
		After induction	After treatment	After induction	After treatment
I	Control	0.31±0.06	0.31±0.04[b]	0.18±0.06	0.21±0.05[b,c]
II	PTZ – intoxicated Control	0.91±0.08	0.61±0.06[a,c]	0.36±0.08	0.36±0.08[a,c]
III	PTZ + AP 100	0.87±0.05	0.24±0.05[a,b,c]	0.41±0.07	0.17±0.03[a,b]
IV	PTZ + AP 200	0.83±0.04	0.51±0.05[a,c]	0.38±0.06	0.29±0.06[a,c]
V	PTZ + Silymarin	0.85±0.07	0.33±0.08[b]	0.37±0.05	0.20±0.03[a,b]

AP: *Andrographis paniculata*; PTZ: Phenylhydrazine. Data are expressed as mean ± SD (n=8)

One-way ANOVA Tukey post hoc: [a]$p < 0.05$ compared with vehicle control. [b]$p < 0.05$ compared with PTZ. [c] $p < 0.05$ compared with silymarin.

Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

8A.3.2 Phase-II study

For assessing the efficiency of BR degradation *in vivo* by *A. paniculata* extract over standard drug silymarin, we performed the second phase of experiment. Here, BR concentration was monitored for a period of 24 h after intoxication. Three weeks PTZ intoxication increased the total serum BR level upto 1.28 ± 0.14 mg/dl. Single dose of *A. paniculata* extract decreases Tsb to 0.40 ± 0.07 mg/dl within 2 h and back to normal level (0.32 ± 0.05 mg/dl) within 6 h compared with the silymarin treated group 1.22 ± 0.16 mg/dl (2 h) and 1.10 ± 0.14 mg/dl (6 h), respectively. The results are represented in Figure 8A.1. Treatment with *A. paniculata* extract decreased Tsb level almost 70% compared with that of silymarin (8%) in 2 h and restored normal Tsb concentration within 6 h.

8A.3.2a Body weight, liver weight and liver index

Administration of PTZ significantly increased gross body weight as compared with control untreated animals (Table 8A.2). Co-administration with *A. paniculata* (100 and 200 mg/kg) gradually decreased gross body weight as compared with PTZ animals. Standard heapato-protective drug silymarin also partially decreased gross body weight levels as compared with PTZ. On the other hand administration of PTZ is significantly increased liver weight and liver index when compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually decreased the liver weight and liver index as compared with PTZ control group. Standard heapato-protective drug silymarin also partially liver weight and liver index as compared with PTZ.

Table 8A.2: Effect of *Andrographis paniculata* (AP) extract on body weight (BW), liver weight and liver weight index in PTZ intoxicated mice.

Parameters	Control	PTZ – intoxicated Control	PTZ + AP 100	PTZ + AP 200	PTZ + Silymarin
Body Weight (g)	36.52±1.18^{b}	26.94±1.22^{a,c}	35.81±2.15^{b}	37.01±1.34^{b}	34.62±1.26^{b}
Liver Weight (g)	1.94±0.82^{b}	3.50±0.28^{a,c}	3.25±0.47^{b}	2.98±0.18^{b}	1.99±0.52^{b}
Liver Index	4.02±0.12^{b}	7.72±0.15^{a,c}	4.62±0.16^{b}	4.65±0.17^{b}	5.11±0.24^{b}

AP: *Andrographis paniculata*; PTZ: Phenylhydrazine. Data are expressed as mean ± SD (n=8)

One-way ANOVA Tukey post hoc: $^{a}p< 0.05$ compared with vehicle control. $^{b}p< 0.05$ compared with PTZ. $^{c}p< 0.05$ compared with silymarin.

Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

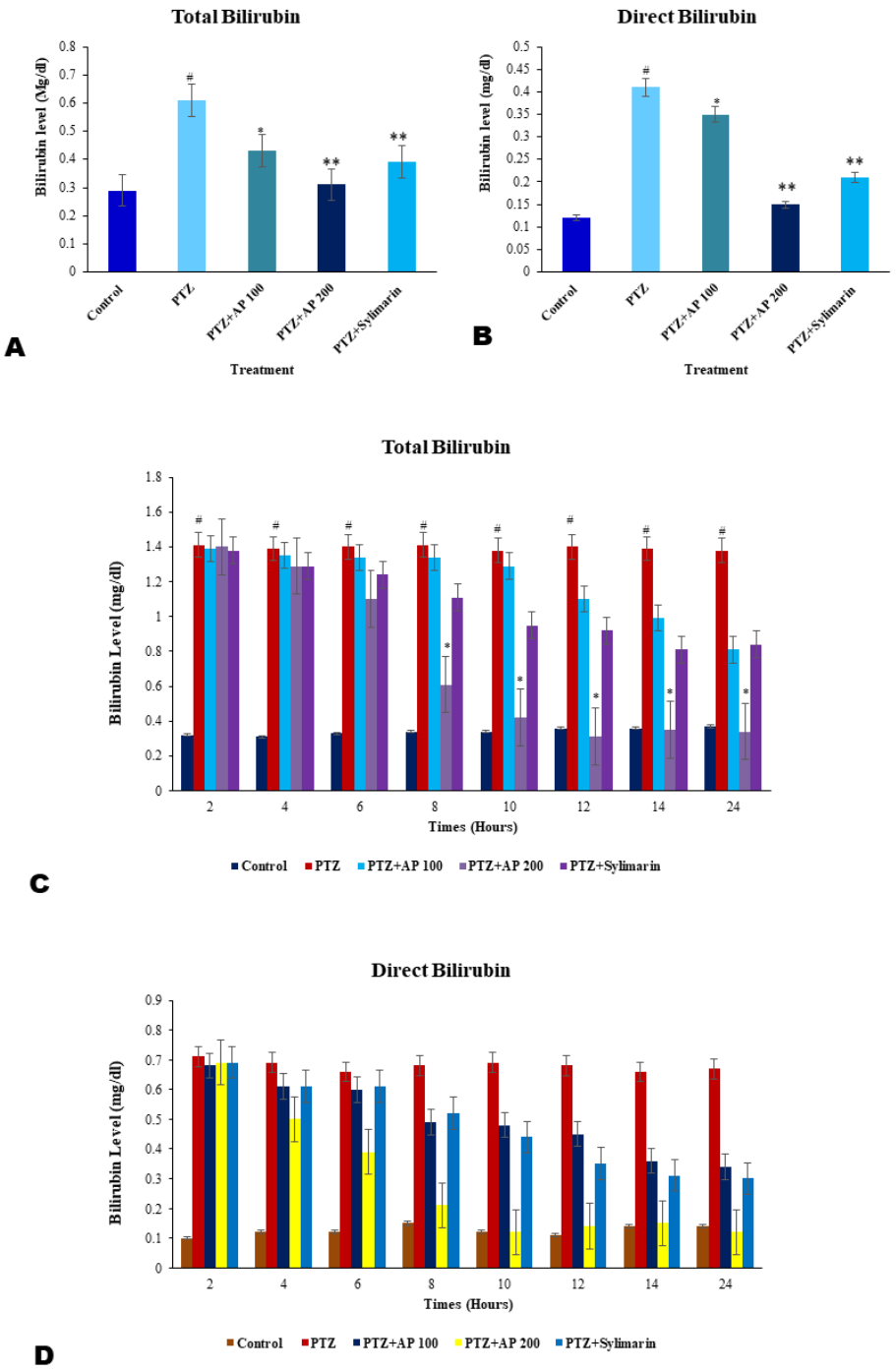


Figure 8A.1. Effect of *Andrographis paniculata* (AP) extract on total and direct bilirubin levels in respect to time. (A) Total bilirubin level (B) Direct bilirubin level (C) Time dependent total bilirubin level (D) Time dependent direct bilirubin level. Data are expressed as mean ± SD (n=8). One-way ANOVA Tukey post hoc: $^{\#}p< 0.05$ compared with vehicle control. $^{*}p< 0.05$ compared with PTZ. $^{**}p< 0.05$ compared with PTZ. Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

8A.3.2b Liver Function test

In this study AST, ALT, ALP, GGT and total protein was measured (Table 8A.3). Administration of PTZ significantly elevated serum AST, ALT, ALP and GGT when compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually decreased the values of AST, ALT, ALP and GGT as compared with PTZ control group. Standard hepato-protective drug silymarin also partially decreased these enzymes levels as compared with PTZ. On the other hand administration of PTZ is significantly decreased total protein content when compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually increased the values of total protein as compared with PTZ control group. Standard heapato-protective drug silymarin also partially increased the total protein levels as compared with PTZ.

Table 8A.3: Effect of *Andrographis paniculata* (AP) extract on serum ALT, AST, ALP, GGT, and Total Protein in PTZ intoxicated mice.

Gr.	Design of treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	GGT (IU/L)	Total Protein (g/dL)
I	Control	79.62±8.51^{b}	18.61±2.65^{b,c}	28.69±4.91^{b}	2.46±0.06^{b,c}	6.88±1.02^{b}
II	PTZ – intoxicated Control	201.54±24.65^{a,c}	61.28±6.69^{a,c}	71.52±8.57^{a,c}	6.91±0.08^{a}	4.16±0.96^{a}
III	PTZ + AP 100	88.34±11.25^{b}	21.33±4.25^{a,c}	26.38±4.28^{a,b}	2.78±0.05^{a}	5.81±0.98^{b}
IV	PTZ + AP 200	76.54±9.67^{b}	24.51±4.98^{a,b,c}	35.81±3.09^{a,b}	3.12±0.04^{a}	5.62±1.04^{b}
V	PTZ + Silymarin	91.91±8.21^{b}	24.69±3.21^{a,b}	31.66±4.01^{a,b}	2.94±0.05^{a,b}	5.39±1.11^{b}

AP: *Andrographis paniculata*; PTZ: Phenylhydrazine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: amma-glutamyl transferase. Data are expressed as mean ± SD (n=8)

One-way ANOVA Tukey post hoc: $^{a}p< 0.05$ compared with vehicle control. $^{b}p< 0.05$ compared with PTZ. $^{c}p< 0.05$ compared with silymarin.

Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

8A.3.2c Haematological study

Different haematological parameters such as Hemoglobin (Hb), Read Blood corpuscle (RBC), Reticulocyte (RT), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), White Blood corpuscle (WBC), prothrombin time (PT), activated partial thromboplastin time (APTT) wear measured. Hemoglobin (Hb) content and monocyte count were significantly decreased when administered PTZ. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually increased the Hemoglobin (Hb) content and monocyte count as compared with PTZ control group. Standard heapato-protective drug silymarin also partially increased these Hemoglobin (Hb) content and monocyte count as compared with PTZ (Table 8A.4).

Table 8A.4: Hematological parameters as studied across the Group I (Control), Group II (PTZ Control), Group III (PTZ + AP 100), Group IV (PTZ + AP 200) Group V (PTZ + Silymarin) is represented.

Hematological Parameters	**Group-I**	**Group-II**	**Group-III**	**Group-IV**	**Group-V**
Hb (g/dL)	11.9±3.2	8.1±3.1	11.8±4.2	10.8±4.5	10.2±6.1
RBC ($x10^6$ μL^{-1})	10.8±4.1	9.2±5.3	10.1±4.2	10.6±5.1	10.2±5.2
RT (%)	2.8±1.1	2.4±1.6	2.8±2.4	2.9±1.6	2.2±2.1
HCT (%)	34.8±1.3	31.8±2.1	32.8±2.1	35.1±3.1	34.9±1.6
MCV (μm^3)	37.0±2.6	36.1±2.3	36.0±1.4	37.2±1.1	37.1±1.4
MCH (pg)	21.1±2.4	21.8±2.8	20.1±1.7	21.4±2.6	21.4±1.6
MCHC (%)	41.4±7.6	41.7±2.4	40.4±1.4	40.6±2.1	41.2±1.8
Platelets ($x10^3$ μL^{-1})	6.6±2.0	6.3±1.2	6.9±1.2	6.6±2.7	6.6±2.8
WBC ($x10^3$ μL^{-1})	8.8±1.1	7.8±1.2	7.8±1.1	8.1±1.2	8.5±1.1
Lymphocyte (%)	76±6.3	72±3.3	73±5.4	76±3.6	74±1.3
Neutrophil (%)	22±4.3	21±5.1	25±6.9	22±6.9	24±5.7
Monocyte (%)	2.3±0.01	1.6±0.01	2.4±0.01	1.8±0.02	2.1±0.01
Eosinophil (%)	9.6±2.6	9.4±2.9	9.1±1.4	9.4±2.5	9.2±3.6
Basophil (%)	1.2±0.05	1.2±0.02	1.2±0.04	1.1±0.02	1.1±0.01
PT(sec)	7.3±2.2	8.2±3.1	7.4±2.6	7.1±4.1	8.5±3..9
APTT (sec)	16.9±4.3	18.7±2.6	16.1±2.5	15.2±2.6	16.4±3.2

Abbreviations: Hb: Hemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Hematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White Blood corpuscle; PT: prothrombin time; APTT: activated partial thromboplastin time. Data are expressed as mean ± SD (n=8)

8A.3.2d Lipid peroxidation

In this study lipid peroxidation (MDA content) was measured upon mice model (Table 8A.5). Administration of PTZ significantly increased lipid peroxidation (MDA content) compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually decreased the values of lipid peroxidation (MDA content) as compared with PTZ control group. Standard heapato-protective drug silymarin also partially increased these lipid peroxidation (MDA content) levels as compared with PTZ.

8A.3.2e Antioxidant enzymes

Antioxidant enzymes SOD, CAT, GSH and GPx was measured (Table 8A.5). Administration of PTZ significantly reduced antioxidant enzymes likes SOD, CAT, GSH and GPx when compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually increased the values of SOD, CAT, GSH and GPx as compared with PTZ control group. Standard heapato-protective drug silymarin also partially decreased these antioxidant enzymes levels as compared with PTZ.

Table 8A.5: Effect of *Andrographis paniculata* (AP) extract on SOD, CAT, ALP, GSH, GPx and Lipid peroxidation in PTZ intoxicated mice.

Gr.	Design of treatment	SOD(U/mg protein)	CAT(U/mg protein)	GSH(U/mg protein)	GPx(U/mg protein)	MDA (nmol/mg protein)
I	Control	6.25 ± 0.18^{b}	$188.61\pm8.65^{b,c}$	6.69 ± 0.74^{b}	$11.46\pm2.56^{b,c}$	22.36 ± 8.02^{b}
II	PTZ – intoxicated Control	$2.61\pm0.21^{a,c}$	$81.02\pm6.69^{a,c}$	$2.52\pm1.37^{a,c}$	6.91 ± 6.38^{a}	56.84 ± 9.96^{a}
III	PTZ + AP 100	6.35 ± 0.25^{b}	$171.35\pm9.25^{a,c}$	$6.38\pm1.28^{a,b}$	12.78 ± 2.05^{a}	25.81 ± 7.98^{b}
IV	PTZ + AP 200	5.54 ± 0.67^{b}	$164.51\pm4.98^{a,b,c}$	$5.81\pm0.99^{a,b}$	11.12 ± 2.04^{a}	29.62 ± 6.04^{b}
V	PTZ + Silymarin	5.91 ± 0.21^{b}	$168.69\pm5.21^{a,b}$	$6.06\pm1.01^{a,b}$	$10.94\pm1.95^{a,b}$	34.39 ± 4.11^{b}

AP: *Andrographis paniculata*; PTZ: Phenylhydrazine; SOD: superoxide dismutase, CAT: catalase; GSH: reduced glutathione; GPx: glutathione peroxidase; MDA: malondialdehyde. Data are expressed as mean ± SD (n=8)

One-way ANOVA Tukey post hoc: $^{a}p< 0.05$ compared with vehicle control. $^{b}p< 0.05$ compared with PTZ. $^{c}p< 0.05$ compared with silymarin.

Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

8A.3.2f Biochemical study

Administration of PTZ significantly increased blood urea nitrogen (BUN) and Protein carbonyl level as compared with control untreated animals (Table 8A.6). Co-administration with *A. paniculata* (100 and 200 mg/kg) gradually decreased blood urea nitrogen (BUN) and Protein carbonyl level as compared with PTZ animals. Standard heapato-protective drug silymarin also partially decreased these blood urea nitrogen (BUN) and Protein carbonyl level (MDA content) levels as compared with PTZ. On the other hand administration of PTZ is significantly decreased total albumin content, albumin-globulin ratio and total thiol content when compared with normal control mice. Pre-treatment with *A. paniculata* (100 and 200 mg/kg) gradually increased the values of total albumin content, albumin-globulin ratio and total thiol content as compared with PTZ control group. Standard heapato-protective drug silymarin also partially increased the total total albumin content, albumin-globulin ratio and total thiol content as compared with PTZ.

Table 8A.6: Effect of *Andrographis paniculata* (AP) extract on BUN, Albumin, Alb/globulin, Protein carbonyl content, and Total thiol in PTZ intoxicated mice.

Gr.	Design of treatment	BUN(mg/dl)	Alb (gr/dL)	Alb/globulin	Protein carbonyl (nmol/mg protein)	Total thiol (µmoles/mg protein)
I	Control	0.45 ± 0.08^{b}	$3.68\pm0.65^{b,c}$	1.18 ± 0.141^{b}	$3.16\pm0.56^{b,c}$	2.66 ± 0.12^{b}
II	PTZ – intoxicated Control	$1.01\pm0.06^{a,c}$	$1.96\pm0.69^{a,c}$	$0.54\pm0.13^{a,c}$	6.91 ± 0.38^{a}	1.14 ± 0.16^{a}
III	PTZ + AP 100	0.95 ± 0.05^{b}	$3.55\pm0.25^{a,c}$	$1.23\pm0.95^{a,b}$	3.78 ± 0.65^{a}	2.81 ± 0.18^{b}
IV	PTZ + AP 200	0.94 ± 0.07^{b}	$3.51\pm0.98^{a,b,c}$	$1.06\pm0.9^{a,b}$	4.12 ± 0.34^{a}	2.62 ± 0.14^{b}
V	PTZ + Silymarin	0.91 ± 0.01^{b}	$2.69\pm0.21^{a,b}$	$0.96\pm0.08^{a,b}$	$3.94\pm0.95^{a,b}$	2.39 ± 0.11^{b}

AP: *Andrographis paniculata*; PTZ: Phenylhydrazine; BUN: blood urea nitrogen. Data are expressed as mean ± SD (n=8)

One-way ANOVA Tukey post hoc: $^{a}p< 0.05$ compared with vehicle control. $^{b}p< 0.05$ compared with PTZ. $^{c}p< 0.05$ compared with silymarin.

Dosage:- Phenylhydrazine hydrochloride (PTZ) was injected intravenously at a dose of 75 mg/kg body weight. *Andrographis paniculata* (AP): 100 mg/kg/day /mice and 200 mg/kg/day /mice. Silymarin: 100mg/kg body weight.

8A.4 DISCUSSION

Hiperbilirubinemia mainly jaundice occurs in newborns, pregnant woman and adults as a result of excessive bilirubin formation and transient inability of the individuals liver to clear bilirubin rapidly enough from the blood. Severe hyperbilirubinemia is toxic to the developing central nervous system [11]. Prolonged and uncontrolled high levels of bilirubin lead to bilirubin encephalopathy and subsequently kernicterus [12]. We used phenylhydrazine to induce nonhepatic neonatal hyperbilirubinemia in mice because it increased unconjugated bilirubin level. The bilirubin level was measured to evaluate the role of *A paniculata* in hiperbilirubinemia. Serum and tissue bilirubin levels were significantly increased after PTZ treatment and the elevated levels was normalized after pre-treatment with *A paniculata*. So our prepared plant extract which is a main ingredient in formulation could be able to protect hiperbilirubinemia and save the body against bilirubin degradation.

Phenylhydrazine (PTZ) induces jaundice conditions because it increases unconjugated bilirubin level without any significant change in the liver function. Liver function was evaluated by assessing serum ALT and AST, since AST and ALT are sensitive indicators of liver cell injury [13]. In the present study, we have confirmed that phenylhydrazine increase AST and ALT levels in serum of mice. Consistent with the result, the liver histopathological observations also show any hepatic damage due to phenylhydrazine administration. As hypothesized, normal activity of the liver function enzymes and absence of any liver damage after *A paniculata* indicated the safety profile of *A paniculata*

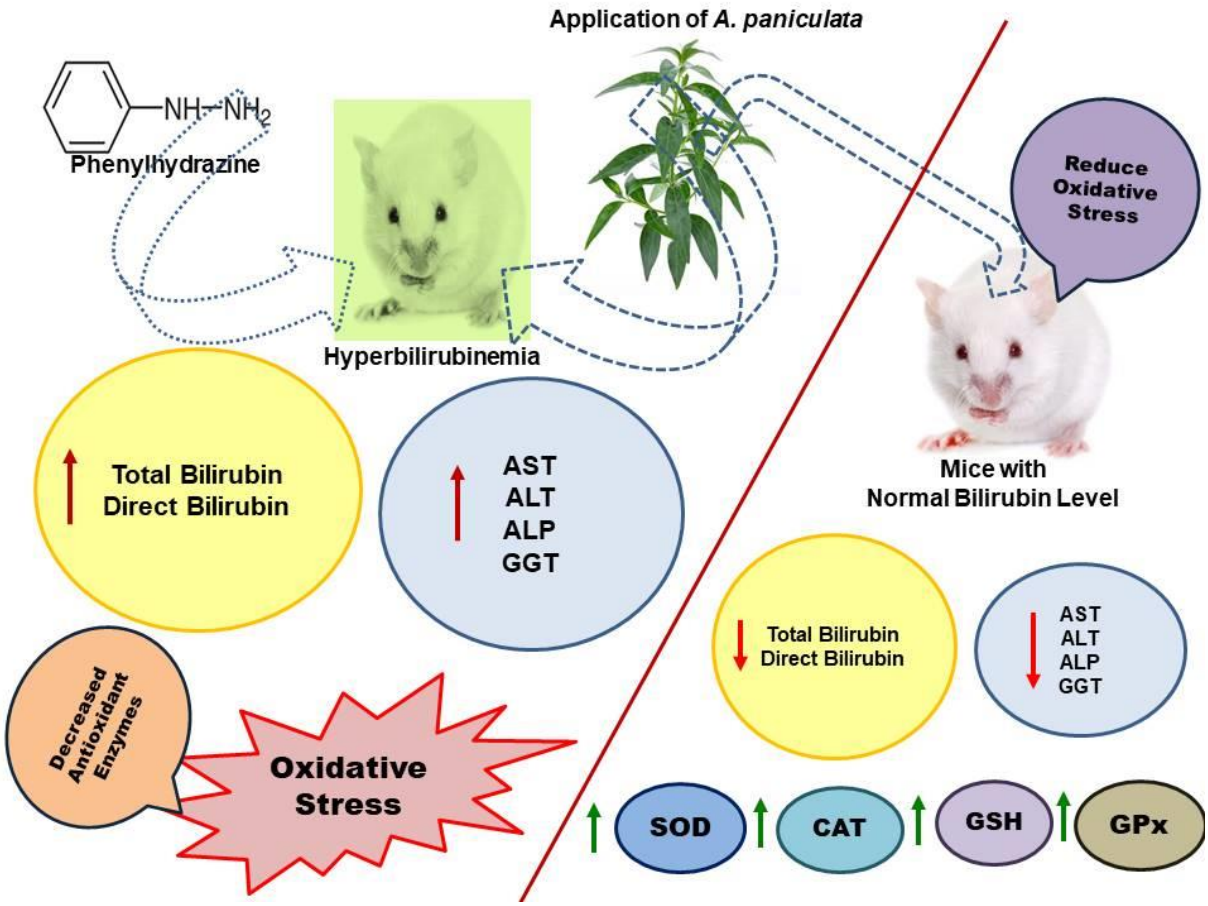
Exposure to Phenylhydrazine (PTZ) cause damage to red blood cells and decrease haemoglobin levels potentially resulting in anemia and consequentially hyperbilirubinemia [14]. Administration of *A paniculata* normalise the red blood cell and haemoglobin levels and inhibit to anaemia development in hiperbilirubinimic condition. PHZ increases reactive oxygen species (ROS). ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induce haemolytic injury seems to be derived from oxidative alterations to red blood cell proteins [15]. *A paniculata* have potentially a huge therapeutic value to control the ROS generation and maintain a homeostasis.

All these results suggest that *A paniculata* extract has the potential to reduce BR concentration to a normal level in jaundiced rats. The possible mechanisms of BR reducing action of *A paniculata* extract might be the increased activity of glucuronyl transferases [16] to facilitate hepatic conjugation of BR or increased BR binding by albumin [17] or Prevention of enterohepatic circulation of bilirubin via enhancement of bilirubin sequestration or degradation in the intestinal lumen[18] or probably due to a shift in the distribution equilibrium of the pigment between serum, skin and other tissues

CONCLUSION

In conclusion, our findings showed that *A paniculata* extract prevented the progression of phenylhydrazine-induced neonatal jaundice in experimental animals. The therapeutic mechanism of *A paniculata* extract not only included efficient bilirubin clearance potential but also reduced oxidative.

SUMMARY



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Chapter - IX

Antioxidant and Sanative effect of Silver nanoparticle coupled with Andrographis paniculata, a component of AKSS16-LIV01 against CCl_4 treated liver dysfunction

9A In Vitro Studies of the Antimicrobial and Free-Radical Scavenging Potentials of Silver Nanoparticles Biosynthesized from the Extract of *Andrographis paniculata*, a component of AKSS16-LIV01

9A.1 INTRODUCTION

Nanotechnology is the study of extremely small structures, having size of 0.1 to 100 nm. There is increasing optimism that nanotechnology applied to medicine and dentistry will bring significant advances in the diagnosis, treatment, and prevention of disease. Growing interest in the future medical applications of nanotechnology is leading to the emergence of a new field called nanomedicine. Nanomedicine needs to overcome the challenges for its application, to improve the understanding of pathophysiologic basis of disease, bring more sophisticated diagnostic opportunities, and yield more effective therapies and preventive properties [1-5].

Recently, among the metal oxide nanoparticles, silver nanoparticles have attained significant importance because of their distinctive properties, and their varied range of applications such as, solar cells [6], gas sensors, catalytic, optical, hydrogen storage materials, and medical applications [7].

Nanotechnology represents innovation and facilitates the platform to fabricate novel nanomaterials for a wide range of biological and biomedical applications [8]. Biosynthesis or green synthesis of nanoparticles/nanomaterials is becoming increasingly popular as safer, cost-effective, easy to use, timesaving, free from toxics and pollutants, and simple without many environmental concerns. It is an alternative to the usual physical and chemical process [9, 10]. Therapeutic nanomaterials for biomedical and pharmaceutical applications are being carried out by different green synthesis technologies using macro- and micro-scopic organisms (bacteria, fungi, microalgae, seaweeds, and plants) [11].

Free radicals are accountable for producing a large number of sicknesses including cardiovascular disease, cancer, Alzheimer's disease, neural disorders mild cognitive impairment, Parkinson's disease; alcohol induced liver disease, ulcerative colitis, aging and atherosclerosis [12, 13]. Defence against free radicals can be improved by plentiful consumption of dietary antioxidants. Antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases [14]. In addition, they have a possible role for savings in the cost of health care delivery.

The present study was to investigate the antioxidant activity of *Andrographis paniculata* leaves extract and AP-AgNPs. The antibacterial activities AP-AgNPs against pathogenic bacteria were also reported in this study.

9A.2 MATERIAL AND METHODS

9A.2.1 Chemicals

All the experiments were conducted at room temperature. Silver nitrate ($AgNO_3$) purchased from Merck, India. Materials used for the synthesis of Ag nanoparticles were AR grade.

9A.2.2 Synthesis of Ag Nanoparticles using *Andrographis paniculata* leaf extract

The fresh leaves of *Andrographis paniculata* were collected in sterilized polyethene bags from the medicinal plant garden of Jadavpur University, Kolkata, West Bengal, India. About 20 gm of fresh leaves were thoroughly washed with de-ionized water to removed dust particles. The leaves were dried at room temperature. About 5gm of the leaves were cut and ground finely in a mortar and pestle. It was extracted with 100ml of distilled water in a conical flask. The extract was heated for about 1 hour and filtered (Whatman 40 filter paper). It was then stored for further use in the refrigerator.

In green synthesis of AgNPs, 0.01M of aqueous solution of $AgNO_3$ (99.99%) was used. 5mL of leaf broth was added to 45 ml of 0.01M $AgNO_3$ aqueous solution and allowed at ambient condition to react. After different time intervals, the color change of reaction mixture is observed from transparent yellow to dark brown indicates that the formation of AgNPs.

9A.2.3 Characterization studies of Ag Nanoparticles

The optical property of the prepared Ag nanoparticles was analyzed by UV-Visible [Shimadzu] absorption spectra. Crystallinity and crystal phases were identified with X-Ray Diffraction [Perkin–Elmer] spectrometer. Functional group analysis was done with FTIR [Shimadzu] Spectrometer. Elemental analysis was done with the Energy Dispersive X-Ray [RONTEC'S EDX system] spectrometer. Particle morphology was analyzed by Scanning Electron Microscope.

9A.2.4 In Vitro Antioxidant Activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical-scavenging activity was determined by the method of Shimada et al., [15]. The scavenging activity of the *Andrographis paniculata* towards superoxide anion radicals was measured by the method of Liu *et al*., [16]. The total antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al., [17]. The chelating activity of the AgNPs and plant extract for ferrous ions $Fe^{2+}$was measured according to the method of Dinis et al., [18]. The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu [19].

9A.2.5 Antibacterial activity of Ag nanoparticles

The antibacterial activity of Ag nanoparticles against bacterial pathogens was determined. *Streptococcus pneumonia*, *Staphylococcus aureus*, and *Klebsiella pneumonia* were provided by Microbiology Department, Jadavpur University, West Bengal, India.In this analysis, the positive control used was tetracycline. The microbes were cultured in nutrient broth. Then it was incubated at a temperature of 37 ° C for about 12 hours. A 100 Microliter (µL) solution of broth microbial culture

was prepared which was then blown out on Muller Hinton agar plates. The plates were left undisturbed for about 10 minutes to enhance culture absorption. With a sterile gel puncher, the wells (5 mm size) were pierced into the agar. Using a micropipette a 100 Microliter (µL) solution (50 µg/ml and 25 µg/ml) of Ag nanoparticles and 100 Microliter (µL) solutions (10 µg/ml) of the positive control (tetracycline) were taken which was then shifted to the wells kept in all plates. The incubation was carried out at a temperature of 37 ° C for about 24 hrs. The size of the inhibition zone was quantified. The same analysis was repeated for three replicates. The results of the mean and standard error of the mean were presented[20].

9A.2.6 Antifungal activity

An analysis of antifungal activity was carried out by well diffusion method according to Magaldi et al. [21]. the fungal pathogens such as *A. niger*, *Aspergillus oryzae*, and *Candida albicans* were used in this assay. Potato dextrose agar (PDA) media were prepared and poured in the plates. Fungal pathogens were inoculated carefully after the solidification of PDA. Five wells (5 mm of size) were cut out on the agar plates. Various concentration of AgNP (25–100 µg/ml) and antifungal agent (positive control) ketoconazole (20 mg/ml) was introduced in well. The plates were incubated for 2–3 days at room temperature. After 3 days, the zone of inhibition was obtained measured in millimeter.

9A.2.3 RESULTS

9A.2.3.1 Characterization of copper oxide nanoparticles

The UV-VIS spectra of both chemically synthesized and green synthesized nanomaterials (Figure 8A.1) showed absorption band–between wavelength range 200-900 nm, with a peak at 360 nm, conforming presence of Ag NPs in the solution. In case of AP conjugated AgNPs (AP-AgNps) the absorption spectra remained almost same with a slight blue shift in the absorption maxima. The XRD pattern of the synthesized nanomaterial (Figure 8A.2) showed diffraction peaks at 2θ = 36.2 º, 40.6 º, 62.6 º and 71.3 º, which respectively corresponded to (111), (200), (220) and (311) planes of pure silver. All peaks were duly assigned by using JCPDS file no. 04-0783 pertaining to pure silver.

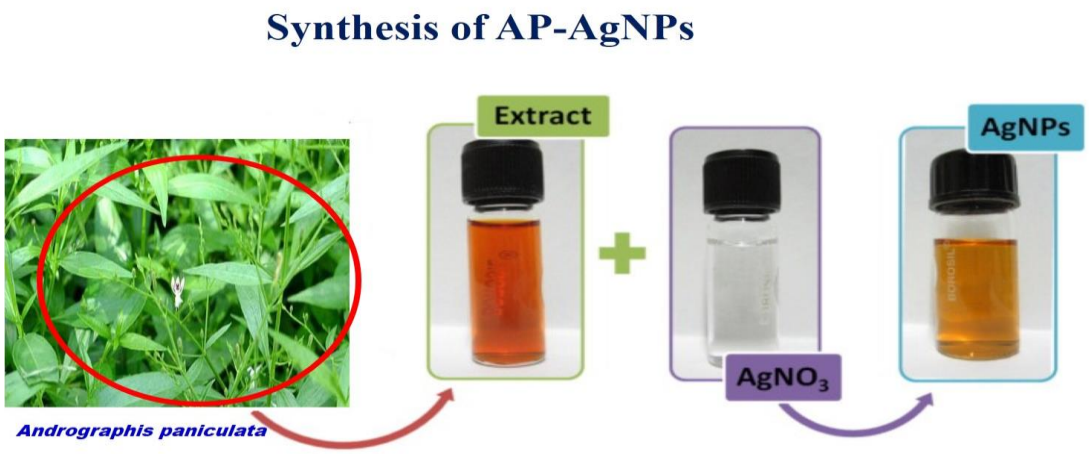

Figure 9A.1: Green Synthesis of AgNPs using *Andrographis paniculata*

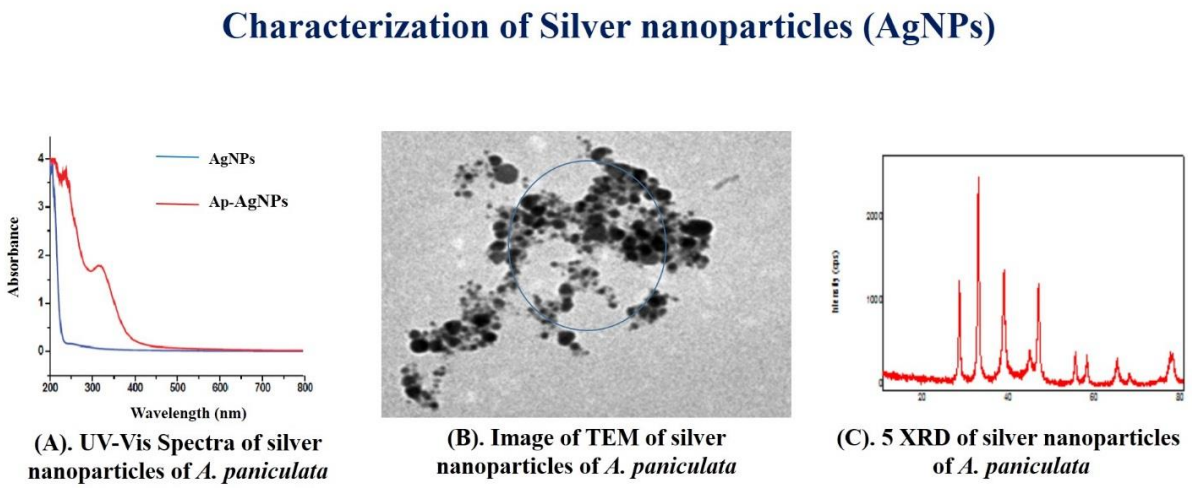

Figure 9A.2: Characterization of AgNPs using *Andrographis paniculata*

9A.2.3.2 Antioxidant activity

The AgNPs exhibited a significant dose dependent inhibition of DPPH activity as compared to *Andrographis paniculata* leaf extract. The potential of Lascorbic acid to scavenge DPPH radical is directly proportional to the concentrations. AgNPs has potential antioxidant activity than *A. paniculata* act and near to standard (Figure 9A.3). The total antioxidant activity of *A. paniculata* leaf extract, AgNPs and ascorbic acid was dose dependent manner. The AgNPs exhibited a significant dose dependent inhibition of DPPH activity. AgNPs has potential antioxidant activity than *A. paniculata ex*tract *a*nd near to standard (Figure 9A.3).

The superoxide scavenging activity of *Andrographis paniculata* leaf extract, AgNPs and ascorbic acid was increased markedly with the increase of concentrations. AgNPs have potential superoxide anion scavenging activity than *A. paniculata* extract and near to standard (Figure 9A.3).The metal chelating assay involves color reduction which in turn determines their chelating ability of synthesized nanoparticles for ferrous ions. The formation of the ferrozine–Fe^{2+} complex is interrupted in the presence of *A. paniculata* leaves extract, AgNPs and ascorbic acid was increased markedly with the increase of concentrations. Thus the decrease in the absorbance at 562 nm indicated high levels of iron binding potential and antioxidant activity of the nanoparticles. AgNPs has potential ferrous ion chelating activity than *A. paniculata* extract and was near to standard (Figure 9A.3)

The reductive effect of *Andrographis paniculata* leaves extract, AgNPs and ascorbic acid increased with increasing dosage (Figure 9A.3). All the doses showed significant activities but the one near to the control exhibited greater reducing power, indicating that *A. paniculata* consist of hydrophilic polyphenolic compounds that cause the greater reducing power. Since the reducing power activity of the compounds could serve as a significant indicator of the antioxidant potential, we assessed this property by measuring the ability of the extract to transform Fe^{3+} to $Fe^{2+}$and to donate an electron. The

ability of the extracts to reduce Fe^{3+}could be attributed either to the reducing agents such as number of phenolic groups or position of the hydroxyl molecule on these groups.

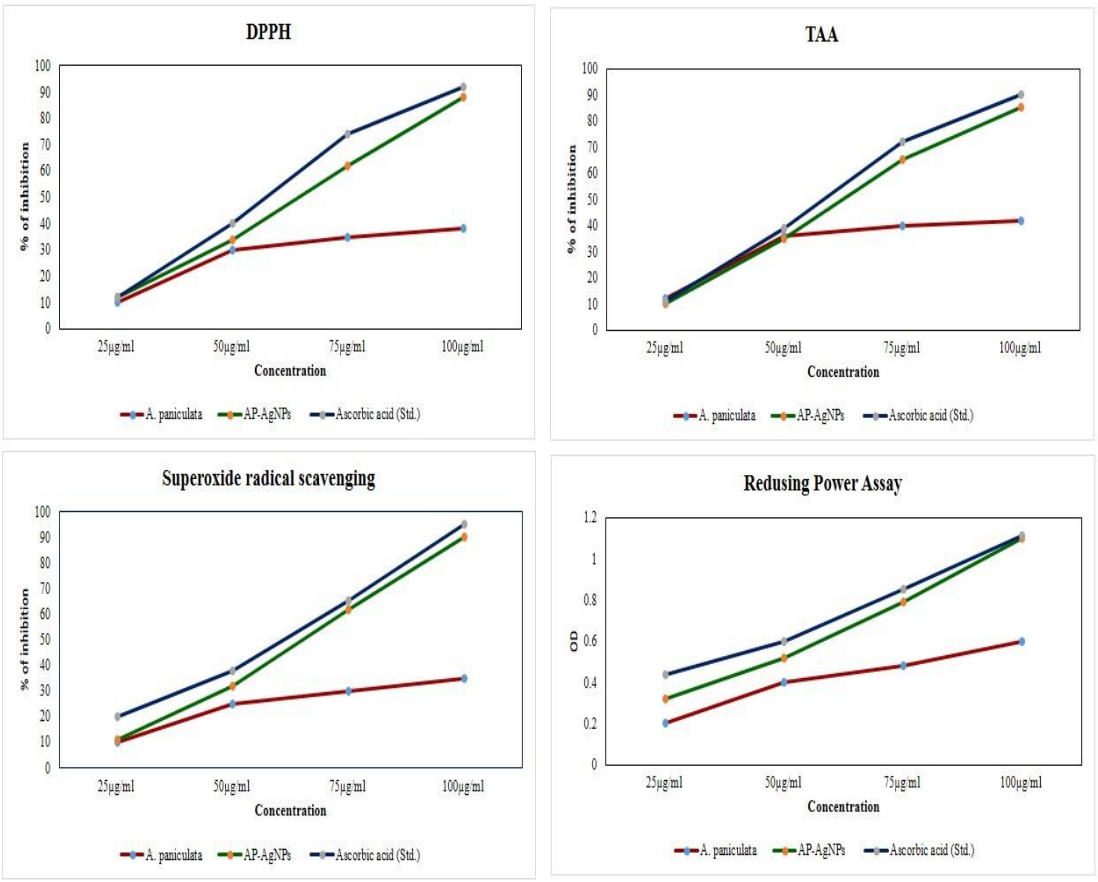

Figure 9A.3: Antioxidant activity of *Andrographis paniculata* mediated silver nanoparticles. Data represent mean ± standard error

9A.2.3.3 Antimicrobial activity

Figure 9A.4 shows the antibacterial activity of the synthesized AgNP against pathogenic bacteria. At different concentration of samples, the distinct zone of inhibition was formed around the wells. Tetracycline used as a control to compare the zones. Significant results were observed in *E. coli*, *S. aureus*, and *K. pneumonia*. *S. typhi* and *S. aureus* showed less inhibition. This result indicates that AgNP can be used as an antibacterial agent. AgNP is a less toxic and low-cost nanoparticle that can be used in many fields that include antibacterial, antifungal, antioxidant, and anticancer ones The antibacterial activity of AgNP was dependent on their capability to activate excess ROS generation such as superoxide anion, hydrogen peroxide, and hydroxyl radical generation.

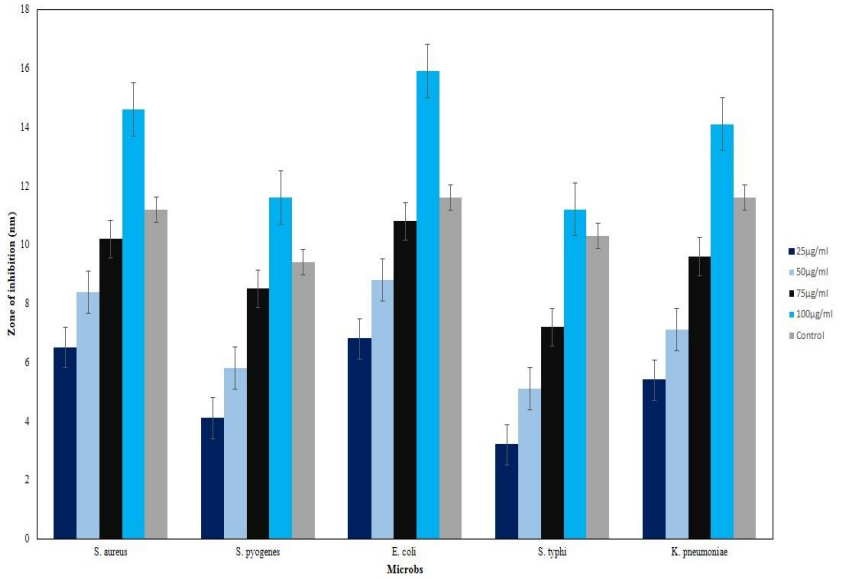

Figure 9A.4: Antibacterial activity of *Andrographis paniculata* mediated silver nanoparticles. Data represent mean ± standard error

9A.2.3.4 Antifungal activity

Figure 9A.5 shows the antifungal activity of AgNP against the chosen pathogenic fungus. Agar well diffusion method was used for this study. In different concentration of zinc oxide, nanoparticles showed efficient antifungal activity for *C. albicans* and *A. orate* than *A. niger*

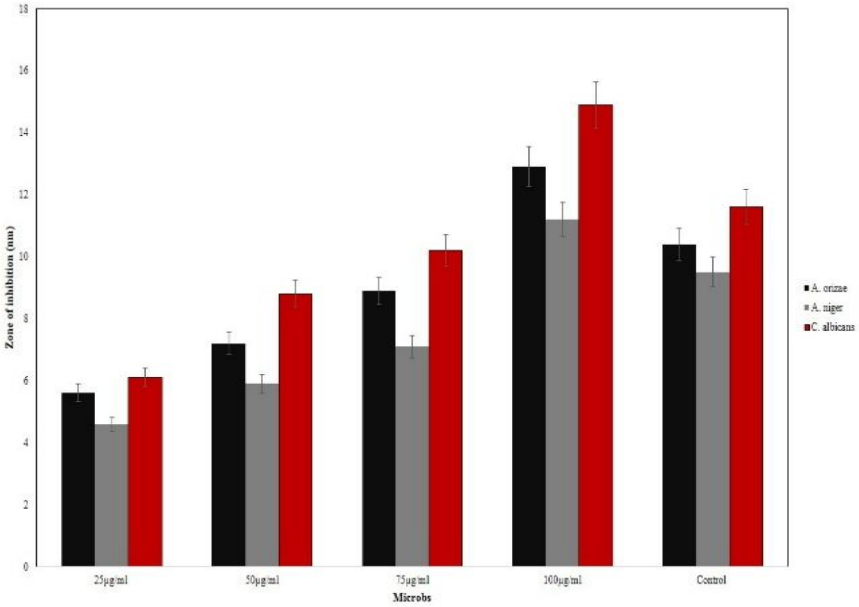

Figure 9A.5: Antifungal activity of *Andrographis paniculata* mediated silver nanoparticles. Data represent mean ± standard error

9A.2.4 DISCUSSIONS

Silver nanoparticles have attracted a lot of research interest because of their significant and important roles as catalyst, ceramic resistor, superconducting material, gas sensor, as well as their roles in biological fields and in the energy sector [22].Green synthesis of metallic and metallic oxide particles has gained great significance in the recent past due to its simplicity, cost effectiveness and environment friendly nature. It has been considered as an alternative method to all existing methods. UV-Visible spectroscopy, XRD, EDS, DLS, SEM, TEM, FTIR, HRTEM, Particle analyzer and Surface Plasmon Resonance are the most applied analytical tools for the characterization of copper and its oxide nanoparticles. Ag and AgNPs were found to exhibit spherical morphology with size range of 2 – 500 nm depending on the concentration of extracts as well as on preparative conditions. Ag nanoparticles proved to be multifunctional in nature with significant applications with great future implications in the fields of catalysis, photocatalysis, organic dye degradation, cosmetics, biomedicine and pharmaceutics [23, 24].

Synthesis of nanoparticles can be performed using a number of routinely used chemicals and physical methods. In this study, synthesis of copper oxide nanoparticles was conducted using an environmentally friendly mechanism in which aqueous leaf extract of *A. paniculata* was utilized with copper salt. The plant-synthesized Ag nanoparticles were found to be more stable with less ion release compared to the engineered AgNPs. Thus, plant-synthesized Ag nanoparticles were found to be more stable when compared with engineered nanoparticles. Acute nanoparticle toxicity tests proved that plant-synthesized CuO NPs are less toxic than engineered Ag NPs, which provides a new way to synthesize more environmentally friendly nanoparticles for various applications.

In the present study synthesized Ag nanoparticles using *Andrographis paniculata* were investigated for antioxidant potential in terms of total antioxidant (TAA), DPPH activity, Superoxide radicle scavenging activity. The AP-Ag nanoparticles at 75 µg/ml and 100µg/ml produced higher antimicrobial effects than the standard drug Tetracycline in the inhibition of growth of *E. coli*, *S. aureus*, *K. pneumonia*. *S. typhi* and *S. aureus*. Thus this superior antimicrobial activity was due to the fact that the copper ions released form Ag nanoparticles permeated the bacterial cell membrane and destroyed the structure of the cell membrane by attaching to the negativelycharged cell wall [25,26]. Copper ions are involved in cross-linkage of nucleic acid strands by binding them with DNA molecule of bacteria. This results in a disordered helical structure of DNA molecule which causes denaturation of proteins and some other biochemical processes in the cell, leading to complete destruction of the bacterial cell [27]. Factors which affect the sensitivity of bacteria to cupric oxide nanoparticle are size of particles, temperature of synthesis of the nanoparticles, structure of bacterial cell wall, and degree of contact of the nanoparticles with bacteria [28].

CONCLUSION

It can be concluded that AgNP synthesized from *Andrographis paniculata* leaf extract possesses strong antibacterial, antifungal, and antioxidant activity based on the above in vitro analysis. In higher concentrations, it has significant antimicrobial activity against pathogenic bacteria and fungi. It is also a good source of antioxidant property.

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9B Toxicological Assessment of Silver Nanoparticle Synthesized through Green Route Using *Andrographis paniculata*, a component of AKSS16-LIV01

9B.1 INTRODUCTION

Nanomedicine is the medical application of nanotechnology. The applications of nanomedicine have been far and widespread. The sizes of nanomaterials are similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. Nanomedicine seeks to deliver a valuable set of research tools and clinically useful devices in the near future [1,2].

In recent years Silver nanoparticles are widely applied in consumer products, food technology and textiles/fabrics due to its unique chemical and biological properties [3]. Silver nanoparticles are the first and most widely commercialized nanomaterial in medical and healthcare sectors. AgNPs has been used in a range of biomedical applications, owing to their antibacterial activity [4-6], antifungal properties [7] and antiviral properties [8-10].

Green synthesis is defined as the use of environmentally compatible materials such as bacteria, fungi and plants in the synthesis of nanoparticles [11]. Alternatively, synthesis from biologically derived extracts offers several advantages such as rapid synthesis, high yields and importantly, the lack of costly downstream processing required producing the particles [12-14]. Hence, nanoparticle synthesis from plant extracts tentatively offers a route for large scale production of commercially attractive nanoparticles.

Toxicity testing is essential in the examination of newly developed drugs before it can be used on humans. It is the limitation of potential hazards a test substance may probably produce and the description of its action, most of the toxicity testing is done on experimental animals [15].Toxicity studies are divided into, acute toxicity, sub-acute toxicity and chronic toxicity studies. Between the different types of toxicological studies, acute toxicity studies supply input about the overall profile of magnitude of a drug toxicity, its activity and overall effects [16]. One of the basic steps in toxicological estimation of a new substance is the account of its toxicity after a single exposure of that substance. So, Current problems for nanomedicine involve understanding the issues related to toxicity and environmental impact of nanoscale materials.

Considering the vast potentiality of plants as sources, this work aims to apply a biological green technique for the synthesis of silver nanoparticles as an alternative to conventional methods. In this regard, leaves extract of *Andrographis paniculata* (commonly known as Kalmegh) a species of family Acanthaceae was used for bioconversion of silver ions to nanoparticles. In this study, silver nanoparticles can be produced at low concentration of leaf extract without using any additional harmful chemical/physical methods. The effect of concentration of metal ions and concentration of leaf extract quantity were also evaluated to optimize route to synthesise silver nanoparticle. The

method applied here is simple, cost effective, easy to perform and sustainable. Optical properties of the synthesized NPs were measured using UV-visible (UV-VIS) spectroscopy. Morphology of the prepared samples was analysed by high resolution transmission electron microscopy (HRTEM), X-Ray Diffraction (XRD) were used to study the crystallinity of the sample. The present work evaluates the toxicity of the prepared samples and ensures their safety upon experimental mice model and also analyzes their behavioural changes if any upon their in vivo application.

9B.2 MATERIAL AND METHODS

9B.2.1 Materials and reagents

Andrographis paniculata was collected from Jadavpur University campus. *Swiss albino mice* (6-7 weeks old) weighing 32 ± 5 g were procured from a CPCSEA registered animal house to conduct the safety toxicity study of the aforesaid NPs. All chemicals and reagents used in this study are analytical grade.

9B.2.2 Synthesis of AP conjugated silver nanoparticles (NPs)

9B.2.2a Plant extracts preparation

About 1gm of AP leaves were washed thoroughly with plenty of distilled water and both surfaces of leaves were sterilized using alcohol by gently rubbing. These leaves were heated for 15 min in 50 ml of distilled water at 50 °C. Then the extract was filtrated with Whatman filter paper no 1 and further filtered using vacuum filter with pore size of 0.2 µm. The final filtrate was stored in cool dry place for further use.

9B.2.2b Green synthesis of silver nanoparticles

Initially, 50 mM of silver nitrate ($AgNO_3$) solution was prepared. Then plant extract was added drop wise to it under stirring. The reaction mixture became yellowish to brownish and precipitate of silver was formed. The reaction mixture was left for 30 min for complete reduction to silver nitrate. Then the precipitate was collected by centrifugation at 15000 rpm for 10 min at 4 °C. The precipitate was vacuum dried at 30 °C and the sample, i.e AP conjugated silver nanoparticle (AP-Ag NP) was stored for further studies.

9B.2.3 Experimental animals

Swiss albino male mice of 6-7 weeks old and weighing approximately (32 ±2.0 gm.) were procured from a CPCSEA approved animal house (Registration No. 50/CPCSEA/1999) and randomly divided into three groups of six mice each (n=6) and all of them received standard laboratory diet (Hindustan Lever, Kolkata) and water *ad libitum*. The animals were housed in large clean polypropylene cages in a temperature-controlled room (20±2ºC) with relative humidity (45–60%) under 12-h light and dark cycles during the whole study period. Prior to experimentation, acclimatization was done for 7 days. The animals were maintained according to the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC)

(Approval No. IAEC/PHARM/1503/03/2015 dated 30.11.15). All procedures complied with the Declaration of Helsinki, as revised in 1996.

9B.2.4 Characterization of synthesized AP-Ag NP

UV-VIS spectra of the aqueous solutions of synthesized Ag NPs were recorded in λ25 spectrophotometer (Perkin Elmer, Germany) within the range of 200 to 800 nm. XRD patterns of the synthesized materials were analyzed in the range of 2θ from 25 º to 80 º using powder diffractometer, Model D8, BRUKER AXS, using Cu Kα radiation (α = 0.15425 nm). For this purpose, solution of Ag NPs was repeatedly drop casted on glass slides to make a thick coating of Ag NPs. Surface morphology and shape distribution was studies with the help of HRTEM.

9A.2.5 Appraisal of acute toxicity

Acute toxicity studies of the nanoconjugate was carried out in mice by using Organisation for Economic Co-operation and Development (OECD) guideline 425 [9]. Before administration of a single dose of the test samples, the mice were deprived of food for 3 h. Doses of 2000 and 5000 mg/kg of the test samples were given to Group II and Group III respectively, whereas Group I served as vehicle control and were orally administered 2ml of distilled water. The respective doses were suspended in water and were administered orally.

The mice were observed after every 30min post nanocomposite treatment for 2hrs.Once daily cage side observations included changes in skin, fur, eyes, mucous membrane (nasal), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation) and central nervous system (drowsiness, gait, tremors and convulsion) changes. Mortality, if any, was determined over a period of 2 weeks. After completion of the treatment, the animals were sacrificed by cervical dislocation and necropsied to facilitate gross pathological examination of organs.

9B.2.6 Clinical Observation

The treated animals were observed for mortality (twice daily) and the clinical signs were recorded to note the onset, duration and reversal (if any) of toxic effect at 2, 4, 6 and 8 hours after the administration of last substances and once daily thereafter for 14 days. The routine cage side observation s included changes in skin and fur, eye and mucus membrane, somatomotor activity, general behavior pattern were noted. Miscellaneous things like arching of the back, alopecia, wound, nasal discharge, lacrimation and loose stool were also recorded during the observation.

9B.3 RESULT AND DISCUSSIONS

9B.3 Behavioural Observations and General appearance

The behavioural parameters of animals were observed 2,4 ,6 and 8 h after the administration of the nanocomposite according to the standard protocol. No marked changes were observed in the wellness parameters observed. There were no mortality and all the mice survived the 14 day study period.

9B.3.1 Body Weight, Daily Food Consumption and Daily Water Intake

Table 9B.1&2 shows the change observed before and after administration of the nanocomposite , there were very slight increase in body weight of the animals but were statistically insignificant (Figure 9B.1). Daily Food Consumption and Daily Water Intake pattern (Figure 9B.1) were almost normal in comparison to normal control animals.

Table 9B.1: Effect of AP-AgNPs on the body weight of mice at 2,000 mg/kg dose

Animals (Mice)	0 Day	7th day	14th day
Control	24.3±2.66	27.5±2.07	30.2±0.99
2000 mg/kg body wt	24.4±1.35	25.4±2.24	27.9±1.22
5000mg/kg body wt.	26.7±0.89	25.9±1.34	27.6±1.54

Table 9B.2: Effect of AP-AgNPs on the body weight of mice at 5,000 mg/kg dose

Animals (Mice)	0 Day	7th day	14th day
Control	24.2±2.15	27.1±1.65	30.6±1.02
2000 mg/kg body wt	24.5±1.55	26.5±1.93	28.2±1.27
5000mg/kg body wt.	24.1±0.98	25.8±1.05	27.1±1.06

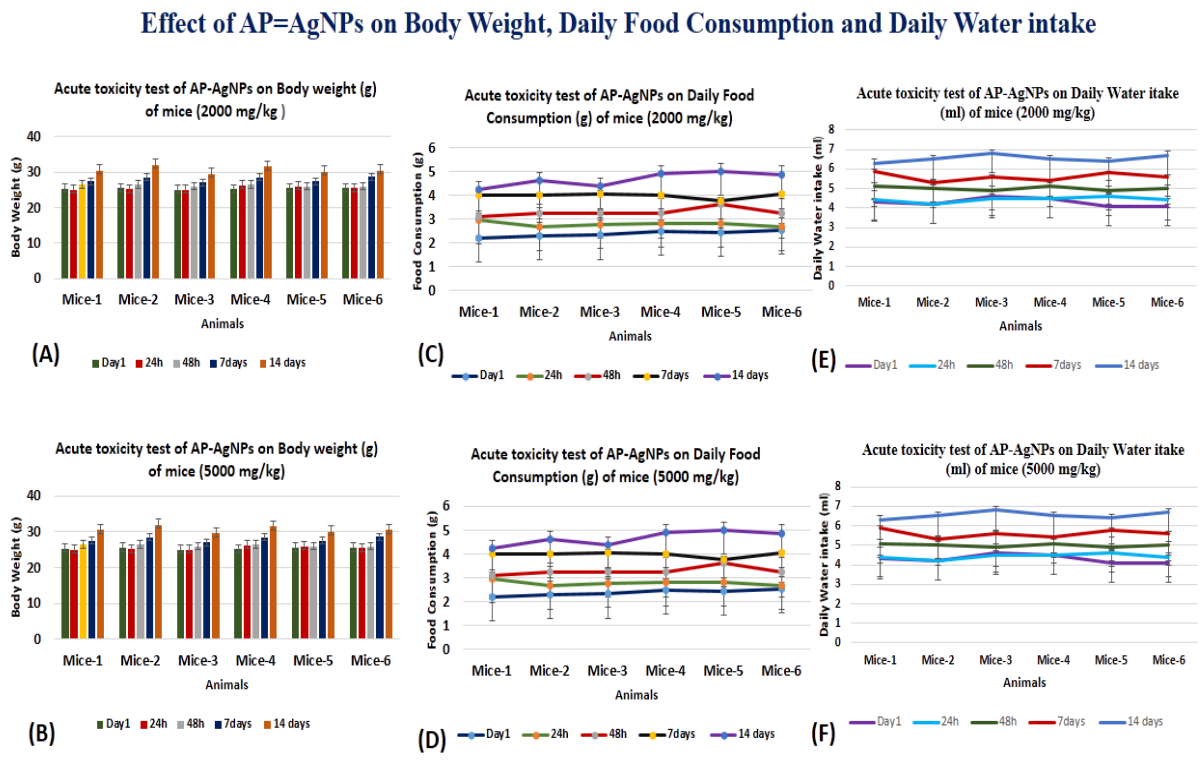

Figure 9B.1: Effect of AP-AgNPs on Body weight, Food Consumption and Water Intake

9B.3.3 General Behavioral Observations and appearance

In this study the behavioral parameters and appearance of animals after drug administration is indicator of the toxicity of the test drug. The behavioral patterns of animals were observed in 2h, 4h, 6h and 8h interval and followed by 14 h after the administration. The behavioral parameters and appearance was observed according to the standard protocol. No significant changes were observed in wellness parameters used for evaluation of toxicity. Skin, fur, eyes, mucous membrane, behavioral pattern, salivation and sleep pattern parameters of the treated animals were found to be normal (Table 9B.3&4). No toxic symptom or mortality was observed in any mice. All treated mice lived up to 14 days after the administration of NPs.

Table 9B.3: Clinical observations of mice at 2,000 mg/kg dose of AP-AgNPs

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Table 9B.4: Clinical observations of mice at 5,000 mg/kg dose of AP-AgNPs

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

9B.3.4 Necropsy

All the animals were sacrificed on day 14 and necropsied. Body cavities (cranial, thoracic, abdominal and pelvic) did not reveal any lesions nor any inflammation or any abnormalities (Table 9B.5).

Table 9B.5: Effect of AP-AgNPs on the Necropsy of mice

Experimental Animals	Observed lesions during study (2000 mg/kg)	Observed lesions during study (5000 mg/kg)
1.	Nil	Nil
2.	Nil	Nil
3.	Nil	Nil
4.	Nil	Nil
5.	Nil	Nil
6.	Nil	Nil

CONCLUSION

The toxicological investigation of Ag-AP nanocojuagate established that the developed nanopartical is safe and does not produce any abnormality upon animals. There was no change in the general health of the animals throughout the study. The results indicate that the nanocomposite did not exhibit any toxicity. These findings may facilitate the development of safe and efficient Ag-AP nanocojuagate as an effective therapy against various health disorders in future.

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9C Redox scavenging signature along with antioxidant action of silver nanoparticle coupled with *Andrographis paniculata* (AP-Ag NP), a component of AKSS16-LIV01 against carbon tetrachloride (CCl_4) induced hepatotoxicity in mice

9C.1 INTRODUCTION

Nanomedicine is the medical application of nanotechnology. The applications of Nanomedicine have been far and widespread. The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. Nanomedicine seeks to deliver a valuable set of research tools and clinically useful devices in the near future [1,2].

In recent years Silver nanoparticles are widely applied in consumer products, food technology and textiles/fabrics due to its unique chemical and biological properties [3]. Silver nanoparticles are the first and most widely commercialized nanomaterial in medical and healthcare sectors. AgNPs has been used in a range of biomedical applications, owing to their antibacterial activity [4-6], antifungal properties[7] and antiviral properties [8-10].

Green synthesis is defined as the use of environmentally compatible materials such as bacteria, fungi and plants in the synthesis of nanoparticles [11]. Alternatively, synthesis from biologically derived extracts offers several advantages such as rapid synthesis, high yields and importantly, the lack of costly downstream processing required producing the particles [12-14]. Hence, nanoparticle synthesis from plant extracts tentatively offers a route for large scale production of commercially attractive nanoparticles.

Radical scavengers (RS) are highly reactive species that can potentially abstract hydrogen atoms from activated bonds of biological materials under physiological environment. RS plays crucial role in neutralizing the direct attack of reactive oxygen species (ROS) that prevents a number of acute and chronic cellular implications by inhibiting the oxidative damage. Chronic diseases such as diabetes, cardiovascular disease and liver fibrosis has increased oxidants and decrease antioxidants quantity in patients [15]. Conditions that increase oxidants and reduce antioxidants also exacerbate these diseases [16-18]. Clinical and experimental studies have shown that disturbing the balance of the oxidant–antioxidant system can contribute to the pathogenesis of liver fibrosis [19]. So, there is a worldwide need to find out a new drug that can fight against these diseases and ameliorate the redox healing.

Considering the vast potentiality of plants as sources this work aims to apply a biological green technique for the synthesis of silver nanoparticles as an alternative to conventional methods. In this regard, leaf extract of *Andrographis paniculata* (commonly known as Kalmegh) a species of family Acanthaceae was used for bioconversion of silver ions to nanoparticles. The effect of concentration of metal ions and concentration of leaf extract quantity were also evaluated to optimize route to synthesise silver nanoparticle. The method applied here is simple, cost effective, easy to perform and sustainable.

The present study was aimed at investigating the efficacy of Silver Nanoparticle Coupled with *Andrographis paniculata* (AP-Ag NP) against CCl_4 induced oxidative injury. Liver & Kidney was selected as the major organ for investigation as it is the major target for free radical attack leading to lipid peroxidation.

9C.2 MATERIALS AND METHODS

9C.2.1 Synthesis of AP conjugated silver nanoparticles (NPs)

9C.2.1a Plant extract preparation

About 1gm of AP leaves were washed thoroughly with plenty of distilled water and both surface of leaves were sterilized using alcohol by gently rubbing. These leaves were heated for 15 min in 50 ml of distilled water at 50°C. Then the extract was filtrated with Whatman filter paper no 1 and further filtered using vacuum filter with pore size of 0.2 µm. The final filtrate was stored in cool dry place for further use.

9C.2.1b *Green synthesis of silver nanoparticles*

Initially, 50 mM of silver nitrate ($AgNO_3$) solution was prepared. Then plant extract was added drop wise to it under stirring. The reaction mixture became yellowish to brownish and precipitate of silver was formed [20]. The reaction mixture was left for 30 min for complete reduction to silver nitrate. Then the precipitate was collected by centrifugation at 15000 rpm for 10 min at 4°C. The precipitate was vacuum dried at 30 °C and the sample, i.e AP conjugated silver nanoparticle (AP-Ag NP) was stored for further studies.

9C.2.2 Experimental animals

Swiss albino male mice of 5-8 weeks old and weighing approximately (35±2.0 gm.) were procured from a CPCSEA approved animal house (Registration No. 50/CPCSEA/1999) and randomly divided into three groups of six mice each (n=6) and all of them received standard laboratory diet (Hindustan Lever, Kolkata) and water *ad libitum*. The animals were maintained according to the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No..IAEC/PHARM/1503/03/2015 dated 30.11.15). All procedures complied with the Declaration of Helsinki, as revised in 1996.

9C.2.3 Experimental Design

	Groups	Treatment
1.	Control	Normal Saline for 28 days
2.	CCl_4 treated	Received 1:1 (v/v) CCl_4 in olive oil for 28 days
3.	CCl_4 + AP-AgNP	Received 1:1 (v/v) CCl_4 in olive oil along with 0.50 g/kg /day for 28 days
4.	AP-AgNP	0.50 g/kg /day for 28 days

9C.2.4 Characterization of synthesized AP-Ag NP

UV-VIS spectra of the aqueous solutions of synthesized Ag NPs were recorded in λ25 spectrophotometer (Perkin Elmer, Germany) within the range of 200 to 800 nm. XRD patterns of the synthesized materials were analyzed in the range of 2θ from 25º to 80º using powder diffract meter, Model D8, BRUKER AXS, using Cu Kα radiation (α = 0.15425 nm). For this purpose, solution of Ag NPs was repeatedly dropped casted on glass slides to make a thick coating of Ag NPs[21].

9C.2.5 Isolation of serum

At the end of the experimental period, the animals were euthanized and decapitated after being fasted. Blood was collected from retro orbital plexus just before sacrifice and centrifuged at 3500 rpm for 20 min using centrifuge 5418R (Eppendorf, Ontario, Canada); the clear serum obtained was used for the determination of serum enzymes.

9C.2.6 Preparation of liver homogenate

After the experimental period, fasted animals were anesthetized with ketamine (50 mg/kg) and prepared serum samples according to standard protocol and stored at −20 °C. Liver tissue was removed and minced with a small scissor in a cold mannitol solution containing 0.225 M D-mannitol, 75 mM sucrose, and 0.2 mM ethylene-diaminetetra-acetic acid (EDTA). The minced liver was gently homogenized, in a homogenizer with a Teflon pestle, and then centrifuged. The supernatant was used to evaluate the parameters [22].

9C.2.7 Determination of liver enzymes

Carefully observed that serum samples were haemolysis-free, sterile and were kept at −20 °C before measurement of liver function enzymes. Serum aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined calorimetrically using commercial kits. Serum alkaline phosphatase activity (ALP), Serum gamma-glutamyl transferase (GGT) were measured calorimetrically using kit obtained from Stanbio Laboratory Kit, USA [23]. Protein concentration in the samples was measured by the Bradford method [24].

9C.2.8 Determination of Renal enzymes

Blood urea nitrogen (BUN), serum Ceratinine (Cr) and Uric acid concentration was assessed as markers of nephrotoxicity [25]. All these nephrotoxic marker parameters were determined spectrophotometrically from serum samples using commercially available kits (Sigma).

9C.2.9 Measurement of liver malondialdehyde (MDA)

Contents of MDA in serum and tissue were measured spectro-fluorometrically as Thiobarbituric acid (TBA) reactive substances. TBA reacts with MDA and is formed TBA reactant substances (TBARs), as biomarkers of oxidative damage to polyunsaturated fatty acids and measured at 532 nm by spectrophotometer.

9C.2.10 Determination of Antioxidant Enzymes

The tissues were chopped into small pieces with scissors and homogenized in ice-cold phosphate buffer (pH 8) at a concentration of 15% (weight by volume). They were then centrifuged in cooling centrifuge (Hettich Zentrifugen, Germany) at 960 g for 5 min. The supernatant was separated and further centrifuged at 7680 g for 40 min at 4°C. The final clear supernatant was used for evaluation of SOD, CAT, GPx and GSH by standard methods [27, 28].

9C.2.11 Histopathological Studies

For the histopathological observations at light microscopic level, fresh tissue pieces of liver were immersion fixed in 10% phosphate-buffered formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax [29]. Blocks were made and 5–7µm thick sections were double stained with hematoxylin and eosin (H&E), Sirius red and collagen deposition and examined under an Olympus BX51 fluorescence microscope (Olympus Optical, Tokyo, Japan).. Masson's trichrome (MT) and Sirius red (SR) staining were performed to quantify the extent of fibrotic damage in liver. Histopathological changes were examined under the microscope (Olympus BX51).

9C.2.12Statistical analysis

Results were expressed as Mean±SE. All data were analyzed with SPSS (Version: 16) and GraphPad Prism version 6.0 (GraphPad Software, San Diego-USA). One-way analysis of variance (ANOVA) followed by post hoc Tukey's test was used to detect the statistical significance between groups using a computer program GraphPad Prism Version 6 (GraphPad Software, San Diego, USA. Differences between groups were considered significant if $P<0.05$.

9C.3 RESULTS

9C.3.1 Characterization of NPs

The UV-VIS spectra of both chemically synthesized and green synthesized nanomaterials (Figure 9C.1A) showed absorption band–between wavelength range 200-900 nm, with a peak at 360 nm, conforming presence of Ag NPs in the solution. In case of AP conjugated AgNPs (AP-AgNps) the absorption spectra remained almost same with a slight blue shift in the absorption maxima. The XRD pattern of the synthesized nanomaterial (Figure 9C.1C) showed diffraction peaks at $2\theta = 36.2^{\circ}$, 40.6°, 62.6° and 71.3°, which respectively corresponded to (111), (200), (220) and (311) planes of pure silver. All peaks were duly assigned by using JCPDS file no. 04-0783 pertaining to pure silver.

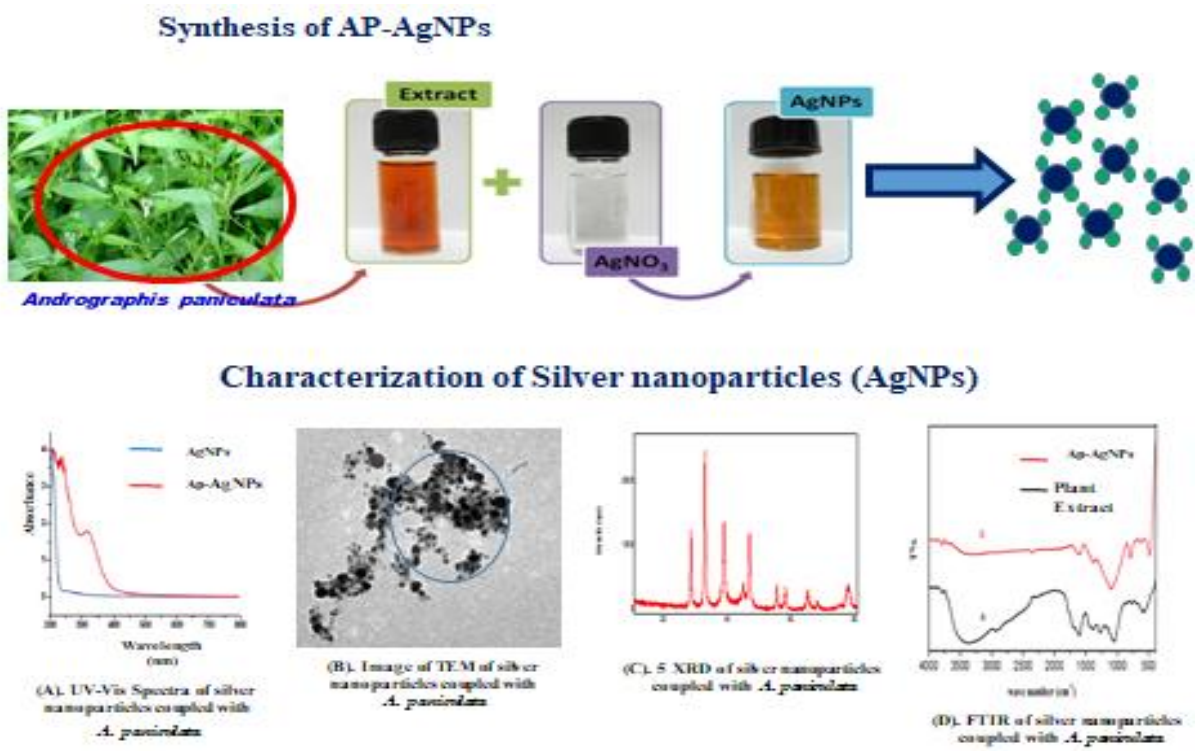


Figure 9C.1: Synthesis and Characterization of NP

9C.3.2 Effect of AP-Ag NP on body weight

Significant reduction in body weight was observed in mice exposed to CCl_4 for 14 days (Table 9C.1). No changes were observed in groups receiving CCl_4 and CCl_4 plus AP-Ag NP simultaneously.

Table 9C.1: Effect of AP-AgNP on body weight changes on CCl_4 Exposure in mice

Groups	Initial Body Weight (g)	Final Body Weight (g)	Body Weight gain or loss (g)
Control	25.14±0.13	29.37±0.11	4.23±0.042
CCl_4	24.98±0.14	26.01±0.14	1.03±0.051
CCl_4+ AP-AgNP	25.22±0.11	28.99±0.12	3.77±0.039
ET- AP-AgNP	25.31±0.12	29.48±0.11	4.17±0.044

Values are means ± SEM (n=6)

9C.3.3 Effect of AP-Ag NP on heme synthesis pathway

Significantly altered activity of ALAD was observed in the CCl_4 group compared to normal (Figure 9C.2). A marked recovery was observed in the group co-exposed to CCl_4 and AP-Ag NP.

9C.3.4 Effect on blood oxidative stress variables

Significant elevation in ROS noted following CCl_4 exposure compared to normal. A significant recovery was observed after co-administration of AP-Ag NP (Table 9C.2). A significant elevation in TBARS was observed, which was effectively reduced by AP-Ag NP pre-treatment (Table 9C.2).

Table 9C.2: Effect of AP-AgNP on blood biochemical and antioxidant variables on CCl_4 Exposure in mice

Blood	Control	CCl_4	CCl_4 + AP-AgNP	AP-AgNP
ROS (FIU)	412.59±14.3	736.13±17.8$^{\#}$	492.81±11.24	462.14±12.19
SOD (U/mg)	106.14±8.1	72.05±6.9$^{\#}$	98.34±7.1**	100.02±6.2**
CAT (U/mg)	232.62±21.3	161.1±19.4$^{\#}$	212.4±16.2**	224.9±13.1**
GSH (mg/g)	38.39±0.49	21.92±0.51$^{\#}$	33.12±0.81**	33.66±0.41**
MDA (nmol/g)	34.16±2.14	76.39±3.02$^{\#}$	40.12±3.44**	36.13±2.91**

Values are means ± SEM (n=6), a $P<0.05$, significant change with respect to control group, changes between ** $P<0.001$ compared with respect to CCl_4 group.

9C.3.5 Effect on antioxidant status

Administration of CCl_4 was significantly decreasing the essential antioxidant effect in the vital organ in the body. A significant recovery in blood GSH, CAT and SOD was observed after co-administration of AP-Ag NP (Table 9C.2).

9C.3.6 Effect on liver enzymes indicative of hepatic damage

ALT, AST, ALPO and GGT activities increased on CCl_4 exposure (Figure 9C.2) which responded favorably to AP-Ag NP. Nanoparticle was again found to attenuate CCl_4 induced alteration in the activities of these enzymes more effectively.

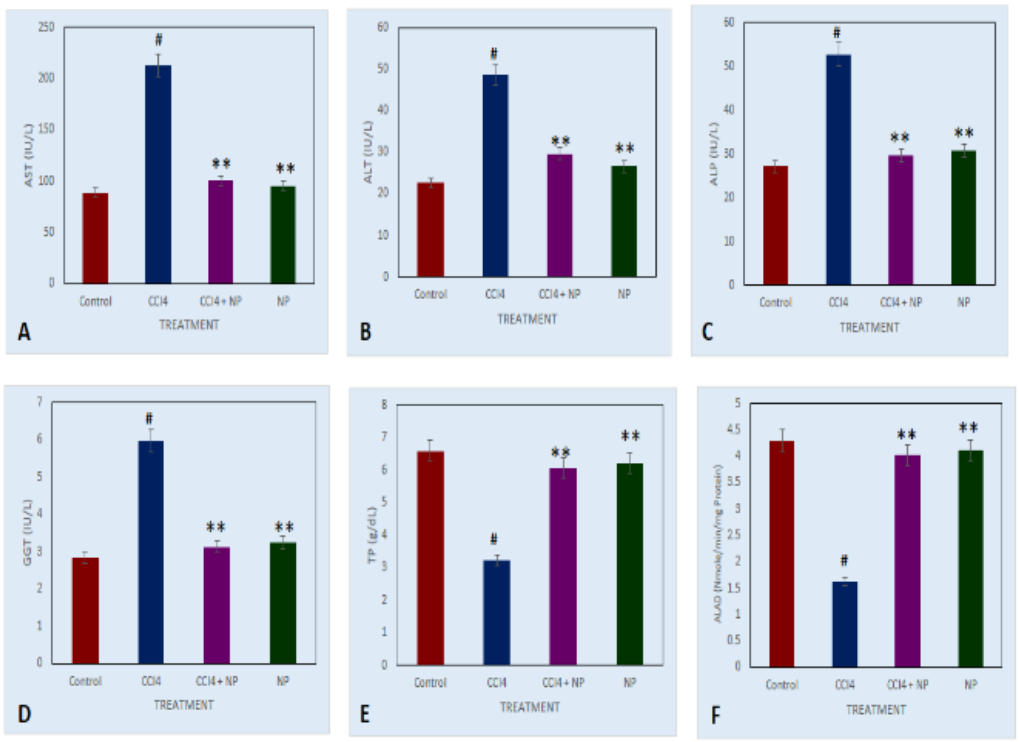


Figure 9C.2: Determination of Liver Biochemical parameters

9C.3.7 Evaluation of renal functions

Blood urea nitrogen (BUN), urea, uric acid and creatinine levels were studied to assess the renal functions. Results showed significant differences between the CCl_4-treated group, and CCl_4 plus AP-Ag NP treated group with regard to blood urea nitrogen, uric acid and creatinine levels (Table 9C.3).

Table 9C.3: Effect of AP-AgNP on renal function test variables on CCl_4 Exposure in mice

Renal Function Test	Control	CCl_4	CCl_4 + AP-AgNP	AP-AgNP
Urea (mg/dl)	32.52±2.5	64.16±3.1$^{\#}$	38.06±2.9	39.16±3.4
Uric acid (mg/dl)	1.410±.24	2.920±0.22$^{\#}$	1.850±0.14**	1.510±0.31**
Creatinine (mg/dl)	0.510±0.05	0.84±0.04$^{\#}$	0.56±0.04**	0.59±0.06**
BUN (mg/dl)	15.2±1.8	35.14±2.3$^{\#}$	17.03±2.1**	16.22±1.8**

Values are means ± SEM (n=6), a P<0.05, significant change with respect to control group, changes between ** P<0.001 comp

9C.3.8 Histology

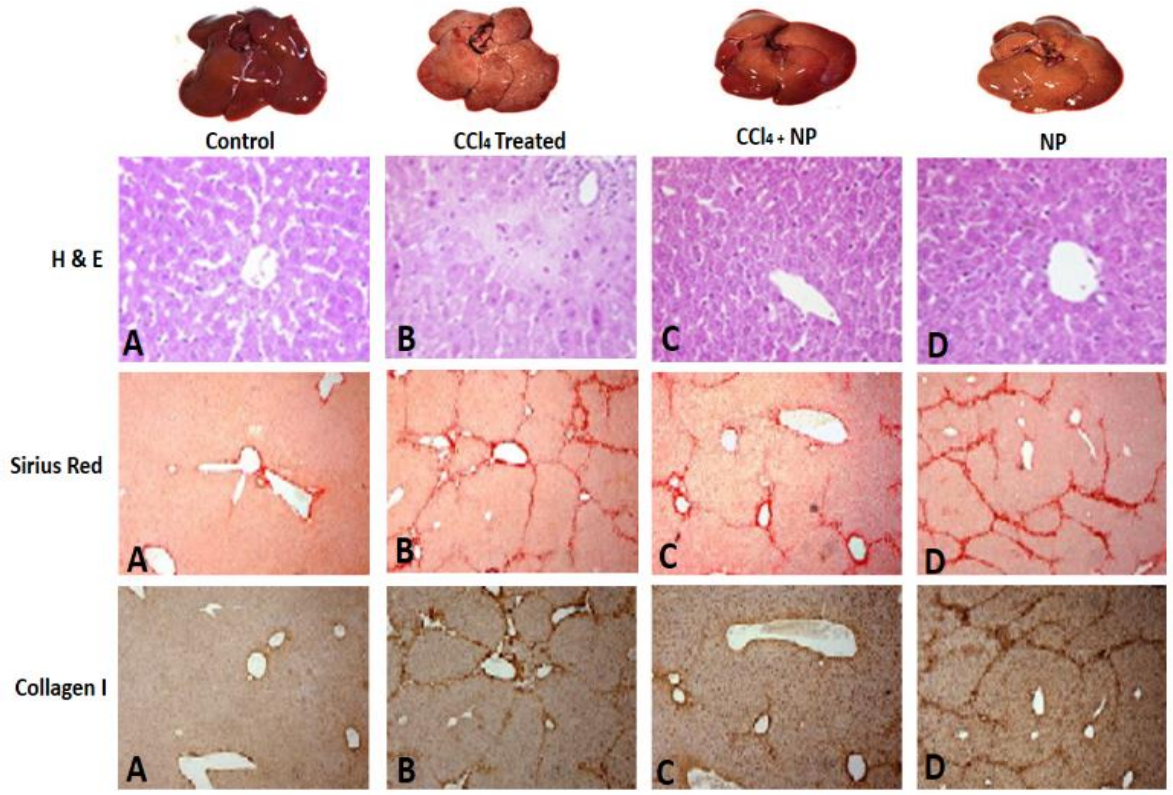


Figure 9C.3: Histopathological Investigation

9C.4 DISCUSSION

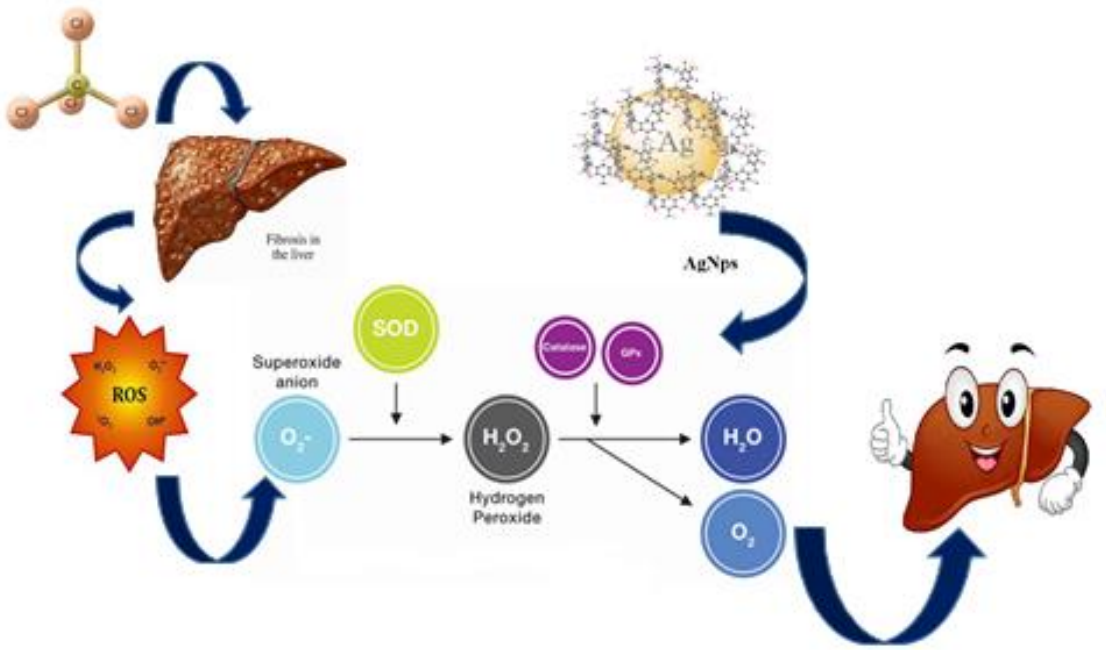
Cellular oxidative stress was evident by elevated ROS level, reduced glutathione level, increased lipid peroxidation and impaired antioxidant defense status [30-34]. Our study reported elevated ROS levels in CCl_4-treated group, suggesting free radical generation leading to oxidative stress conditions. Increased lipid peroxidation like elevated TBARS and reduced GSH, further signifies oxidative stress condition. Concomitant administration of Ag nanoparticle coupled with *Andrographis paniculata* led to pronounced recovery, suggesting it become a more effective scavenger of free radicals. ROS generation leads to impaired cellular antioxidant defence system. GSH levels decreased after CCl_4 treatment [35], possibly to its increased utilization in neutralizing free radicals. Glutathione is the major form of cellular glutathione and earlier reports support our results for the protective efficacy of developed silver nanoparticle. Elevated ROS levels are implicated in the damage of biological molecules such as lipids, which are altered by peroxidation. Elevation in TBARS is an indicator of lipid peroxidation under oxidative stress condition [36]. We observed a significant elevation in TBARS level following CCl_4 exposure and back to normal level after NP administration. Interestingly the level of plasma AST, ALT, ALP and GGT were restored to normal in the animals co-exposed to AP-Ag NP.

We also determined the antioxidant profile. SOD prevents the harmful effects of superoxide ion by converting them into less toxic hydrogen peroxides [37] which subsequently splits into nontoxic water and oxygen molecule by catalase activity. Catalase is another major antioxidant enzyme whose activity decreases during oxidative stress, leading to H_2O_2 accumulation and finally peroxidation of lipids [38]. We observed decreased hepatic SOD activity in our study. SOD is one of the components of intrinsic antioxidant defense system, and is responsible for dissemination of Superoxide radicals. During oxidative stress the body uses its defense mechanism to minimize the process of lipid peroxidation by using the antioxidant enzymes such as SOD, thus, the activity of this enzyme become higher in early stages of insult, but if the insult continue, the enzyme become depleted which means that in advance stages of per-oxidation the activity of SOD declined. We observed a significant increase in hepatic SOD and Catalase activity on NP exposure which responded favorably to the co-administration of antioxidants.

CONCLUSION

In this study, AgNPs were synthesized successfully using the leaf extract of *Andrographis paniculata* as a novel bio-reductant. Plant extract being very eco-friendly and cost effective can be used for the large scale synthesis of AgNPs in nanotechnology processing industries. The present study synthesized AgNPs from *Andrographis paniculata* leaf extract seem to be promising antioxidant activity which can be useful for the development of a new drug for biomedical applications. This biological chemistry approach towards the synthesized AgNPs through green route is highly essential effort being addressed in nanomedicine because of its varied advantages.

SUMMARY



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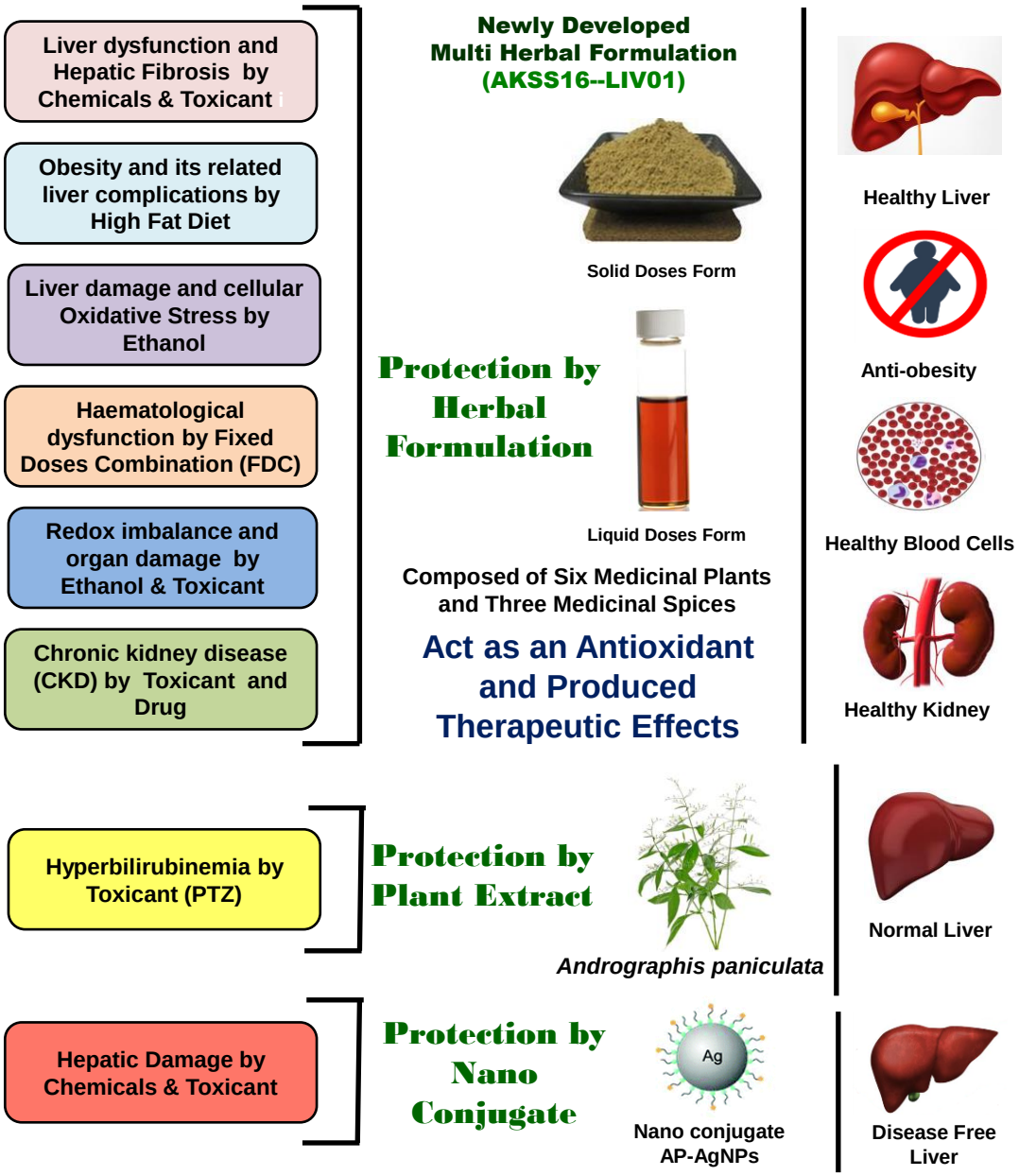
Summary and Conclusion

In this thesis work we have synthesized first time a novel multi herbal formulation (AKSS16-LIV01) which is composed of six Indian medicinal plants and three medicinal spices. The amounts of each ingredient mainly medicinal plants present in the composition are comparatively lower in comparison to the available marketed products as it reduces the bio-burden. Addition of medicinal spices have increased the therapeutic efficacy and produced the optimum curative effects upon the performed preclinical studies. We prepare both solid and liquid doses forms that are why the formulation has huge medicinal impact in future. The formulation complies with all the standard analytical parameters based on Indian Pharmacopeia (IP) and also abides by the 12 months long term stability study as per WHO guidelines. In vitro study established that the formulation have potent antimicrobial and antioxidant property. The developed formulation (ASSS16-LIVo1) are enriched with polyphenols, flavonoids, tannins, proteins, amino acids and saponin which play a key role in protection organs against various diseases. To confirm the safety and efficacy of the developed formulation 14 days acute and 28 days dose dependent acute and subchronic toxicity studies on mice model were conducted which revealed that AKSS16-LIV01 have completely safe, no toxic and adverse health effects on mammalian system.

The developed phytomedicine (AKSS16-LIV01) have proved to be very effective against various types of chronic hepatic and renal complications. Chemical intoxicated (CCl_4) chronic liver dysfunctions (hepatic fibrosis) were successfully prevented by the application of this formulation. High fat diet (HFD) induced Obesity which showed the higher lipid profile value in the mice along with various complications within the body. The therapeutic administration of AKSS16-LIV01 reduced the body fat and normalized the obesity related various alterations and complications. Long term alcohol (ethanol) consumption produced fatty liver as well as liver oxidative stress which increased various liver function parameters mainly transaminase activity, alkaline phosphatase activity and gamma-glutamyl transferase concentration in the blood stream. Long term use or overdose of tramadol hydrochloride/paracetamol (THP) a fixed dose combination (FDC) damages the liver cell in the mice which is prevented by the application of this novel formulation. The formulation (AKSS16-LIV01) has the ability to protect the haematopoietic cells from the damaging effects of exposure to Tramadol hydrochloride/paracetamol (THP) and this protection might be attributed to the anti-oxidative power of multi herbal formulation (AKSS16-LIV01). All the above hepatic and haematological dysfunctions generate reactive oxygen species (ROS) which is produced by the liver and alter inflammatory parameters (Pro-inflammatory cytokines). It is established that due to herb-herb interactions herbal formulations enriched with polyphenols, flavonoids, lignin, tannin etc. produce synergistic action, and are capable to reduce the oxidative stress. Synthesized formulation (AKSS16-LIV01) reduced the total ROS production and elevated the various antioxidant levels which converted the free radicals to produce water and oxygen thereby protecting the organ against ROS damage. On the other hand the formulation protects the cell membrane by decreasing lipid peroxidation (MDA content), Nitric oxide (NO) content and Nitric oxide syntheses (iNOS) levels. Apart from this our developed formulation is beneficial against CCl_4 induced renal dysfunction and protects the kidney against external chemical injury. The developed formulation has also improved the various haematological parameters specially those which were altered by toxicant (CCl_4) and fixed dose combination (FDC).

On the other hand, several chronic kidney diseases (CKDs) are reported to be due to redox imbalance in mitochondria. Our study suggests that multi herbal formulation (AKSS16-LIV01) could be an efficient medicine to attenuate renal injury and tubule intestinal fibrosis as evidenced by the improved renal functions, reduction in biochemical markers of nephrotoxicity, reduced fibrotic content, and down regulated pro-inflammatory cytokines. The molecular mechanism involves regulation of the redox balance through synchronization of the causal relationship between mitoprotection and ROS scavenging by newly developed multi herbal formulation (AKSS16-LIV01).



Andrographis paniculata (AP) is one of the primary active ingredients present in the formulation. We prepared various solvent extract with ethanol, methanol, chloroform, ether etc. from where methanolic extract of the herbs (AP) showed best therapeutic efficacy upon phynylehydrazine (PTZ) induced hyperbilirubenia in Swiss albino mice. Time dependent study established that the application of methanolic AP extract was able to reduce the elevated bilirubin levels within 6 hours. All the supportive parameters those were altered during hyperbilirubinemia returned to normalcy

Finally we synthesized and charecterized a metal silver (Ag) nanoconjugate with *Andrographis paniculata* (AP) as the reducing agent via the green synthesis route (Ap-AgNP). UV-VIS Spectra, TEM, SEM, FTIR, XRD clearly established the characteristics of the formed silver nanoconjugate. The synthesized nanoconjugate (AP-AgNP) has potent in-vitro antioxidant and antimicrobial activity. 14 days acute and 28 days subchronic toxicity studies upon mice model revealed that the nanoconjugate has no toxicity and adverse health effects on mammalian system. In preclinical disease model the nanoconjugate potrayed the capability to protect liver and kidney damage against chronic CCl_4 induction on experimental mice. Moreover AP-AgNP acts as a nanomedicine and maintain the homeostasis of the human body Altered inflammation is also restored by this nanoparticle.

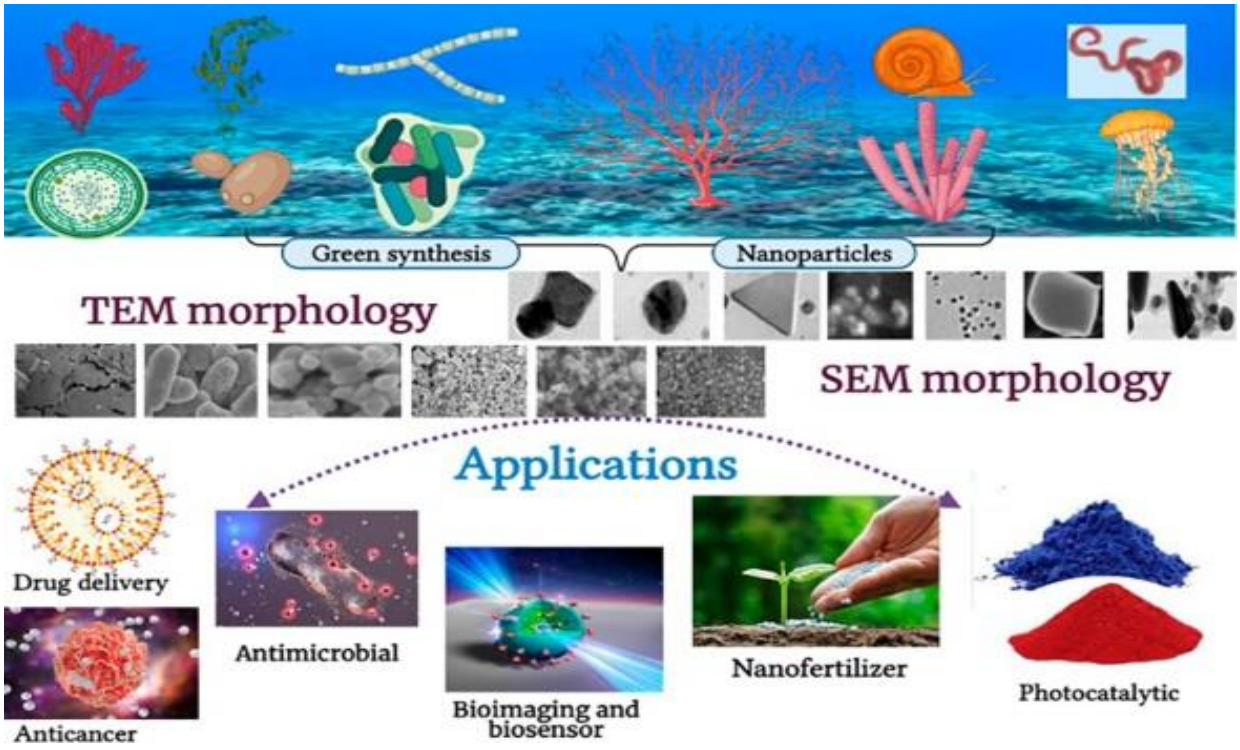
Invention of hepatic and renal phytomedication is the serious challenge in the 21st century. Here we have tried to develop a multi herbal phytotherapeutic formulation which may be an effective drug in the near future to prevent hepatic fibrosis, fatty liver, drug induced liver injury (DILI), hyperlipidemia (nonalcoholic fatty liver disease - NAFLD) and chronic kidney diseases (CKD). The amalgamations of medicinal plants and spices in the formulation have made it biologically more potent in comparison to marketed products due to the synergistic action of multiple active constituents of the active pharmaceutical ingredients (API). On the other hand methanolic extract of *Andrographis paniculata* (AP) one of the main active ingredients of the formulation has the potency as a future medication against hyperbilirubinemia (Jaundice). Prevention of disease with the aid of nanomedicine is now a new avenue of target specific nano therapy which is the future route medicine will partake and be a bench to bedside solution. Our synthesized plant based nanoconjugate may be the answer for chronic liver and kidney disease in the upcoming era.

Future Prospects

The work presented in this thesis emphasizes various disease models and the therapeutic aspect of synthesized herbal formulations along with nanoconjugates which is aimed to target the common people, being a bench to bedside success story. But, the story unfolds in a new aspect in the future frame. The future will prosper by raising the bar by partnering with industries and bringing the formulation as a product in commercial scale. Moreover, for the easy bioavailability the nanoconjugates could be suitable candidate for targeted drug delivery vehicles.

In the coming days various marine biomes and algae are going to take the center stage in the drug development research arena. The formulations with marine components and exploring the herb-mineral combination will play a role in treating various life threatening diseases, which will be a personal challenge which I wish to partake.

Furthermore, the pandemic has taught us that the indigenous ingredients not only increase the immunity but also has preventive action in the long run. So utilizing them along with nanomaterials as formulations to open new avenues in combatting with different diseases, including neonatal diseases like hyperbilirubinemia will be a new path of research.



Publications

Clinical Phytoscience

ORIGINAL CONTRIBUTION Open Access

Antioxidant and immunomodulatory effect of AKSS16-LIV01 – a multi herbal formulation against ethanol induced liver dysfunction in mice

Soumendra Darbar[1], Srimoyee Saha[2], Kausikisankar Pramanik[3] and Atiskumar Chattopadhyay[1*]

Abstract

Background: Liver complication arises commonly due to high alcohol consumption rate. Majority of the people residing in both developed and under developed countries consuming alcohol face various liver complications such as liver fibrosis, fatty liver, liver cirrhosis and even hepatocellular carcinoma. Invention of safe and symptomatic medication to overcome this situation is a new challenge worldwide. The main objective of the study is to deliver a safe and symptomatic medication to reduce the ethanol induced liver dysfunction.

Methods: In this study we have developed a multi herbal formulation (AKSS-16-LIV01) which minimised liver damage against various toxicants. Swiss albino mice were divided into seven groups where ethanol induced damage was observed for weeks followed by sanative response observation by our herbal formulation. The groups are normal control group, ethanol treated group (50% v/v), AKSS16-LIV01 low dose (75 mg/kg/day) pre-treated group, AKSS16-LIV01 middle dose (150 mg/kg/day) pre-treated group, AKSS16-LIV01 high dose (300 mg/kg/day) pre-treated group, Sylimarin pre-treated group (100 mg/kg/day) and only AKSS16-LIV01 (300 mg/kg/day) treated group.

Results: The results potrayed significant elevation of various biochemical parameters, lipid profile parameters, lipid peroxidation, nitric oxide (NO) concentration, nitric oxide synthase level and pro inflammatory cytokines level i.e. tumor necrosis factor (TNF-α) and transforming growth factor (TGF-β1) in the ethanol induced mice. On the other hand serum total protein, total albumin, albumin globulin ratio and level of tissue antioxidant enzymes activity (SOD, CAT, GSH and GPx) were significantly reduced by ethanol. Dose depended therapeutic application of the formulation (AKSS16-LIV01) significantly suppressed all the relevant above parameters and protected the liver from ethanol induced fibrogenesis. Apart from this gross morphology of the liver, H&E liver histology and massontrichrome&serius red examination of the liver section strongly supported the hepatoprotive effect of the formulation as compared with standard drug Sylimarin. Result of the study implies that developed multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg/day gave the best optimum response to reduce the ethanol intoxication.

Conclusion: Result clearly depict that AKSS16-LIV01 may be a safe and nontoxic medication which protect the liver against ethanol induced oxidative injury and maintained pro inflammatory cytokines level in the future.

Keywords: Multi herbal formulation, Liver damage, Liver function test, Lipid profile, Oxidative stress, Liver histology

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Introduction

Our body continuously exposed with various harmful toxicants as a result liver detoxifying the toxicant and maintains cellular homeostasis [1, 2]. People in both developed and underdeveloped countries when consume excesses alcohol or taken alcohol on a continuous basis leading to alcoholic liver disease (ALD). Individuals those are suffering with ALD facing lots of liver complications such as fatty liver disease, hepatic fibrosis, hepatic cirrhosis and hepatic cellular carcinoma even death [3, 4]. World health organization (WHO) published a report that stated death due to the liver failure stands the fifth position after cancer, cardiac failure, nervous disorder and respiratory infection [5]. Scientific study stated that consumption of ethanol elevate the ratio of $NADH/NAD^+$ in the liver cell which create disruption of oxidation of fatty acids in mitochondria developed liver dysfunction [6, 7]. Other causes of liver disease deposition of lipid molecule in the liver cells which stop the normal hepatic functions. Alcohol enhances the transportation process of lipids towards the liver from the small intestine which elevate the fatty acids mobilization from adipose tissue, taken up by the liver [8]. This causes damage of the liver cell membrane which releases the transaminases enzymes (AST and ALT) in the blood stream. On the other hand this damaged cell membrane also release alkaline phosphatase (ALP) and gamma glutamyltransferase (GGT) which indicate hepatic damage and inhibit the intercellular homeostasis [9–12].

Superoxide (O_2^-), hydroxyl radical (·OH), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2) are the common reactive oxygen species (ROS) were generated in the liver cell [13] when exposed with certain chemicals, environmental pollutants, xenobiotics etc. Firstly, these free radicals are generated from the oxidation procedure within the cell developed oxidative stress which destabilizes the normal cellular homeostasis [14, 15]. Secondly, Chronic intake of alcohol generates ROS via cytochrome P450 2E1 in the liver cell producing DNA damage, loss of membrane integrity, amino acid oxidation and inactivation of specific enzymes through oxidation of their cofactors [16, 17]. Important antioxidant enzymes like super oxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) mainly converts these harmful radicals through a series of biochemical reaction into oxygen and water molecule and protect the hepatic cell from the oxidative stress injury. It is evident that excessive intake of alcohol may develop cellular oxidative stress; indirectly disrupting cellular antioxidant defense system, which eventually produces cell death (apoptosis) and tissue damage. On the other hand nitric oxide (NO) is an important mediator of many physiological functions responsible for pathogenesis of many diseases. Chronic alcohol consumption increases nitric oxide (NO) levels which may lead to toxicity by peroxynitrite and destroy the membranous integrity. Free radicals i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS) when generates excessively in cell may not be neutralized in the system and cannot be eliminated from the body [18].

From the ancient time people from various countries depends on traditional system of medicine for curing the diseases as a safe and symptomatic medications. In the modern world Indian medicinal herbs and medicinal spices have been extensively used as an alternative medicine because of their promising medicinal property and lesser side effects in comparison to the allopathic drugs [19]. These medicinal herbs and spices are enriched with various constituents likes polyphenols, flavonoids, alkaloids, glycosides, tannins, proteins, amino acids, saphonin etc., which play a key role in cellular protection from the toxicants. Herbal formulations composed of some medicinally sound plants, now have garnered greater interest throughout the world due to its synergistic action. Recently people have a greater interest in herbal medicines because of their lesser side effect in clinical experience, pronounced effectiveness, safe for long term use and relatively low cost [20–22].

Currently throughout the globe fight against various liver dysfunctions such as fatty liver, liver fibrosis, liver cirrhosis etc. through symptomatic and safe medication is a new challenge. Presently, there is no effective treatment for hepatic dysfunctions. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and hygiene. Our previous reports stated that the formulation have no adverse side effect and no toxicity in mice [23, 24]. So, in the present study we formulated a new novel phytomedicine (AKSS16-LIV01) composed of six indigenous medicinal herbs and three medicinal spices those were mentioned in Ayurveda. Commercially available formulations composed of more than 12 medicinal herbs without medicinal spices. On the other hand in composition of the formulation the concentration of the individual herbs is less in comparison to marketed products. So, the developed formulation is an unique one and produced better therapeutic effects on animal models. Therefore, the present study was undertaken to evaluate the ethanol induced oxidative stress and hepatic injury of adult mice and its deleterious recovery by the application of unique novel multi herbal formulation (AKSS16-LIV01). The results of this study are expected to supply a transparent picture about the role of our newly formulated AKSS16-LIV01 in ethanol-induced hepatic damage, and should shed light on an achievable ethno-botany driven solution for serious liver problems.

Materials and methods

Chemicals

Trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethanol, TRIS buffers were purchased from SISCO laboratories, India. PBS buffer (pH 7.2) was taken from Sigma-Aldrich, Germany. All the biochemical kits (ALT, AST, GGT, ALP, Cholesterol, Triglyceride, Phospholipid, HDL, LDL etc.) were procured from Span Diagnostic, Surat, India. Hydroxyproline assay kit was procured from Bio Vision, Milpitas, CA. Antioxidant kits (SOD, CAT, GSH and GPx) were obtained from Boehringer, USA. ELISA kit TNF-α and TGF-β_1 were procured from Sigma Aldrich, USA. All others reagents utilized in this study are laboratory grade.

Collection and authentication of the herbs

In this study all the required medicinal herbs and medicinal species used for the preparation of novel formulation were collected from registered (authorized by West Bengal AYUSH department) medicinal herbs supplier. The plants and spices were identified and authenticated by renounced taxonomist, department of Pharmaceutical Technology, Jadavpur University, Kolkata, India and properly kept as voucher specimen (Table 1). The plants and plant parts used in extract preparation are listed in Table 2.

Preparation of extract

At the onset of the experiment collected plants and spices were cleaned by double distilled water until and unless those are properly cleaned. The cleaned spices and plants were air dried through normal temperature under sunlight. After that the samples were kept in calibrated and validated hot air oven at 75 °C for 20 min and 55 °C for 30 min. To obtain fine powder the plant parts and the spices were ground by a blade mill. A standard established protocol was followed for the preparation of extract with slight modification. After collection of the final extract, it was stored at 4 °C for further use. The extract was prepared with the ratio of 2:2:5:5:5:2:1:1:1 based on the individual plants ingredients. Individual yield of the each extract were *Tinospora sinensis* (Lour.) Merr. 11.80%, *Terminalia chebula* Retz. 10.52%, *Azadirachta indica* A. Juss. 9.75%, *Andrographis paniculata* (Burm.f.) Nees 12.52%, *Aloe barbadensis* Mill. 11.48%, *Curcuma longa* L. 14.06%, *Trigonella foenum-graecum* L. 15.05%, *Piper nigrum* L. 13.60% and *Elettaria cardamomum* (L.) Maton 12.64%. After the final collection of the working extract we obtained 92% of the plant extract which is commercially sound.

Preparation of Tinospora cordifolia extract

The plants were collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. Two hundred grams of *Tinospora cordifolia* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 11.80%.

Preparation of Terminalia chebula extract

The collected plants were identified and authenticated by a well-known botanist. Plants were cleaned with double distilled water. After that the sample was dried under sunlight. The plants were kept in a hot air oven at 75 °C for 20 min and 55 °C for 30 min. Two hundred grams of *Terminalia chebula* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary

Table 1 List of ingredients and their voucher specimen number

Sl. No.	Plant Ingredients	Voucher specimen number
1.	*Tinospora sinensis* (Lour.) Merr.	JU/AKC/16/151
2.	*Terminalia chebula* Retz.	JU/AKC/16/152
3.	*Azadirachta indica*A.Juss.	JU/AKC/16/153
4.	*Andrographis paniculata* (Burm.f.) Nees	JU/AKC/16/154
5.	*Aloe barbadensis* Mill.	JU/AKC/16/155
6.	*Curcuma longa* to *Curcuma longa* L.	JU/AKC/16/156
7.	*Trigonella foenum-graecum* L.	JU/AKC/16/157
8.	*Piper nigrum* to *Piper nigrum* L.	JU/AKC/16/158
9.	*Elettaria cardamomum* (L.) Maton	JU/AKC/16/159

Table 2 Composition of ingredient(s) present in AKSS16-LIV01

Sl No.	Botanical Name	Common Name	Family	Part Used	Quantity used in extract[a]
1.	*Tinospora sinensis* (Lour.) Merr.	Guduchi	Menispermaceae	Stem	20 mg
2.	*Terminalia chebula* Retz.	Haritaki	Combretaceae	Fruit	20 mg
3.	*Azadirachta indica*A.Juss.	Neem	Meliaceae	Leaves	50 mg
4.	*Andrographis paniculata* (Burm.f.) Nees	Kalmegh	Acanthaceae	Leaves &Steam	50 mg
5.	*Aloe barbadensis* Mill.	Aloevera	Liliaceae	Leaves &Steam	50 mg
6.	*Curcuma longa* to *Curcuma longa* L.	Curcuma,Haldi	Zingiberales	Rhizome	20 mg
7.	*Trigonella foenum-graecum* L.	Methi	Fabaceae	Seed	10 mg
8.	*Piper nigrum* to *Piper nigrum* L.	Blackpepper	Piperaceae	Seed	10 mg
9.	*Elettaria cardamomum* (L.) Maton	Cardamom	Zingiberaceae	Seed	10 mg

[a]Amount required for preparation of 5ml extract

evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 10.52%.

Preparation of Azadirachta indica extract

Azadirachta indica was collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. Five hundred grams of *Azadirachta indica* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process was repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 9.75%.

Preparation of Andrographis paniculata extract

The plants were collected, identified and authenticated by a experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. Five hundred grams of *Andrographis paniculata* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process was repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 12.52%.

Preparation of Aloe barbadensis miller extract

The plants were collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. Five hundred grams of *Aloe barbadensis miller* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 11.48%.

Preparation of Curcuma longa extract

The plants were collected, identified and authenticated by a experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. Two hundred grams of *Curcuma longa* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage of yield of the extract is 14.06%.

Preparation of Trigonella foenum-graecum extract

The plants were collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at 75°C for

20 min and 55 °C for 30 min. One hundred grams of *Trigonella foenum-graecum* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 15.05%.

Preparation of Piper nigrum extract

The plants were collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hotairovenat75°C for 20 min and 55 °C for 30 min. One hundred grams of *Piper nigrum* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 13.60%.

Preparation of Elettaria cardamomum extract

The plants were collected, identified and authenticated by an experienced botanist. Then the plants were cleaned with double distilled water and dried under sunlight. The plants were kept in a hot air oven at75°C for 20 min and 55 °C for 30 min. 100gmo of *Elettaria cardamomum* powder were mixed with 1200 ml methanol and sonicated for 30 min using an ultrasonic bath at room temperature, centrifuged at 3000 rpm for 15 min and the supernatant was collected, process was repeated four times, finally supernatant was evaporated under reduced pressure at 35 °C in a rotary evaporator. Three milliliters of methanol was used to reconstitute the residue. The extract was filtered using Whatman filter paper and kept at 4 °C for further use. The percentage yield of the extract is 12.64%.

Phytochemical screening

Various essential plant secondary metabolites such as sterols and triterpenes, Mg^{2+} turning test of flavonoids, alkaloids, saponins, glycosides, tannins, phenolic content, total flavonoids content in the developed multi-herbal formulation (AKSS16-LIV01) were detected through quantitative analysis with slight modification as described by Evans and Gueverra [25–27]. Detailed procedure as follows:

Test for tannins

0.30 g grinding powder sample was weighed into a test tube and boiled for 10 min in a water bath containing 30 ml of water. Standard filter paper was used for filtration. To 5 ml of the filtrate was added 3 drops of 0.1% ferric chloride. A brownish green or a blue black colouration showed positive test [28].

Test for Saponin

Distilled water (30 ml) was added to grinding powder samples (0.30 g) and boiled for 10 min in a water bath and filtered using Whatman filter paper number 42 (125 mm). A mixture of distilled water (5 ml) and filtrate (10 ml) was agitated vigorously for a stable persistent froth. The formation of emulsion on addition of three drops of olive oil showed positive result [29].

Test for steroid

Weighed 0.03 g sample into a beaker was mixed with 20 ml of ethanol; the component was extracted for 2 h. Five milliliters of plant extract (ethanolic) was taken and 2 ml acetic anhydride added within it. After that 2 ml of concentrated tetraoxosulphate (VI) acid was added to obtained the colour. A violet to blue or rather bluish green colour change in sample(s) indicated the presence of steroids [30].

Test for Terpenoids

Powder sample (0.30 g) was weighed into a beaker with 30 ml ethanol and component extracted for 2 h. Two milliliters of chloroform and 3 ml of concentrated tetraoxosulphate (VI) acid was taken and mixed vigorously. Then added 5 ml of plant extract to it. A reddish brown colour in the sample indicates the positive results and presence of terpenoids [31].

Test for flavonoids

0.30 g powder was taken in a cleaned beaker and added 30 ml of distilled water. The mixture was stayed for 2 h and filtered with standard filter paper number. Ten milliliters of the filtrate extract (aqueous) was taken and 5 ml of 1.0 M dilute ammonia solution added to it. After that 5 ml of concentrated tetraoxosulphate (VI) acid was added. Appearance of yellow colouration which disappeared on standing shows the presence of flavonoids [32].

Test for alkaloids

Two grams powder sample was in a cleaned conical flask for extraction by using 5% tetraoxosulphate (VI) acid (H_2SO_4) (20 ml) in 50% ethanol by boiling for 2 min and

filtered through Whatman filter paper. The filtrate was made alkaline using 5 ml of 28% ammonia solution (NH_3) in a separating funnel. Two sets of chloroform (5.0 ml) was for further solution extraction where chloroform solution was extracted with two 5 ml portions of 1.0 M dilute tetraoxosulphate (VI) acid. This final acid extract was then accustomed perform the subsequent test: zero.5 ml of Dragendorff's chemical agent (Bismuth iodide solution) was mixed with 2 ml of acid extract and precipitated orange color infers the presence of organic compound [33].

Test for glycoside

To 2.00 g of sample 20 ml of water was supplementary, heated for 5 min on a water bathtub and filtered through Gem paper (12.5 cm). The subsequent tests were administrated with the filtrate: (a) zero.2 ml of Fehling's solutions A and B was mixed with 5 ml of the filtrate till it became base-forming (tested with acid-base indicator paper). A brick-red colouration on heating showed a positive result. (b) Instead of water, 15 ml of 1.0 M sulphuric acid was used to repeat the above test and the quantity of precipitate obtained compared with that of (a) above. High precipitate content indicated the presence of glycoside while low content showed the absence of glycoside [34].

Test for phenolic content

One milliliter of plant sample was taken in a test tube few drops of 10% ferric chloride solution was added to it. Violet colour appeared which indicated the presence of phenolic compounds [35].

Animals

Adult male swiss albino mice weighing 26 g ± 3 g were obtained from our CPCSEA registered central animal house facility. The animals were divided into seven experimental groups with 10 animals in each group. For acclimatization mice were kept in the environment controlled animal room for 1 week before the onset of the experiment. The animals were maintained at 12 h light/dark cycle with constant temperature (22 ± 2 °C) and humidity (54 ± 4%). Standard pellet diets (Procure from Hind liver India Limited, Mumbai) were given to the animal with and water ad libitum. The whole experimental procedure were carried out according to the new revised guidelines (2018) of CPCSEA, Ministry of Agricultural and Animal Husbandry, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC), Jadavpur University having approval number (IAEC/PHARM/1503/03/2019 dated 30.11.19).

Experimental protocol

Swiss albino mice were divided into seven groups where ethanol induced damage was observed for weeks followed by sanative response observation by our herbal formulation. The groups are normal control group, ethanol treated group (50% v/v), AKSS16-LIV01 low dose (75 mg/kg/day) pre-treated group, AKSS16-LIV01 middle dose (150 mg/kg/day) pre-treated group, AKSS16-LIV01 high dose (300 mg/kg/day) pre-treated group, Sylimarin pre-treated group (100 mg/kg/day) and only AKSS16-LIV01 (300 mg/kg/day) group. A detail of the protocol was given in Table 3.

Body weight gains and feed efficiency

Routine body weights of the each animal were measured and recorded in every week from the initial day to the final day of experiment and then determined the body weight alteration. Regular food consumption was calculated by measuring food residue on the basis food given at the fix time. Feed conversion was obtained by dividing total feed intake by weight gain.

Blood collection

After the experimental period 200 μL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%) from the retro orbital plexus of the mice. Blood collected from animals were taken in heparinized tube. The tubes were stay for 3 h in 45° angle at room temperature (27 °C). After that the blood samples were centrifuged at 3500 g for 15 min to obtained serum. Developed light yellow colour serum was collected with caution and stored − 4 °C for further biochemical analysis.

Table 3 Experimental Design

Groups	Treatment
I	Normal control received only the vehicle (1 ml/kg olive oil twice a week for 8 weeks)
II	Received Ethanol (50% v/v) daily for 8 weeks
III	Treatment with ethanol (50% v/v) for 4 weeks and then pre-treated with herbal formulation AKSS16-LIV01 (75 mg/kg bw/day) for next 4 weeks
IV	Treatment with ethanol (50% v/v) for 4 weeks and then pre-treated with herbal formulation AKSS16-LIV01 (150 mg/kg bw/day) for next 4 weeks
V	Treatment with ethanol (50% v/v) for 4 weeks and then pre-treated with herbal formulation AKSS16-LIV01 (300 mg/kg bw/day) for next 4 weeks
VI	Treatment with ethanol (50% v/v) for 4 weeks and then pre-treated with Silymarin standard hepatoprotective drug at a dose (100 mg/kg bw) for next 4 weeks
VII	Treated with multi herbal formulation (MHF) AKSS16-LIV01 (300 mg/kg bw/day all over the experiment).

Hematological parameters

Blood samples were taken from the retro orbital plexus of experimental mice and collected in heparinized tubes for determination of haematological parameters. Blood parameters were studied in this experiment i.e. haemoglobin (Hb), mean corpuscular volume (MCV), reticulocyte, hematocrit, mean corpuscular hemoglobin concentration (MCHC), total red blood cell (TRBC), mean corpuscular hemoglobin (MCH), total white blood cell (TWBC), platelets, and differential count using Sysmax-K1000 automatic Cell Counter.

Assessment of liver function parameters

All biochemical tests were carried out by using commercial kits with little modification of manufactures instruction. Liver function test (LFT) parameters like different aminotransferase (AST & ALT), [36, 37] alkaline phosphatase (ALP), [38] gamma-glutamyl transferase (GGT), [39] globulin, [40] bilirubin (total & direct) [41] were analysed using biochemical assay kits (Merck, India) with manufacturer's instruction. Total protein concentration was determined in the serum by the method of Lowry et al. [42].

Assessment of serum lipid profile

Serum lipid profile like Cholesterol, [43] Triglyceride, [44] Phospholipids, [45] Free fatty acids, [46] LDL-cholesterol [47] and HDL-cholesterol [48] were measured using enzymatic calorimetric kits (ELITech Diagnostic, France) according to manufacture instructions.

Preparation of tissue homogenate

Prior to tissue biochemical analysis, 100 mg/mL of whole liver was homogenized in 50 mM phosphate buffer (pH 7.0). After homogenization the homogenate was centrifuged at 11000 rpm for 12 mins and the supernatant was collected and used for different parameters. Protein concentrations of liver supernatant were determined [49] using commercially available kit (Span Diagnostics Ltd., India) following procedure prescribed by manufacturer.

Hydroxyproline assay

Hydroxyproline assay to quantify collagen content were performed by established protocol. In details, 10 mg of freshly collected liver was homogenized in 100 ml of sterile MQ water followed by hydrolysis in 12 N HCl (100 ml) at 120 °C. Four hours later 5 ml of tissue lysate became transferred to a 96-well plate. The whole sample was incubated at 37 °C for 18 h to evaporate the acid. Test Samples have been incubated with including equal amounts of chloramine T and Ehrlich's reagents for 40 min at 70 °C. Absorbance was recorded at 560 nm by using an ELISA plate reader (Synergy BioTek, Winooski, VT). Three to five mL blood sample was required to conduct the whole study.

Assessment of lipid peroxidation, NO and iNOS

The level of lipid peroxidation (MDA content) was measured from tissue sample and serum sample according to the method of Ohkawa [50] with slight modification. The level of NO and iNOS activity were determined from the liver homogenate through ELISA method using the commercial kit [51]. The detailed methods are as follows:

Lipid peroxidation (LPO)

The measurement was carried out using lipid peroxidation (MDA) assay kit (Sigma-aldrich Ltd., UK) in accordance to the manufacturer's instructions. Here lipid peroxidation is determined by the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) as a reactor to form a colouring product, proportional to the MDA present. To form the MDA-TBA adduct, the TBA solution (600 mL) was added into each sample and incubated at 95 °C for 60 min, after cooling to room temperature in an ice bath for 10 min. Two hundred milliliters of each mixture was transferred into a 96-well plate for analysis. The absorbance was measured at 532 nm [50].

NO activity

Nitrite is estimated through established "Griess Reaction" method. In this method, two-step diazotization reaction during which acidified NO^{2-} produces a nitrosating agent, which chemically reacts with sulphanilic acid to supply the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine [NEDD] to make the chromophoric azo-derivative which absorbs light at 540 nm. In brief equal volumes of tissue sample and Griess reagent (5% phosphoric acid containing 0.1% NEDD, 1% sulfanilamide) was mixed and incubated in dark for 10 min at room temperature. After that the reaction mixture is measured at 540 nm. The concentration of nitrite in the sample can be determined from a sodium nitrite ($NaNO_2$) standard curve [51].

iNOS activity

Forty microliters of Reaction Mixture (mixture contains 10 μL Diluted NOS Cofactor 1 + 20 μL NOS Cofactor 2 (1X) + 5 μL NOS Substrate + 5 μL Nitrate Reductase) was taken in sample, standard and control tube and mix properly. Incubate it at 37 °C for 1 h. Ninety microliter of NOS Assay Buffer was added to sample, standard and control tube. Mixed well and then add 5 μL of Enhancer to all. All the tubes were kept in room temperature for 10 min after proper mixing. Finally 50 μL of Griess Reagent 1 and 50 μL of Griess Reagent 2 were then added

to all with proper mixing and incubated all the tubes at room temperature for 10 min. Measured the OD at 540 nm and calculated the reading through the standard curve [51].

Assessments of antioxidant enzymes

Tissue antioxidant enzymes activities were measured according to standard protocol with slight modification. These are as follows:

Determination of superoxide dismutase (SOD)

The activity of superoxide dismutase (SOD) was measured according to a well-established reported method [52]. In brief, 2.5 mL reagent solution (xanthine 0.3 mM, EDTA 0.65 mM, 140 μM NBT), sodium carbonate 0.4 M, and bovine albumin (35 mg/30 mL) was added to 0.1 mL sample and 50 μL xanthine oxidase (10 μL in 2 M ammonium sulphate), incubated at 25 °C for 20 min and mixed with,0.1 mL 8 M copper chloride. The developed color was measured at 560 nm and calculates the result with using co-factor.

Determination catalase (CAT)

Catalase activity was measured according to the method of Maehly (1955) [53, 54]. Briefly, on the aftermath of the addition of 5 μL liver homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm up to 1 min to determine catalase activity. The enzymatic activity was expressed as U/mg protein.

Determination of glutathione (GSH) and GPx content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method [55]. GPx activity was assayed using a modified method of Hafeman. al [56].

Determination TNF-α TGF 1β

Levels of TNF and TGF in the liver were measured following the procedure provided with the purchased kit [57]. Protein levels were also measured according to the literature provided with the kit.

TNF alpha

All reagents, samples and standards were first prepared as instructed in the manual. Then 100 μl of standard and tissue sample was added to the specific well. Incubated for 2 h 30 min at room temperature. After that 100 μl freshly prepared biotin antibody was added to each well and incubated for 1 h at room temperature. Then 100 μl freshly prepared streptavidin solution was added to each well and incubated for 45 min at room temperature. After that 100 μl of TMB was added to each well and incubate for 30 min at room temperature. Finally 50 μl of stop solution was added to each well and immediately read the reading at 450 nmusing a microplate reader.

TGF-beta 1

All reagents, samples and standards were freshly prepared according to manual. Ten microliters balance solution was added to each 100 μl tissue sample and mixed with caution. One hundred microliters standard and sample was added to specific selected well. Then 50 μl of 1x HRP-conjugate was added to each well except blank. Mixed gently and incubated for 60 min at 37 °C. Aspirated the liquid from each well and washed carefully. Approximately 350 μl of 1x wash buffer was added through multi-channel pipette. All the tubes were kept for 15 min before completely aspirating. After the remaining wash, aspirating eliminated any closing Wash Buffer after which inverted the plate and tapped towards smooth absorbent paper.50 μl of substrate A and 50 μl of substrate B was added with gentle shaking. All the wells were kept in the dark for 15–20 min at 37 °C. Then 50 μl of stop solution was added to each well. The blue color changed to yellow immediately. Blank contained only 100 μl of PBS (0.02 mol/L pH 7.0–7.2). Optical density of each well immediately determined at 450 nm using a microplate reader.

Hematoxylin and eosin staining

Freshly liver sample was taken in a 4% formaldehyde saline solution and stored 72 h. The tissue was embedded and prepares paraffin blocks. Five micron thin sections were deparaffinised and washed with water. After washing tissue sections were treated with Mayer's hematoxylin solution and kept for 5 min. Observed the staining quality under microspore. If staining was perfect then counter stained with 1% eosin for 2 min. The sections stained by the eosin and used dehydrated alcohol to remove any residual stain [58]. Mount the section with Canada balsam by using cover slip. Visualized the liver section under microscope and taken photographs through camera (Olympus BX51 fluorescence microscope) for further histological scoring analysis.

Sirius red staining

After the experimental period liver slices were fixed in 10% neutral buffered formalin for overnight and then transferred to 70% ethanol before imbedding in paraffin blocks. A paraffin-embedded liver tissue block was taken and cut into 5 mm thick sections. Paraffin-free sections were soaked for approximately 1 h in Pico-Sirius red solution (Abcam, Cambridge, MA) followed by a short rinse with acetic acid (0.05%). Liver sections were dehydrated by washing with absolute alcohol [59]. Sections were observed with a light microscope (Olympus BX51 fluorescence microscope).

Statistical analysis

Two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. *p*-values lower than 0.05 (p<0.05) were considered statistically significant.

Results

Determination of phytochemical constituents

Table 4 represents qualitative analysis of the plants secondary metabolites in water extract of multi herbal formulation (AKSS16-LIV01) revealed the presence of sterols in trace amount. After the closing wash, aspirating eliminated any last Wash Buffer after which inverted the plate and tapped towards smooth absorbent paper. Triterpenes weren't detected within the chloroform, ethanol, methanol and aqueous extract (Table 4). Saponins were detected in trace amount, flavonoids, alkaloids and glycosides were found to be moderately plethoric and therefore the presence of sterols was found to be abundant. This implies that a lot of secondary metabolites were found within the liquid extract that could be a sensible sign to determine the extract as a drug.

Determination of physical morphology, body weight, liver weight, liver index and food consumption

Figure 1 showed that chronic administration of ethanol retarded the overall growth and development (Fig. 1C) of the mice in compared with control untreated animal. Novel herbal formulation (AKSS16-LIV01) recovered the normal growth. Interestingly ethanol intoxication decreased gross body weight (Fig. 2B), food consumption (Fig. 2C) as well as daily water intake. Therapeutic treatment with AKSS16-LIV01 (150 & 300 mg/kg) prevent the toxic effects of ethanol and maintained the normal increasing body weight pattern. Moreover significantly increased liver weight and liver index (Fig. 2A) by ethanol intoxication was normalized through natural therapy (Fig. 2).

Table 4 Qualitative analysis of the phytochemical constituents of multi herbal formulation (AKSS16-LIV01)

Phytochemicals	Ethanol	Methanol	Chloroform	Aqueous
Sterols	(+)	(+)	(+)	(++)
Triterpenes	(+)	(++)	(++)	(+++)
Flavonoids	(++)	(++)	(++)	(+++)
Alkaloids	(++)	(+)	(+)	(++)
Saponins	(+)	(+)	(+)	(+)
Glycosides	(+)	(+)	(+)	(++)
Tannins	(+)	(+)	(+)	(++)

(+) = traces, (++) = moderate, (+++) = abundant, (–) = absence of constituents

Determination of serum AST, ALT, ALP, GGT and total protein level

Result presented in Fig. 3 indicated that levels of serum enzymes such as AST, ALT, ALP and GGT were significantly elevated ($P < 0.001$) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study serum enzymes like AST, ALT, ALP and GGT were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Moreover, Serum total protein level was significantly decreased ($P < 0.001$) in ethanol treated mice compared with normal control mice. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly increased the serum total protein level at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Determination of serum BUN, total bilirubin, direct bilirubin, albumin level and albumin-globulin ratio

Result presented in Table 5 indicated that levels of serum biochemical hepatotoxic marker such as blood urea nitrogen (BUN), total bilirubin and direct bilirubin were significantly elevated ($P < 0.001$) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study serum enzymes like BUN, total bilirubin and direct bilirubin were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Moreover, Serum total albumin level and albumin-globulin ration was significantly decreased ($P < 0.001$) in ethanol treated mice compared with normal control mice. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly increased the serum total albumin level and albumin-globulin ratio at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 10% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Determination of liver non enzymatic markers

The total cholesterol, triglycerides, phospholipids, free fatty acids, LDL level of untreated ethanol control group

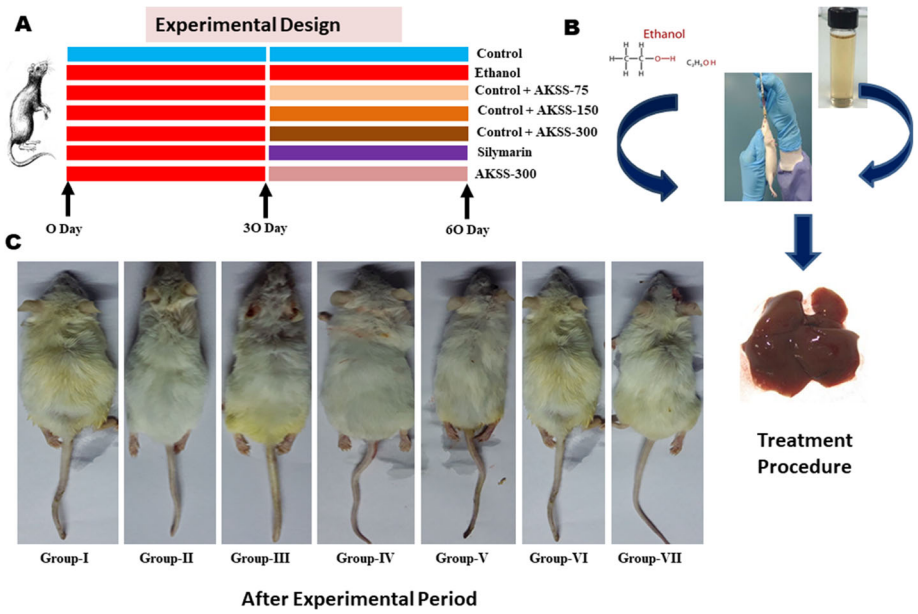


Fig. 1 In-vivo experiments upon experimental mice model. **A** Experimental design, **B** Experimental procedure, **C** External views of mice after experimental period

was significantly higher ($P < 0.001$) and HDL level significantly lower ($P < 0.001$) than the control groups presented in Table 6. In contrast, the levels of cholesterol, triglycerides, phospholipids free fatty acids and LDL of the 75 mg/kg and 150 mg/kg body weight of multi herbal formulation (AKSS16-LIV01) with ethanol groups were significantly lower ($P < 0.05$) and HDL level significantly higher ($P < 0.05$) than the ethanol control group. Administration of multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg body, significantly normalise ($P <$

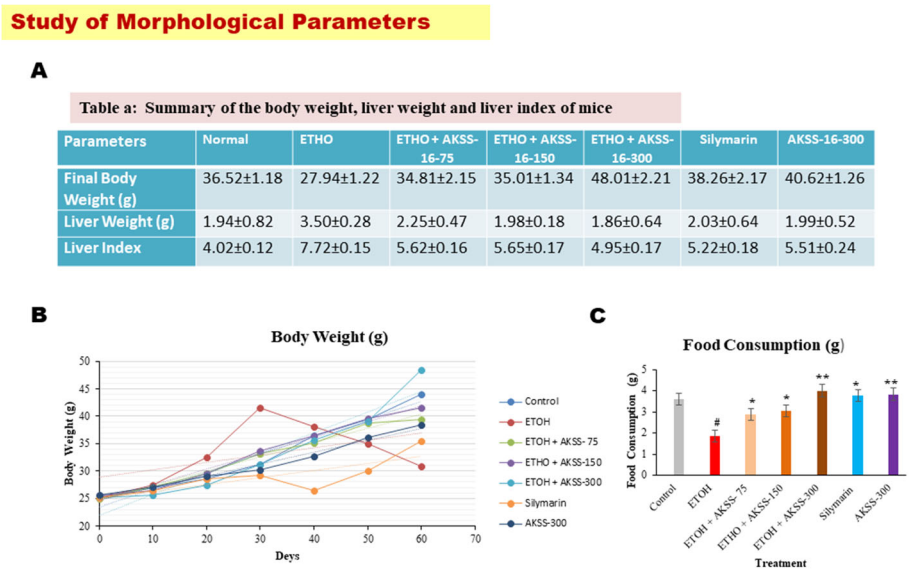


Fig. 2 Study of the morphological parameters. **A** Table showed summary of the body weight, liver weight and liver index in mice, **B** Day wise body weight, **C** Mean food consumption. The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p < 0.001$ and significantly different from ethanol $^{*}p < 0.05$, $^{**}p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test

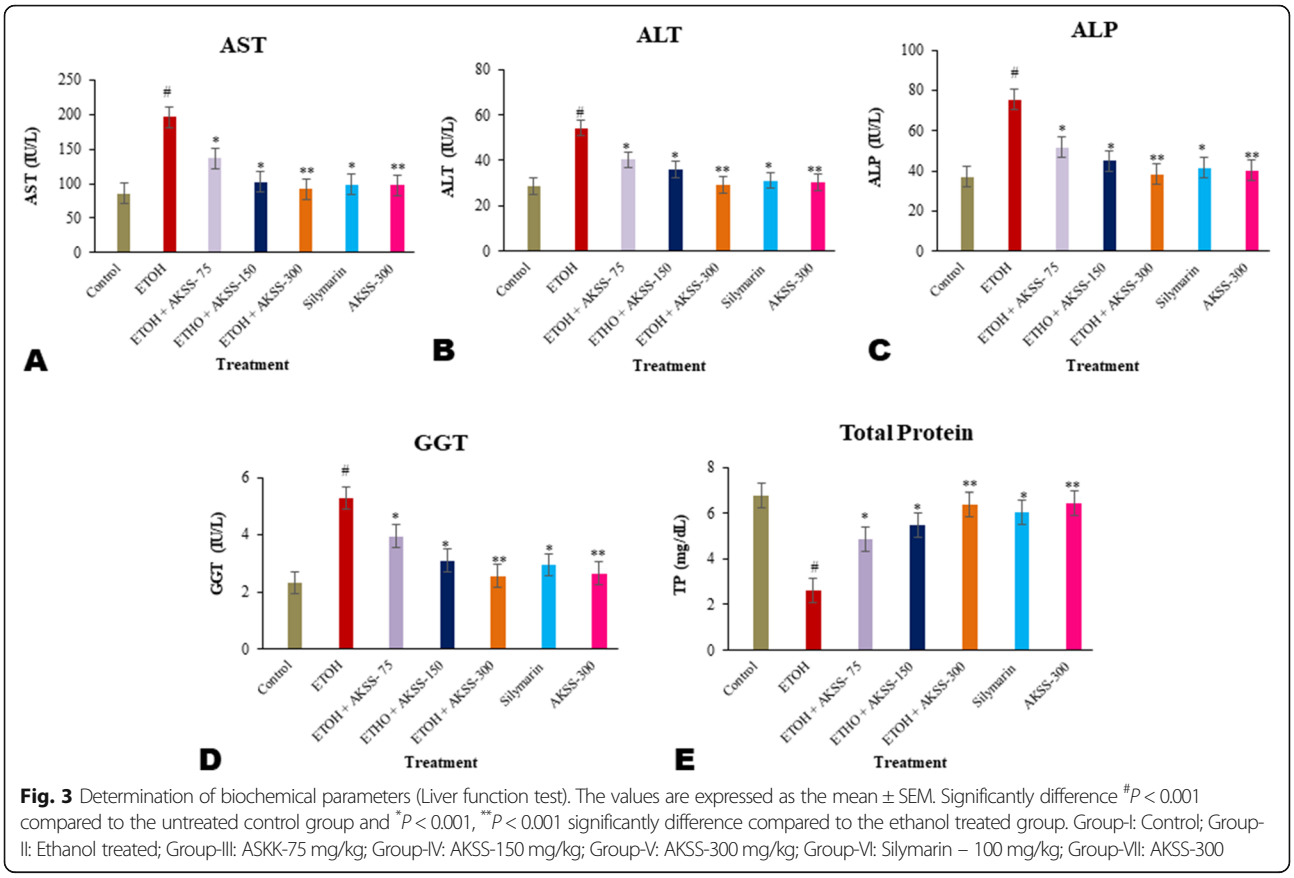


Fig. 3 Determination of biochemical parameters (Liver function test). The values are expressed as the mean ± SEM. Significantly difference $^{\#}P < 0.001$ compared to the untreated control group and $^{*}P < 0.001$, $^{**}P < 0.001$ significantly difference compared to the ethanol treated group. Group-I: Control; Group-II: Ethanol treated; Group-III: ASKK-75 mg/kg; Group-IV: AKSS-150 mg/kg; Group-V: AKSS-300 mg/kg; Group-VI: Silymarin – 100 mg/kg; Group-VII: AKSS-300

0.001) the deleterious effect (Table 6) caused by ethanol. Multi herbal formulation (AKSS16-LIV01) showed 15% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Determination of Haematological parameters

The hematopoietic system is one of the target organs of ethanol toxicity. The results concerning hematologic parameters depicted the Table 7 showed a significant ($p < 0.001$) decline in total erythrocyte count, total leukocyte count, hemoglobin concentration, Mean corpuscular haemoglobin concentration, neutrophil content and monocyte content in the ethanol treated animals. On the other hand, reticulocyte content content insignificantly increased in ethanol treated group, when compared with control animals. Gradually all the above mentioned parameters recover in the dose dependent AKSS16-LIV01 treated groups as compared with standard drug silymarin.

Determination of liver hydroxyproline level

Table 8 showed the hydroxyproline level of control and experimental group. In our study, long term ethanol intoxication produced deleterious effects which was clearly indicated when we found that hydroxyprolein level significantly elevated ($P < 0.001$) in experimental mice with compared with normal untreated group. Pre-treatment

Table 5 Effect of AKSS16-LIV01 on liver function test parameters across the groups in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol+AKSS16-LIV01 (150)	Ethanol+AKSS16-LIV01 (300)	Ethanol +Silymarin(100)	AKSS16-LIV01 (300)
BUN (mg/dl)	0.41 ± 0.02	0.72 ± 0.04$^{\#}$	0.58 ± 0.04*	0.67 ± 0.02*	0.46 ± 0.02**	0.54 ± 0.03*	0.46 ± 0.03*
Total Bilirubin (mg/dl)	0.12 ± 0.2	0.62 ± 0.11$^{\#}$	0.22 ± 0.09*	0.34 ± 0.08*	0.16 ± 0.09**	0.24 ± 0.08*	0.19 ± 0.11*
Direct Bilirubin (mg/dl)	0.06 ± 0.001	0.33 ± 0.07$^{\#}$	0.19 ± 0.002*	0.09 ± 0.003*	0.09 ± 0.002**	0.11 ± 0.005*	0.07 ± 0.002*
Alb (gr/dL)	3.48 ± 0.186	1.97 ± 0.036$^{\#}$	2.85 ± 0.12*	3.70 ± 0.11*	4.02 ± 0.14**	3.32 ± 0.15*	3.16 ± 0.13*
Alb/globulin	1.18 ± 0.141	0.54 ± 0.013$^{\#}$	0.81 ± 0.091*	0.91 ± 0.096*	1.21 ± 0.95**	1.04 ± 0.091*	0.98 ± 0.135*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p < 0.001$ and significantly different from ethanol $^{*}p < 0.05$, $^{**}p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test

Table 6 Effect of AKSS16-LIV01 on concentrations of nonenzymatic biochemical parameters in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol+AKSS16-LIV01 (150)	Ethanol+AKSS16-LIV01 (300)	Ethanol +Silymarin(100)	AKSS16-LIV01 (300)
Cholesterol (mg/dL)	80.17 ± 5402	138.99 ± 6.23$^{\#}$	91.25 ± 2.99*	91.47 ± 2.37*	79.36 ± 4.35**	856.11 ± 2.98*	88.05 ± 3.16*
Triglyceride (mg/dL)	42.55 ± 3.56	75.66 ± 4.28$^{\#}$	68.61 ± 3.24*	47.98 ± 1.97*	37.25 ± 1.87**	44.75 ± 3.25*	51.23 ± 3.01*
Phospgolipids (mg/dL)	78.48 ± 5.29	141.51 ± 5.14$^{\#}$	91.72 ± 2.66*	98.51 ± 3.28*	82.69 ± 4.87**	88.03 ± 2.84*	92.37 ± 2.66*
Free fatty acids (mg/dL)	14.07 ± 0.79	34.87 ± 1.87$^{\#}$	24.33 ± 1.91*	21.22 ± 1.69*	14.09 ± 1.22**	19.27 ± 2.01*	20.14 ± 1.96*
LDL-cholesterol (mg/dL)	38.11 ± 1.91	76.94 ± 1.77$^{\#}$	44.61 ± 2.88*	45.78 ± 1.65*	36.85 ± 1.25**	42.52 ± 2.28*	43.61 ± 1.88*
HDL-cholesterol (mg/dL)	19.52 ± 0.88	9.21 ± 0.68$^{\#}$	16.25 ± 0.62*	16.24 ± 0.41*	10.28 ± 0.28**	21.57 ± 0.99*	15.28 ± 0.55*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p < 0.001$ and significantly different from ethanol$^{*}p < 0.05$, $^{**}p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test

with multi herbal formulation (AKSS16-LIV01) significantly reduce at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice (Group-II). Multi herbal formulation (AKSS16-LIV01) showed 15% better protective effect compared with standard drug silymarin (Group-VI). Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone (Group-VII) showed no such toxic effect in mice compared with control mice.

Determination of NO and iNOS

In this study result showed that in Table 8 ethanol intoxication significantly elevated ($P < 0.001$) NO content and iNOS level in mice as compared with normal untreated group. Pre-treatment with multi herbal formulation (AKSS16-LIV01) significantly reduce at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice (Group-II). Multi herbal formulation (AKSS16-LIV01) showed 12% better protective effect compared with standard drug silymarin (Group-VI). Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone (Group-VII) showed no such toxic effect in mice compared with control mice.

Determination of MDA and ROS level

Result presented in Table 8 and Fig. 4 indicated that level of lipid peroxidation (MDA level) and the tissue ROS levels. Both lipid peroxidation and ROS content

Table 7 Effect of AKSS16-LIV01 on haematological parameters in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol+AKSS16-LIV01 (150)	Ethanol+AKSS16-LIV01 (300)	Ethanol +Silymarin(100)	AKSS16-LIV01 (300)
Hb (g %)	12.1 ± 1.05	9.03 ± 0.89$^{\#}$	12.0 ± 1.02*	11.05 ± 0.99*	12.51 ± 0.95**	10.96 ± 0.74	11.21 ± 0.82
RBC ($\times 10^6$ cm^2)	10.8 ± 0.82	8.1 ± 0.71$^{\#}$	10.5 ± 0.77*	9.44 ± 0.71	10.02 ± 0.85*	9.85 ± 0.79	9.62 ± 0.84
RT (%)	2.7 ± 0.12	4.9 ± 0.26$^{\#}$	2.6 ± 0.14*	3.1 ± 0.14	2.8 ± 0.15*	3.0 ± 0.12*	3.6 ± 0.16
HCT (%)	34.6 ± 0.48	39.4 ± 0.55$^{\#}$	34.1 ± 0.44*	35.8 ± 0.51	34.9 ± 0.56*	34.4 ± 0.51*	35.1 ± 0.77
MCV (μm^3)	37.8 ± 0.32	31.0 ± 0.68	36.7 ± 0.29*	36.5 ± 0.44	35.9 ± 0.79	36.2 ± 0.43*	35.5 ± 0.36
MCH (pg)	21.2 ± 0.15	22.2 ± 0.14$^{\#}$	22.8 ± 0.23*	21.1 ± 0.12*	21.4 ± 0.11*	21.2 ± 0.14	21.1 ± 0.12
MCHC (%)	41.2 ± 1.06	32.4 ± 0.95$^{\#}$	40.2 ± 1.07	37.1 ± 0.92	39.6 ± 0.87*	38.6 ± 0.99	36.2 ± 0.91
Platelets	6.5 ± 0.02	5.5 ± 0.03	6.5 ± 0.04	5.8 ± 0.05	6.1 ± 0.07	5.5 ± 0.05	5.4 ± 0.06
WBC ($\times 10^5$ $cm^{2)}$)	9.2 ± 0.09	12.4 ± 0.11$^{\#}$	9.1 ± 0.08	10.8 ± 0.12	9.2 ± 0.11**	10.1 ± 0.13	10.7 ± 0.11
Lymphocyte	74 ± 2.98	79 ± 3.04$^{\#}$	72 ± 2.54*	73 ± 3.06*	74 ± 2.58*	72 ± 3.08*	71 ± 3.11
Neutrophil	26 ± 1.12	15 ± 0.49$^{\#}$	24 ± 1.09*	20 ± 0.56*	25 ± 0.69**	24 ± 0.51*	21.52 ± 2.09

Data are expressed as mean ± standard deviation ($N = 6$). *Hb* Haemoglobin, *RBC* Read Blood corpuscle, *RT* Reticulocyte, *HCT* Haematocrit, *MCV* Mean corpuscular volume, *MCH* Mean corpuscular haemoglobin, *MCHC* Mean corpuscular haemoglobin concentration, *WBC* White Blood corpuscle

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p < 0.001$ and significantly different from ethanol$^{*}p < 0.05$, $^{**}p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test

Table 8 Effect of AKSS16-LIV01 onliver Lipid peroxidation, NO, iNOS levels and Hydroxyproline concentration in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol+AKSS16-LIV01 (150)	Ethanol+AKSS16-LIV01 (300)	Ethanol +Silymarin(100)	AKSS16-LIV01 (300)
Lipid Peroxidation (nm/100 g tissue)	68.16 ± 3.52	182.16 ± 3.09	92.35 ± 2.14*	77.92 ± 1.47*	62.58 ± 2.52**	82.57 ± 1.67*	66.57 ± 0.99*
NO (μmol/mg protein)	0.64 ± 0.004	2.29 ± 0.002#	102.77 ± 0.005*	0.84 ± 0.004*	0.69 ± 0.006**	0.88 ± 0.007*	0.65 ± 0.002*
iNOS (U/mg protein)	0.32 ± 0.003	1.11 ± 0.002#	0.62 ± 0.005*	0.46 ± 0.006*	0.38 ± 0.003**	0.45 ± 0.005*	0.39 ± 0.003*
Hydroxyproline (mq/g)	0.42 ± 0.004	0.99 ± 0.004#	0.76 ± 0.006*	0.55 ± 0.003*	0.47 ± 0.002**	0.56 ± 0.004*	0.44 ± 0.005*

The values are expressed as the mean ± SEM. Significantly different from control #$p < 0.001$ and significantly different from ethanol*$p < 0.05$, **$p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test

were significantly elevated ($P < 0.001$) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study MDA and ROS content were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12–13% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Determination of TNF-α and TGF 1β

Inflammation is commonly associated with liver dysfunction and fibrosis during chronic liver injury. The values of proinflammatory cytokines like TNF-α were determined from the hepatic tissue sample. Table 9 shows significant increase ($p < 0.001$) of TNF-α in ethanol treated mice when compared to control untreated animals. The elevated level of TNF-α was significantly inhibited ($p < 0.05$, $p < 0.001$) by the application of multi herbal formulation (AKSS16-LIV01) in dose dependent manner 150 and 300 mg/kg). This observation was comparable to that of the standard drug silymarin.

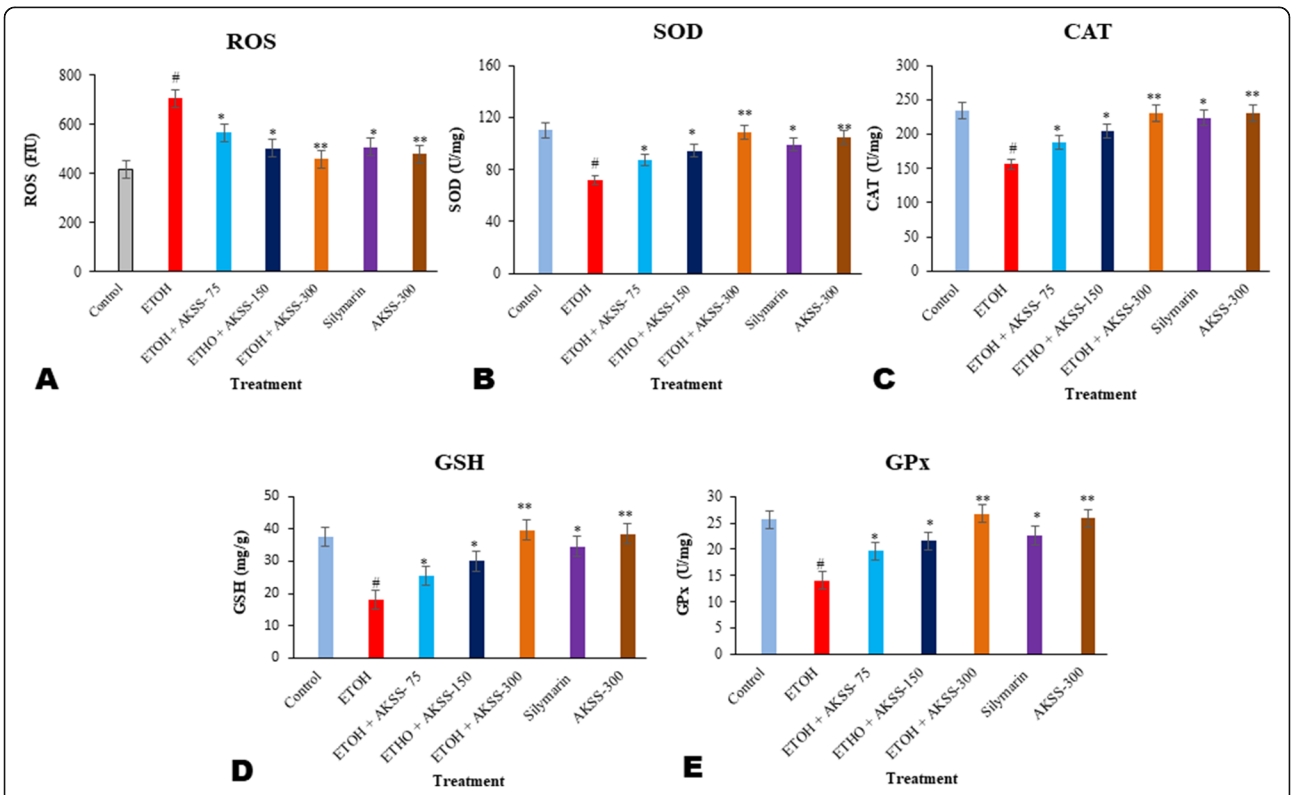


Fig. 4 Determination of the total reactive oxygen species (ROS) contents and levels of various antioxidant enzymes. The values are expressed as the mean ± SEM. Significantly difference #$P < 0.001$ compared to the untreated control group and *$P < 0.001$, **$P < 0.001$ significantly difference compared to the ethanol treated group. Group-I: Control; Group-II: Ethanol treated; Group-III: ASKK-75 mg/kg; Group-IV: AKSS-150 mg/kg; Group-V: AKSS-300 mg/kg; Group-VI: Silymarin – 100 mg/kg; Group-VII: AKSS-300

Therapeutic application of AKSS16-LIV01at a dose of 300 mg/kg showed optimum protective potential against ethanol induce liver intoxication. TGF 1β is the major profibrogenic cytokine. As shown in Table 9 significantly increased level of TGF 1β was observed in ethanol treated mice when compared to control untreated animals. On the other hand, treatment with AKSS16-LIV01 both 150 mg/kg and 300 mg/kg significantly decreased ($p < 0.05$, $p < 0.001$) the level of TGF 1β when compared with ethanol treated animals. This observation was comparable to that of the standard drug silymarin. Therapeutic application of AKSS16-LIV01at a dose of 300 mg/kg alone showed optimum protective potential against ethanol induce liver dysfunction.

Determination of SOD, CAT, GSH and GPx level

Result depicted in Fig. 4 indicated that levels of different antioxidant enzymes such as SOD, CAT, GSH and GPx were significantly reduce ($P < 0.001$) in ethanol intoxicated mice compared with normal control mice. However in dose dependent study the above antioxidant enzymes i.e. SOD, CAT, GSH and GPx were significantly reduced in multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg ($P < 0.05$), 150 mg/kg ($P < 0.05$) and 300 mg/kg ($P < 0.001$) respectively compared with ethanol treated mice. Multi herbal formulation (AKSS16-LIV01) showed 12–13% better protective effect compared with standard drug silymarin. Administration of Multi herbal formulation (AKSS16-LIV01) at a dose of 300 mg/kg alone showed no such toxic effect in mice compared with control mice.

Histopathological examination

Histologic examination Fig. 5 shows normal morphological architecture under light microscope of H&E in the control group. In the ethanol treated intoxicated groups (50:50 v/v) necrosis, hyperemia, vacuolar degeneration and infiltration of the inflammatory cells were observed which indicated hepatocellular damage (Fig. 5). In this regards, administration of multi herbal formulation (AKSS16-LIV01) at a dose of 75 mg/kg showed less damages compared with ethanol intoxicated mice (Fig. 6). Mild necrosis vacuolar degeneration and infiltration of the inflammatory cells were observed in the group 4 (150 mg/kg). In the group 5 (300 mg/kg) only mild vacuolar degeneration and infiltration of the inflammatory cells were seen which may indicate that the treatment of multi herbal formulation (AKSS16-LIV01) was very effective (Fig. 5). Histopathologic parameters of the liver tissues were graded in Fig. 5e. Masson trichrom and serius red staining photographs showed that liver's normal architectures completely massed by chronic administration of ethanol and deleterious effect was completely restored by AKSS16-LIV01 (300 mg/kg) which fully supported the above results.

Discussion

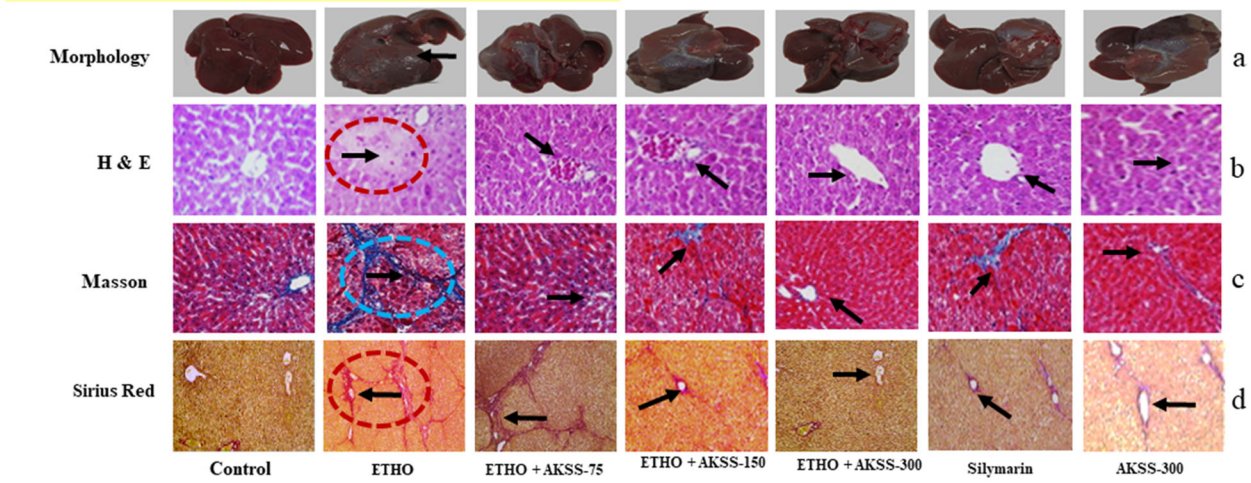
People all over the globes widely consume alcoholic drinks and consequently suffer from various diseases such as liver cirrhosis, liver fibrosis, fatty liver and, hepatic cell carcinoma (liver cancer). The condition produces hepatic dysfunctions that alter the body's normal homeostasis [60–62]. From ancient times, Indian medicinal herbs and spices are very useful for liver complications by boosting the antioxidant system and make a balance between antioxidants and prooxidants. These herbs and spices are enriched with polyphenols, flavonoids, tannins, proteins, amino acids, saponin, etc. which play a key role in liver protection against various toxicants. It also increases the body's antioxidant ability which further increases the immune power for fighting against disease. In the present study, we developed a multi herbal formulation (MHF) composed of six Indian medicinal herbs and three Indian medicinal spices (AKSS16-LIV01) have cumulative actions in comparison with the single herbal extract. We try to establish its antioxidant and immune-suppressive effect against ethanol intoxication and find out a new safe and symptomatic medication in liver dysfunction.

The preclinical and clinical studies have already established that ethanol is a potent hepatotoxicant which produces severe liver complications [63]. Liver damage by ethanol is closely associated with the generation of reactive oxygen species (ROS) such as peroxide, singlet oxygen, superoxide anion, hydroxyl ions which elevates MDA (malondialdehyde), NO (nitric oxide) and iNOS (nitric oxide synthase), suppress the cellular integrity. Ethanol intoxication showed the function of the various antioxidant enzymes gets severely reduced causing cell

Table 9 Effect of AKSS16-LIV01 on TNF α and TGF 1β levels in liver tissue in chronic ethanol-induced hepatic damage in mice

Parameters	Normal	Ethanol	Ethanol + AKSS16-LIV01 (75)	Ethanol+AKSS16-LIV01 (150)	Ethanol+AKSS16-LIV01 (300)	Ethanol + Silymarin (100)	AKSS16-LIV01 (300)
TNF-α (pg/mg protein)	1411.91 ± 206.53	2344.77 ± 371.98$^{\#}$	1636.77 ± 220.97*	1572.25 ± 236.93*	1437.11 ± 285.83**	1478.26 ± 288.17*	1422.87 ± 198.54*
TGF-1β (pg/mg protein)	485.90 ± 91.34	1237.05 ± 125.78$^{\#}$	702.38 ± 98.77*	598.11 ± 212.45*	474.03 ± 102.76**	552.03 ± 203.84*	492.11 ± 88.16*

The values are expressed as the mean ± SEM. Significantly different from control $^{\#}p < 0.001$ and significantly different from ethanol $^{*}p < 0.05$, $^{**}p < 0.001$using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test



e **Table: Lesion Scores of Different Liver Alterations among all Experimental Groups**

Histopathological lesions	Normal	ETHO	ETHO + AKSS-75	ETHO + AKSS-150	ETHO + AKSS-300	Silymarin	AKSS-300
Fibroblast proliferation in the capsule	–	+++	++	–	–	–	–
Fibroblast proliferation in the portal triad	–	+++	++	+	–	+	–
Focal hepatic necrosis	–	++	+	+	–	–	–
Steatosis of hepatocytes	–	+++	+	–	–	–	–
Cytoplasmic vacuolization of hepatocytes	–	++	–	–	–	–	–
Kupffer cell activation	–	+++	–	–	–	+	–

Fig. 5 Representative photomicrographs of livers in different experimental groups. **a** liver morphology **b** H & E stained liver sections **c** Masson trichrom stained liver sections **d** Sirius red stained liver sections. **e** Table showed histological scoring of the liver section in different groups

apoptosis [64, 65]. Increased and decreased enzymatic and non-enzymatic markers of serum were also associated with this condition. Alcoholic liver disease (ALD) was normally found in liver histology, which disrupts the normal liver architecture and reduces regular functions. Our developed formulation AKSS16-LIV01 was enriched with antioxidants that could revert and lower the free radicals level. It had shown that the beneficial effects of this phytomedicine in preventing the ethanol-induced hepatotoxicity caused by free radicals.

In the present study, we evaluated the ameliorative effects of AKSS16-LIV01 against ethanol-induced hepatotoxicity in the animal model. Dose-dependent administration of ethanol increased the gross liver weight and liver-body weight ratio and decreased the cumulative body weight, which caused the changes in the behavior of mice. Administration of AKSS16-LIV01 with three specific doses gradually normalized the changes. Ethanol intoxication elevated the concentrations of key cellular enzymes like AST, ALT, ALP, and GGT present in the liver cells that leak into the serum during liver damage [66–70]. This happens because of a higher concentration of alcohol dehydrogenase in the liver, which catalyzes alcohol to its corresponding aldehyde [71]. Therefore, administration of AKSS16-LIV01 at a dose of 150 mg and 300 mg body weight could help to normalize the AST, ALT, ALP, and GGT enzyme levels. So, the developed multi herbal formulation AKSS16-LIV01 could reduce the level of these enzyme markers.

These results are also consistent with the protective effects of the developed formulation correlating with its antioxidant ability against alcohol-induced hepatocyte cells of the liver [72–74]. Our results also showed that administration of ethanol increased the serum essential enzymes blood urea nitrogen (BUN), total and direct bilirubin which was reverted with the treatment of AKSS16-LIV01. On the other hand, it was found that ethanol toxicity reduces the body's different protein concentration and breaks the normal homeostasis. Our results showed that reduced total protein, albumin, and albumin globulin ratio were normalized by AKSS16-LIV01.

Ethanol administration increased serum total cholesterol (TC), total triglyceride (TC), phospholipids, LDL,

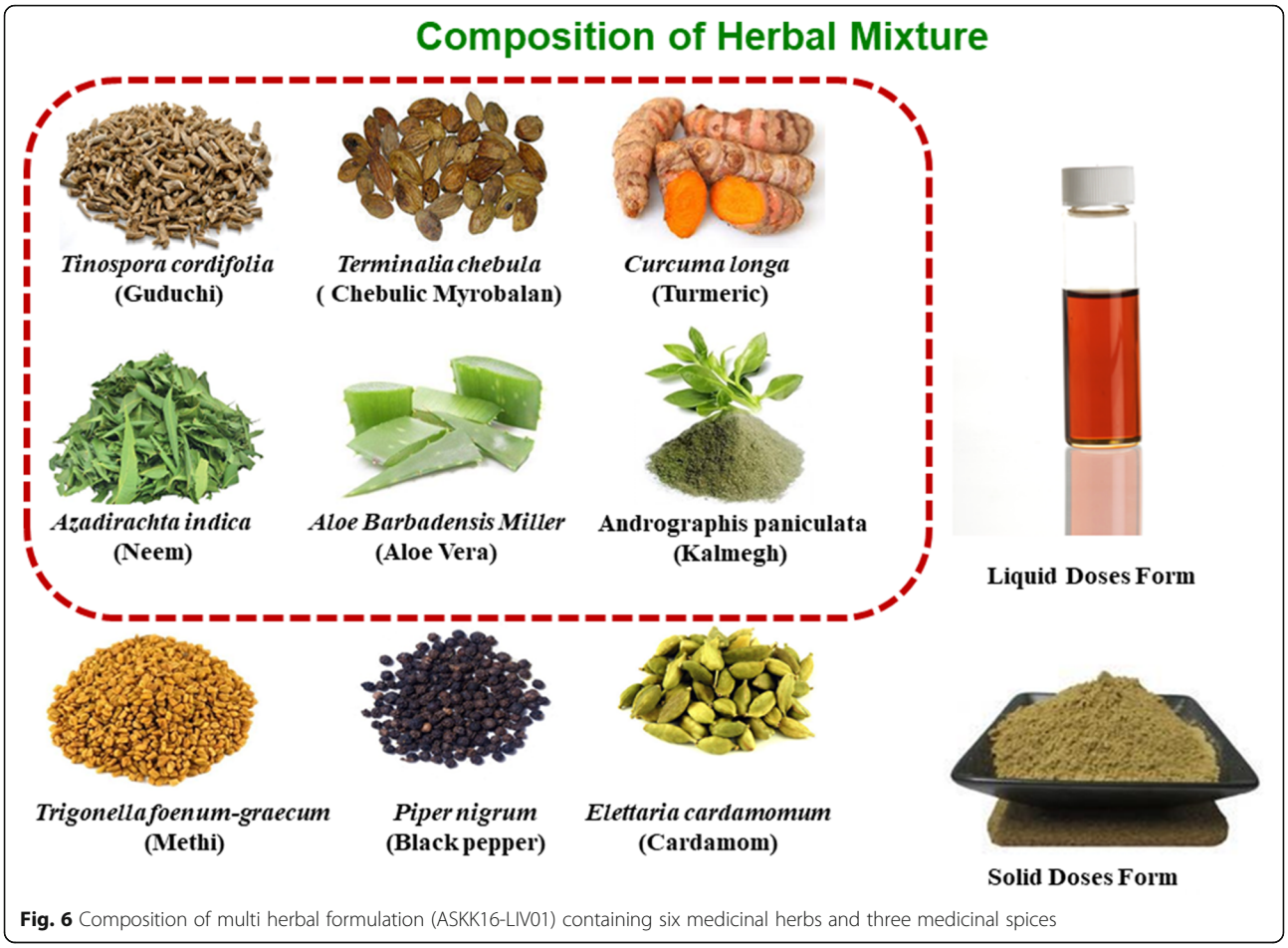


Fig. 6 Composition of multi herbal formulation (ASKK16-LIV01) containing six medicinal herbs and three medicinal spices

VLDL, and HDL levels of non-enzymatic markers which caused liver damage. This damage is attributed to the higher concentration of alcohol dehydrogenase enzyme which catalyzes alcohol to aldehyde and accumulation of export type proteins due to inhibition of the secretion of the proteins from the liver of alcoholics [75]. Both doses of AKSS16-LIV01 restored the higher level of lipid profile parameters in a dose-dependent manner to normal levels [74, 76, 77].

It is reported that in cirrhotic mice caused by ethanol produced significantly higher reactive oxygen species (ROS) and malondialdehyde (MDA) as compared to the control animals [1, 2]. In this study our result also supported the above reports. On the other hand the levels of MDA and ROS content were markedly low in the AKSS16-LIV01 treated group (Table 7). It is predicted that polyphenol, flavonoids, and other essential constituents rich multi herbal formulation (MHF) inhibit lipid peroxidation and reactive oxygen level in experimental mice [78]. During this study, the antioxidant system of liver fibrotic mice was extensively impaired, causing a high level of MDA and ROS. However, in this study higher levels of liver nitrite/nitrate indicating significantly increased production of hepatocellular NO content in response to chronic alcohol administration via induction of inducible nitric oxide synthase (iNOS) [79]. Nitric oxide (NO) and its metabolite i.e. peroxynitrite ($ONOO^-$) is penetrate the cell membranes through anion channels, which generates nitration of tyrosine and inactivation of biologically important proteins and enzymes [80]. In this study treatment with AKSS16-LIV01 decreased the levels of nitrite/nitrate significantly which was altered by ethanol intoxication. Increasing nitrites/nitrates production in alcoholic mice restored after receiving AKSS16-LIV01, which might be mainly due to inhibition of the hepatic cytosolic iNOS enzyme activity by the newly developed novel multi herbal formulation [81].

Oxidative stress (OS) induced by ethanol intoxication causes liver ailment in alcoholism [82, 83] and produced various health complications. Indian traditional healthcare system is extensively used for a long time to cure liver dysfunctions and augment body's immune system. Two lines of oxidative defense (first and second order) protect cells against oxidative stress. Various scavenging

enzymes such as SOD, CAT, GPx, and GSH are neutralized and save the cell against oxidative injury. Superoxide ions were scavenged by SOD and catalase converts hydrogen peroxide (H_2O_2) to water.

In this study chronic administration of alcohol reduced CAT, SOD, GPx as well GSH level in hepatic cell in mice. Oxidative inactivation of the enzyme is the main causes of decreased SOD activity responsible for cell damage. This condition generates massive reactive oxygen species (ROS) and break cellular homeostasis [84]. Reduced glutathione (GSH) is one of the major non-protein thiol and takes a provital role in coordinating the antioxidant defense process. Normal cell structure & functions through its detoxification process requires equilibrium of GSH and GPx [85]. Glutathione (GSH) in combination with glutathione peroxidase (GPx) helps to metabolize hydrogen peroxide (H_2O_2) to water via biochemical pathways, thereby protecting cells against oxidative injury. Administration of our developed multi herbal formulation against ethanol intoxicated mice normalized these essential antioxidant enzymes concentrations in the liver tissue. The formulation thus protects the liver against ethanol-induced redox healing.

TNF-α is a key factor that initiates a cascade of immune responses involving the induction of cytokines after liver damage. In the damaged liver, predominantly Kuffer cells and infiltrating macrophages and neutrophils will produce TNF-α. TNF-α plays a dichotomous role in the hepatic tissue, where it not only induces hepatocyte proliferation, apoptosis, and inflammation but also is known to suppress collagen α1 gene expression. Our results showed that the administration of multi herbal formulation (AKSS16-LIV01) with ethanol reduced the level of TNF-α in the liver as compared to ethanol administration alone. This result suggested that AKSS16-LIV01 is capable to suppress TNF-α production against ethanol-induced liver damage.

Hepatic stellate cells (HSC) activation was triggered by TGF-β1, which was released from Kuffer cells as well as oxidative stress caused by ethanol. TGF-β1 regulates the production, degradation, and accumulation of the extracellular matrix (ECM) in liver fibrosis [86]. TGF-β1 leads fibrogenesis through the autocrine and paracrine effects of HSC. Our results showed that the administration of AKSS16-LIV01 with ethanol reduced the level of TGF-β1 in liver tissue as compared with ethanol administration alone. This suggested that the ameliorative effect of AKSS16-LIV01 on ethanol-induced hepatotoxicity was associated with their abilities to inhibit HSC activation by reducing TGF-β1 production.

Histopathological examination was an indication of hepatic damages after administration of ethanol. Chronic administration of ethanol developed cellular necrosis, vacuolar degeneration, hyperemia and infiltration of the inflammatory and lymphocyte cells [87–90]. The histopathology of the liver confirmed the protective effect of multi herbal formulation (AKSS16-LIV01). Application of the newly developed unique formulation restored the hepatic damage in mice and inhibits the damaging effect of ethanol. The efficacy of the developed formulation was dipicted by the reduction of livers damages such as necrosis, hyperemia, vacuolar degeneration, and infiltration of the inflammatory cells; the effects were especially evident at the dose of 300 mg/kg. All the above study was compared with standard drug silymarin and it is showed that AKSS16-LIV01 has more potent hepatoprotective effect instead of standard drug.

Conclusion

The newly developed multi herbal formulation (AKSS16-LIV01) has shown protection by ethanol-induced liver injury by ameliorating oxidative stress in mice. The protective action was evidenced by liver histopathological studies, various hepatic enzymes, reduced lipid peroxidation, NO levels, and elevated antioxidant status. Apart from this AKSS16-LIV01 can suppress pro-inflammatory cytokines TNF-α and inhibit HSC activation by reducing profibrogenic cytokines TGF-β1. The presence of various constituents such as tannins, polyphenols, flavonoids in AKSS16-LIV01 could be contributed to the above mechanism.

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Authors' contributions

Soumendra Darbar (SD) and Atiskumar Chattopadhyay (AKC): Both authors conceived and designed the experiment. Soumendra Darbar and Srimoyee Saha (SS): Both authors conducted the animal and biochemical experiments, analysed the samples and compiled the data. SD, SS, AKC and Kausikisankar Pramanik (KP): All the authors wrote and revised the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

Research data and materials can be provided on request.

Declarations

Ethics approval and consent to participate

The animals were maintained according to the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Chennai, India which was approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. AEC/PHARM/1503/03/2019 dated 30.11.19). All procedures complied with the Declaration of Helsinki, as revised in 1996.

Consent for publication

All authors totally agreed for the publication of this research.

Competing interests

The authors declare they have no competing interest.

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Research Article

SANATIVE EFFECT OF MULTIHERBAL FORMULATION – AKSS16-LIV01 ON CCL_4-INDUCED HEPATIC DYSFUNCTION IN MICE

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ABSTRACT

Objectives: Hepatic dysfunction is a critical public health problem affecting the global population, characterized by excessive deposition of extracellular matrix components due to increased matrix production and decreased matrix degradation. The present work was aimed to evaluate hepatoprotective effect of AKSS16-LIV01 a newly developed multiherbal formulation against carbon tetrachloride (CCl_4)-induced liver dysfunction in Swiss albino mice to establish it as a bench to bedside formulation catering to the various facets of hepatic malfunction.

Methods: Thirty-six Swiss adult albino Wister mice divided into six groups. Group-I control untreated animals, Group-II received AKSS16-LIV01 (400 mg/kg), Group-III received CCl_4 (1 ml/kg-bw), Group-IV received AKSS16-LIV01 (200 mg/kg) after 2 weeks CCl_4 induction, Group-V received AKSS16-LIV01 (400 mg/kg) after 2 weeks CCl_4 induction, and Group-VI received standard drug silymarin (100 mg/kg). At the end of the experimental period, all the animals were fasted overnight and blood was collected through retro-orbital plexus for preparation of serum and was analyzed for biochemical parameters, lipid profile, and total plasma protein. Liver tissue was collected for histological study.

Results: The combined plant extract including six Indian medicinal herbs and three medicinal spices (AKSS16-LIV01) showed significant hepatoprotective effect by controlling the various essential biochemical parameters in serum. Moreover, treatment with AKSS16-LIV01 raised the level of serum total protein, normalizes the serum biochemical and lipid profiles parameters. Pre-treatment with AKSS16-LIV01 in mice also restored the alteration of various liver parameters such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, blood urea nitrogen, total bilirubin, and direct bilirubin on CCl_4-induced liver damage. Gross liver morphology and normal histological examination of the liver also supported hepatic protection by AKSS16-LIV01.

Conclusion: Taken together, these results suggest that AKSS16-LIV01 may induce remarkable protective effects against hepatic injury induced by CCl_4 treatment.

Keywords: Hepatoprotective, Natural therapy, Herbal formulation, Liver function test, Histopathological studies.

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INTRODUCTION

Hepatocytes, sinusoidal cells, Kupffer cells, and hepatic stellate cells (HSCs) are the main cells those are involved in normal liver functions. Several pathogens when attack the liver cells they alter the normal liver functions and damage the liver cells produce liver fibrosis [1,2]. Inflammatory reactions take place when hepatocytes cause damage which lead to the activation of HSCs [3]. During the abnormal situation, activated Kupffer cells release a number of soluble agents, including cytokines, such as platelet-derived growth factor, transforming growth factor-β, and tumor necrosis factor-α, generate reactive oxygen species (ROS), and other factors causing inflammation and damage to hepatocytes leads to liver fibrosis [4,5]. In an experimental setting, carbon tetrachloride (CCl_4) is able to induce hepatic fibrosis by stimulating the formation and generation of various free radicals, ROS, and lipid peroxidation products [6] and thus is widely used to generate hepatic fibrosis in model animals [7,8].

At present, global attention has been paid to the protective effects of natural antioxidants against chemically induced toxicities [9]. Herbal extracts could significantly contribute to recovery processes of the intoxicated liver and kidney [10-14]. The World Health Organization defines traditional medicine as "diverse health practices, approaches, knowledge, and beliefs incorporating plant, animal and/or mineral-based medicines, spiritual therapies, manual technique, and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose, or prevent illness" [15,16]. In third world countries including India, up to 90% of the populace still relies entirely on plants as a resource of medicines [17].

Herbal medicines have progressively become prevalent due to rising costs of treatments with synthetic western medicine, numerous side effects of allopathic drugs, drug resistance, unregulated purchase options for consumers on most herbal drugs, and easy availability of these medicines [18]. Therefore, studies with plant extracts are useful to know their efficacy and mechanism of action and safety. Natural remedies from medicinal plant are considered to be effective and alternative treatment for hepatotoxicity [19].

In the present study, we formulated a new phytomedicine composed of nine indigenous medicinal and dietary herbs which were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and happiness. The results of the present study are expected to provide a clear picture about the role of our newly formulated AKSS16-LIV01 in CCl_4-induced hepatic damage, and may shed light on an achievable ethnobotany driven solution to the serious liver problems.

METHODS

Chemicals

CCl_4, trichloroacetic acids, and TRIS buffer were obtained from Merck, India. Phosphate-buffered saline pH 7.4 was procured from

Sigma-Aldrich. Biochemical determination kits, that is, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and lipid profile kits were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

Collection and authentication of the herbs

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by taxonomists of the Department of Botany, Uluberia College, University of Calcutta, India, and kept as voucher specimen. Ayurvedic identification parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti (shape), and Parimana (size). The plants and plant parts used in preparation of the extract are listed in Table 1.

Preparation of extract

At first, collected plant parts were air dried and then clean with double distilled water and kept in a hot air oven at 80°C for 10 min and 60°C for 30 min. All the plants and spices were placed in a blade mill to obtained fine powder. Aqueous extract of the polar fraction was performed according to the method of Adhikari *et al.* (2018) with slight modifications. Five grams of dry plant parts were taken and dissolved it using 10 ml of methanol. The extract then sonicated at room temperature for 30 min using an ultrasonic bath, centrifuged at 4000 rpm for 20 min, and finally, the supernatant was removed. This procedure was repeated 4 times, collecting all the supernatants, which were finally evaporated in a rotary evaporator under reduced pressure at 35°C. Finally, the residue was reconstituted in 3 ml of methanol, filtered using Whatman filter papers (GE Healthcare and Life Sciences, MA, USA) and kept at 4°C for further use [20]. The composition of the formulation is presented in Table 1.

Animals

Healthy adult Swiss albino mice weighing 28±5 g taken from our registered animal house were divided into six experimental groups with six animals per group. The animals were maintained at 12 h light/dark cycle, at constant temperature (22±2°C) and humidity (55±5%). Mice were feed standard pellet diet (Purchase from Hind Unilever India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture, and 2.6% by weight of lipids and water *ad libitum*. Mice were kept under observation for 1 week before the onset of the experiment for acclimatization and to exclude any insercurrent infection. All the experimental procedures were carried out according to the guidelines of CPCSEA, Government of India, New Delhi, and approved by the Institutional Animal Ethics Committee (IAEC) of Jadavpur University having approval number 261/JU/s/IAEC/Pharma/2018.

Acute toxicity studies

The acute toxicity studies were carried out for AKSS16-LIV01 following the general principles of OECD guideline 423. Overnight fasted healthy female mice were divided into four (one control and three test) groups and orally given the extract at doses up to 2000 mg/kg body weight

Table 1: Composition of ingredient(s) present in AKSS16-LIV01

S. No.	Botanical name	Common name	Family	Part used	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	Stem	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	Fruit	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	Leaves	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	Leaves and steam	50 mg
5.	*Aloe barbadensis* Miller	Aloe vera	Liliaceae	Leaves and steam	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	Rhizome	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	Seed	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	Seed	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	Seed	10 mg

*Amount required for preparation of 5 ml extract

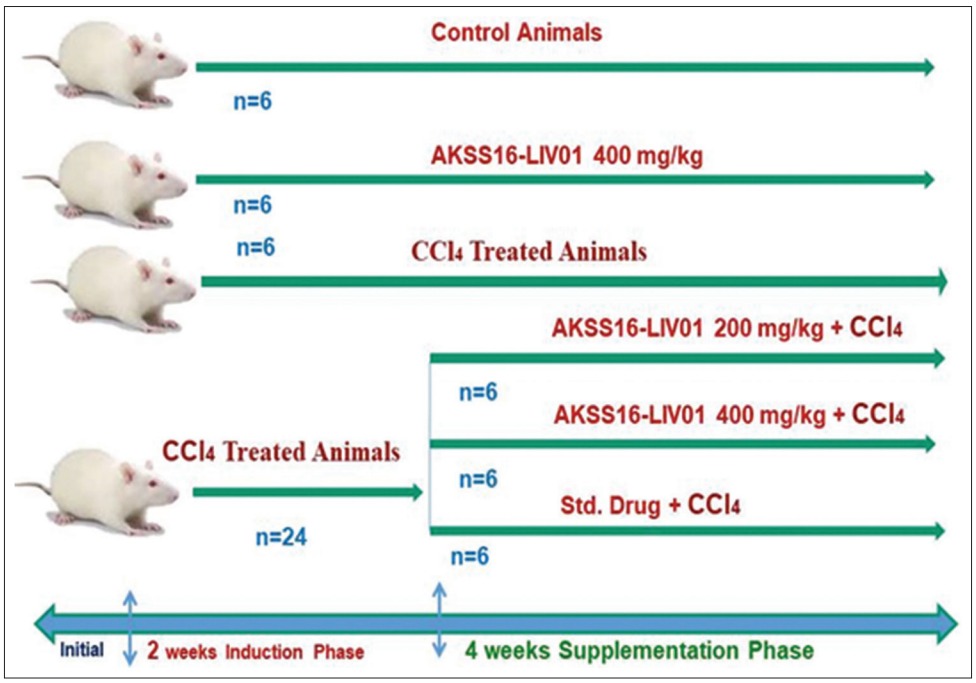

Fig. 1: Experimental design

(BW). They were observed continuously for 14 days for behavioral changes and mortality.

CC_{l4} induction and treatment

Male adult mice were divided into six groups as follows: Group I served as normal control received only the vehicle (1 ml/kg olive oil twice a week for 6 weeks), Group II treated with multi herbal formulation (MHF) AKSS16-LIV01 (400 mg/kg bw/day all over the experiment), Group III received 1 ml/kg bw of CCl_4 diluted 20% in olive oil twice a week for 6 weeks, Group IV pre-treated with herbal formulation AKSS16-LIV01 (200 mg/kg bw/day) low dose (MHF) for 4 weeks after 2 weeks CCl_4 induction and ensure occurrence of liver injury, Group V pre-treated with MHF AKSS16-LIV01 high dose (400 mg/kg bw) for 4 weeks after 2 weeks CCl_4 induction and ensure occurrence of liver injury, and Group VI pre-treated with silymarin standard hepatoprotective drug at a dose (100 mg/kg bw) for 2 weeks after CCl_4 induction and ensure occurrence of injury (Fig. 1).

BW gains and feed efficiency

BWs were measured on weekly basis from the initial day to the final day of experiment to calculate BW alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by BW gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

Blood collection

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. Two hundred microliters of blood sample were collected into microcentrifuge tubes with and without ethylenediaminetetraacetic acid (2%). Collected bloods were placed in slanting position at room temperature for 2 h. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow color serum was separated and used for further analyses.

Hematological parameters

For hematological studies, the blood was collected in heparinized tubes. Blood cell count was done using blood smears in Sysmex K1000 Cell Counter. Parameters studied were hemoglobin, total red blood cell, reticulocyte (RT), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, total white blood cell, and differential count.

Assessment of liver function parameters

The biochemical parameters such as serum enzymes: AST, ALT, serum ALP, GGT, albumin, globulin, total and direct bilirubin, and blood urea nitrogen (BUN) along were assayed using assay kits (Thermo Scientific, USA) following the protocol prescribed by manufacturers. Total protein concentration was determined in the serum by the method of Lowry *et al*. [21].

Lipid profile

Serum levels of total cholesterol (TC), triglycerides (TGs), phospholipids, free fatty acids (FFA), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were assayed using assay kits (ELITech Diagnostic, France) following the protocol prescribed by manufacturers.

Hematoxylin and eosin staining

Five micron paraffin-embedded liver sections were deparaffinized and washed with water. Hydrated tissue sections were incubated with Mayer's hematoxylin for 5 min followed by vigorous washing in running tap water. Sections were counter stained with 1% eosin for 2 min. Eosin stained sections were washed with water and dehydrated with alcohol. Dehydrated sections were washed with xylene. Images were taken with a microscope (Olympus BX51 fluorescence microscope).

Statistical analysis

All quantitative data are expressed as mean±standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), GraphPad Software, California, USA. p and lt; 0.05 was considered statistically significant.

RESULTS

BW, liver weight, liver index, food consumption, and water intake

In this study, we determined the BW, liver weight, liver index, food consumption, and water intake of mice treated with CCl_4 and pretreated with MHF-AKSS16-LIV01 (Fig. 2). BW and liver index of CCl_4-treated

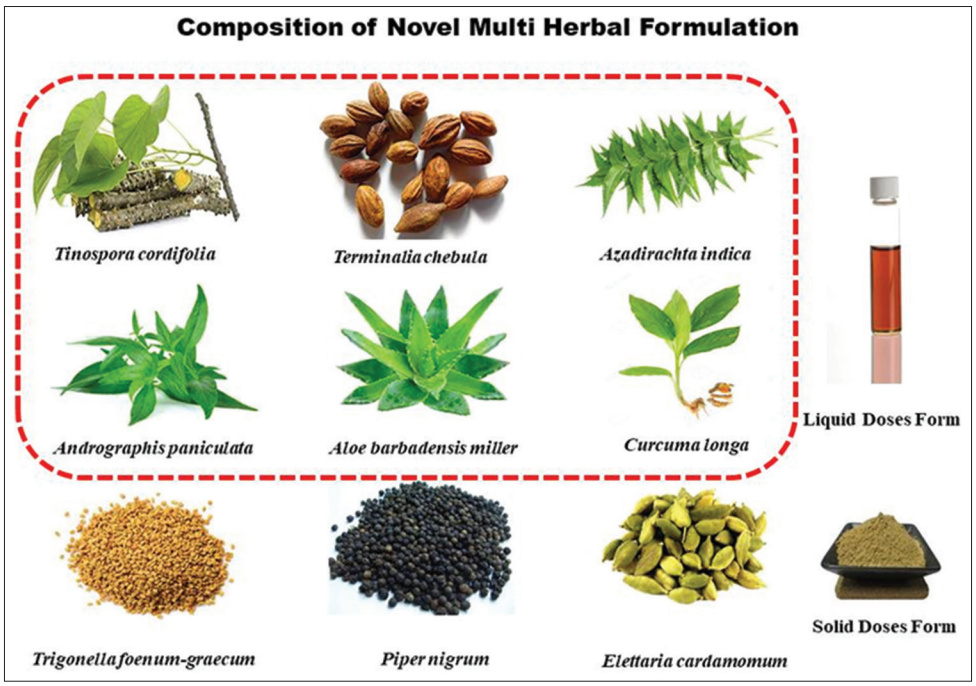

Fig. 2: Composition of multiherbal formulation (AKSS16-LIV01)

animals were significantly reduced (p<0.001) when compared with normal control animals. Liver weight of CCl_4-treated animals was significantly elevated (p<0.001) when compared with normal control animals. Treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly increased (p<0.05 and p<0.001) BW and liver index whereas significantly decrease the liver weight (p<0.001) when compared with CCl_4-treated animals. Silymarin showed less positive effect in comparison with MHF. On the other hand, food consumption and water intake capacity of CCl_4-treated animals were significantly reduced (p<0.001) when compared with normal control animals. Treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly increased (p<0.05 and p<0.001) the food consumption and water intake capacity when compared with CCl_4-treated animals. Treatment with MHF – AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity (Table 2).

Liver function test

Liver function parameters such as serum AST, ALT, GGT, ALP, BUN, total bilirubin, direct bilirubin, total protein, albumin, and albumin-globulin ratio were measured in mice treated with CCl_4 and pretreated with AKSS16-LIV01 after CCl_4 induction which are presented in Table 3. AST, ALT, GGT, ALP, BUN, total bilirubin, and direct bilirubin of CCl_4-treated animals were significantly elevated (p<0.001) when compared with normal control animals. Serum total protein, albumin, and albumin globulin ratio of CCl_4-treated animals were significantly reduced (p<0.001) when compared with normal control animals. The Pre-treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly reduced (p<0.05 and p<0.001) AST, ALT, GGT, ALP, BUN, total bilirubin, and direct bilirubin whereas significantly increased (p<0.05 and p<0.001) the serum total protein, albumin, and albumin-globulin ratio when compared with CCl_4-treated animals. Silymarin showed less positive effect in comparison with MHF. Results of the biochemical study stated that treatment with MHF – AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity.

Lipid profile test

Lipid profile parameters such as serum TC, total TG, phospholipids, FFA, LDL, and HDL were measured in mice treated with CCl_4 and pretreated with AKSS16-LIV01 which are presented in Table 4. Cholesterol triglyceride, phospholipids, FFA, and LDL of CCl_4-treated animals were significantly elevated (p<0.001) when compared with normal control animals. Serum HDL level of CCl_4-treated animals was significantly reduced (p<0.001) when compared with normal control animals. The pre-treatment with AKSS16-LIV01 both 200 mg/kg and 400 mg/kg significantly reduced (p<0.05 and p<0.001) serum cholesterol triglyceride, phospholipids, FFA, and LDL level and significantly increased (p<0.05 and p<0.001) the serum HDL when compared with CCl_4-treated animals. Silymarin showed less positive effect in comparison with MHF. Results of the serum lipid profile study

Table 2: Summary of the body weight (BW), liver weight, and liver index of mice

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + silymarin 100
BW (g)	36.52±1.18	35.01±1.34	26.94±1.22#	34.62±1.26*	38.01±2.21**	33.26±2.17*
Liver weight (g)	1.94±0.82	1.98±0.18	3.50±0.28#	1.99±0.52*	1.86±0.64**	2.03±0.64*
Liver index	4.02±0.12	5.65±0.17	7.72±0.15#	5.51±0.24*	4.95±0.17**	5.22±0.18*
Food consumption (g)	3.98±0.05	3.95±0.04	2.91±0.05#	3.74±0.06*	3.99±0.02**	3.92±0.05*
Water intake (ml)	4.11±0.02	4.02±0.04	2.95±0.05#	3.99±0.02*	4.04±0.05**	3.91±0.03*

CCl_4: Carbon tetrachloride. Values are mean of six individual observations in each group±SD. *Significantly different from control # (p<0.001) and significantly different from CCl_4 *(p<0.05) **(p<0.001) using ANOVA followed by.

Table 3: Summary of the liver function test parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + silymarin 100
AST (IU/l/min/mg protein)	137.25±2.62	135.25±3.02	255.49±1.98#	166.27±2.19*	140.27±2.02**	164.02±1.44*
ALT (IU/l/min/mg protein)	55.16±2.22	58.61±1.81	118.03±3.16#	82.58±1.02*	61.29±1.63**	72.58±1.85*
GGT (IU/l/min/mg protein)	0.26±0.02	0.26±0.09	2.11±0.51#	1.06±0.08*	0.34±0.12**	0.41±0.02*
ALP (IU/l/min/mg protein)	232.05±3.11	237.84±2.91	461.27±4.96#	279.55±2.06*	250.44±2.71**	262.11±2.04*
BUN (mg/dl)	0.41±0.02	0.46±0.03	0.72±0.04#	0.67±0.02*	0.46±0.02**	0.55±0.03*
Total bilirubin (mg/dl)	0.12±0.2	0.19±0.11	0.62±0.11#	0.34±0.08*	0.18±0.09**	0.25±0.08*
Direct bilirubin (mg/dl)	0.06±0.001	0.07±0.002	0.33±0.07#	0.09±0.003*	0.09±0.004**	0.24±0.005*
Total protein	6.51±0.65	627±0.21	2.56±0.32#	4.58±0.41*	6.22±0.27**	5.39±0.21*
Alb (gr/dL)	3.48±0.186	3.16±0.13	1.97±0.036#	3.70±0.11*	4.12±0.12**	3.30±0.15*
Alb/globulin	1.18±0.141	0.98±0.135	0.54±0.013#	0.91±0.096*	1.23±0.95**	1.06±0.091*

CCl_4: Carbon tetrachloride, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transferase, ALP: Alkaline phosphatase, BUN: Blood urea nitrogen, Alb: Albumin. Values are mean of six individual observations in each group±SD. *Significantly different from control #(p<0.001) and significantly different from CCl_4 *(p<0.05) **(p<0.001) using ANOVA followed by Dunnett's multiple comparison test.

Table 4: Summary of the lipid profile parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + silymarin 100
Cholesterol (mg/dL)	81.03±5.02	88.05±3.16	135.69±6.15#	91.47±2.37*	79.36±4.35**	85.11±2.98*
Triglyceride (mg/dL)	40.58±2.05	51.23±3.01	72.58±3.28#	47.98±1.97*	37.25±1.87**	41.75±3.25*
Phospholipids (mg/dL)	76.59±6.28	92.37±2.66	142.97±4.69#	98.51±3.28*	82.69±4.87**	88.03±2.84*
Free fatty acids (mg/dL)	15.97±0.58	20.14±1.96	31.87±1.67#	21.22±1.69*	14.09±1.22**	16.27±2.01*
LDL cholesterol (mg/dL)	39.65±1.96	43.61±1.88	76.94 ±1.77#	45.78±1.65*	36.85±1.25**	44.52±2.2*
HDL cholesterol (mg/dL)	19.58±0.69	15.28±0.55	10.28±0.28#	16.24±0.41*	10.28±0.28**	22.67±0.9*

Values are mean of six individual observations in each group±SD. *Significantly different from control # (p<0.001) and significantly different from CCl_4 *(p<0.05) **(p<0.001) using ANOVA followed by Dunnett's multiple comparison test

stated that treatment with MHF – AKSS16-LIV01 at a dose 400 mg/kg showed optimum protective capacity.

Hematological study

All the different hematological parameters such as hemoglobin (Hb): Hb, red blood corpuscle, RT, HCT, MCV, MCH, MCHC, and white blood corpuscle were studied in mice treated with CCl_4 and pretreated with AKSS16-LIV01. Hemoglobin level was significantly reduced ($p<0.05$) and WBC count was significantly elevated ($p<0.05$) in CCl_4 group as compared with normal control group. The pre-treatment with AKSS16-LIV01 (400 mg/kg) was recovered these alteration when compared with CCl_4 group (Table 5).

Histology

H and E staining

Fig. 3 shows histological photographs of the liver tissue both control and different experimental groups. The normal control group animals showed the typical architecture of liver tissue with a central vein and chords of hepatocytes radiating, whereas CCl_4 treatment produced extensive necrosis of hepatocytes which was more pronounced in the centrizonal (zone 3) area. The fatty changes were of macrovesicular type which was evident in centrizonal and portal areas with inflammatory reactions (Fig. 3). Partial hepatic protection with reduction in the extent of hepatic necrotic areas, fatty infiltration, and mild portal inflammation was visualized in the liver section of AKSS16-LIV01 (200 mg/kg) treated animals. On the other hand administered with AKSS16-LIV01 (400 mg/kg) almost completely protected the liver as evidenced by restoration of a normal histoarchitecture of the liver.

DISCUSSION

CCl_4 is a well-known hepatotoxin which is widely used to induce toxic liver injury and to study the cellular mechanisms behind oxidative damage in laboratory animals [22]. Fight against various liver dysfunctions such as liver fibrosis, fatty liver, and liver cirrhosis through safe and symptomatic medicine is a new challenge. At present, there is no effective treatment for hepatic dysfunctions. To overcome this worldwide health complication, we formulated a novel herbal drug composed of nine indigenous medicinal and dietary herbs those were mentioned in Ayurveda. These herbs are natural resources of antioxidants that serve as the first line of defense against free radical damage and are considered to be important in maintaining optimum health and happiness. Our previous reports stated that the formulation has no adverse side effect and no toxicity in mice [23,24].

AST and ALT are the most sensitive indicators for the diagnosis of liver cell damage. During amino acid synthesis and catabolism, AST and ALT play vital roles as endoenzymes in hepatocytes. Under the circumstance of the normal working condition of the body, ALT and AST levels in the blood are very low and, thus, the activity of these two enzymes in

Table 5: Summary of the hematological parameters studied across the groups

Parameters	Normal	AKSS16-LIV01 (400)	CCl_4	CCl_4 + AKSS16-LIV01 (200)	CCl_4 + AKSS16-LIV01 (400)	CCl_4 + silymarin 100
Hb (g %)	12.1±1.05	11.21±0.82	9.03±0.89#	11.05±0.99	12.51±0.95*	10.96±0.74
RBC ($x10^6$ cm^2)	10.8±0.82	9.62±0.84	9.1±0.71	9.44±0.71	10.02±0.85	9.85±0.79
RT (%)	2.7±0.12	3.6±0.16	4.9±0.26	3.1±0.14	2.8±0.15	3.0±0.12
HCT (%)	34.6±0.48	35.1±0.77	39.4±0.55	35.8±0.51	34.9±0.56	34.4±0.51
MCV (μm^3)	37.8±0.32	35.5±0.36	31.0±0.68	36.5±0.44	35.9±0.79	36.2±0.43
MCH (pg)	21.2±0.15	21.1±0.12	22.2±0.14	21.1±0.12	21.4±0.11	21.2±0.14
MCHC (%)	41.2±1.06	36.2±0.91	32.4±0.95	37.1±0.92	39.6±0.87	38.6±0.99
Platelets	6.5±0.02	5.4±0.06	5.5±0.03	5.8±0.05	6.1±0.07	5.5±0.05
WBC ($x10^5$ $cm^{2)}$)	9.2±0.09	10.7±0.11	12.4±0.11#	10.8±0.12	9.2±0.11*	10.1±0.13
Lymphocyte	74±2.98	71±3.11	79±3.04	73±3.06	71±2.58	72±3.08
Neutrophil	26±1.12	21±0.55	15±0.49	20±0.56	25±0.69	24±0.51

Data are expressed as mean±standard deviation (N=6). Hb: Hemoglobin, RBC: Red blood corpuscle, RT: Reticulocyte, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, WBC: White blood corpuscle. Values are mean of six individual observations in each group±SD. *Significantly different from control group #($p<0.05$) and significantly different from CCl_4 group *($p<0.05$) using ANOVA followed by Dunnett's multiple comparison test

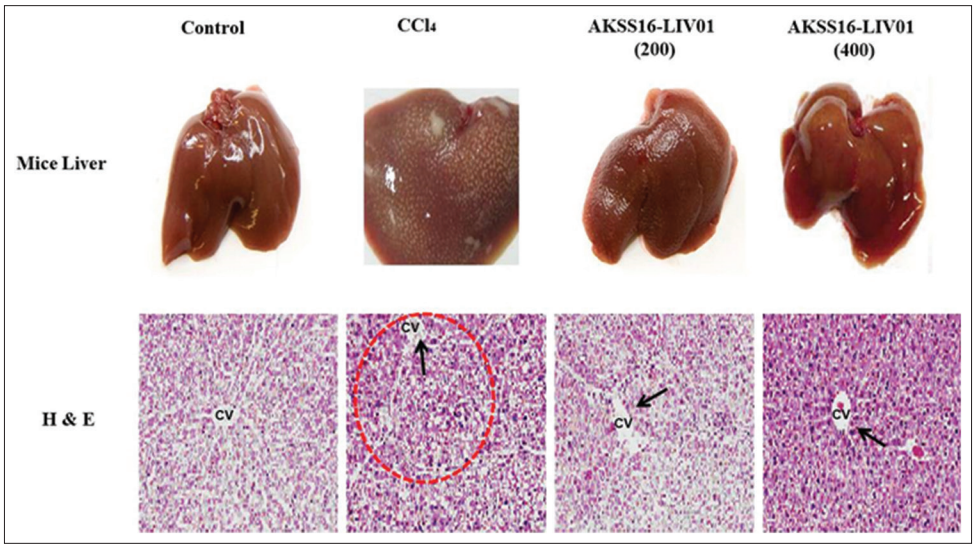

Fig. 3: Morphological and histopathological photographs of liver tissue. Lane 1: Morphological photographs of whole liver tissue; lane 2: H and E staining section of liver tissue

normal serum is very low. When the liver tissue is damaged and the cell membrane permeability increases, these two enzymes penetrate into the blood in large quantities, leading to a significant increase in the activity of the enzymes in the sera. Therefore, an increase in serum AST and ALT can reflect the extent of liver cell damage [25]. When CCl_4 enters the animal body, liver microsomal lipids and hepatocyte membrane phospholipid molecules are attacked by free radicals generated by CCl_4, which, in turn, trigger changes in the TC and TG levels in the liver [26,27]. The increase in the AST, ALT, ALP, GGT, and bilirubin levels indicates an exaggeration of liver damage. The experimental data from this study also confirmed that CCl_4 resulted in an increase in the AST, ALT, ALP, GGT, and bilirubin levels in mice. On the other hand, serum lipid profile, that is, total cholesterol, triglyceride, FFA, phospholipids, and LDL significantly elevated by the deleterious action of CCl_4.

The damage to the hepatic parenchymal cells due to accumulated toxins acts on the liver leading to the formation of reactive oxygen species which further leads to oxidative stress augmenting hepatic damage and dysfunction altering the liver transaminase enzyme parameters [28,29].

Therapeutic application of novel MHF AKSS16-LIV01 significantly reduced the serum biochemical and lipid profile levels in the serum and thus exerted a preventive effect on liver damage. This is further confirmed by our histopathological analysis of liver tissues.

CONCLUSION

The protective effect of MHF (AKSS16-LIV01) in CCl_4-induced liver injury was established in this study in mice. The newly developed MHF AKSS16-LIV01 normalized biochemical enzymes levels, lipid profile parameters, and blood parameters from CCl_4-induced liver injury. Liver histology strongly supported that AKSS16-LIV01 (44 mg/kg) able to protect liver cell form CCl_4-induced liver damage. Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of liver dysfunction.

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AUTHORS' CONTRIBUTIONS

Soumendra Darbar and Atiskumar Chattapadhyay conceived and designed the experiment. Soumendra Darbar and Srimoyee Saha conducted the animal and biochemical experiments. Soumendra Darbar, Atiskumar Chattapadhyay, and Kaushikisankar Pramanik wrote and revised the manuscript.

CONFLICTS OF INTEREST

All authors report no conflicts of interest regarding this manuscript.

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Original Article

Ameliorative Effect of Multi Herbal Formulation on Lipid Peroxidation and Redox Dysfunction in Ethanol Induced Hepatic Imbalance

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ABSTRACT

Objectives: Traditional medicine is a potent antioxidant. In the present study, we developed a multi herbal formulation and examined its anti-oxidative activities and possible protective effect of it on serum and liver lipid peroxidation and glutathione in ethanol-induced hepatic dysfunction. **Materials and Methods:** In this experimental study 40 Swiss albino mice were divided into 4 groups randomly; group I as control, group II as sham treated with multi herbal formulation (MHF) (300mg/kg orally, daily), group III as liver damage control and group IV as ethanol treated with multi herbal formulation (MHF) (300mg/kg orally, daily) after induce liver damage, respectively. Liver damage was induced in the 3rd and 4th groups by ethanol administration (50% v/v). **Results:** After sixty days, animals were anaesthetized, liver was then removed immediately and used fresh or kept frozen until analysis. Blood samples were also collected before killing of the mice to measure the lipid peroxidation and antioxidant enzymes level. Serum and Liver lipid peroxidation level (MDA level) were significantly increased in ethanol treated group compared with untreated group. However, content of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) in serum and liver tissue were significantly decrease in ethanol treated group compared with untreated group. Alteration of serum and liver lipid peroxidation and antioxidant enzymes content were significantly inhibited by the administration of developed multi herbal formulation (MHF) (300mg/kg orally, daily). **Conclusion:** In conclusion the study showed that developed multi herbal formulation (MHF) might be a potent antioxidant and exert beneficial effects on the lipid peroxidation level and maintained the antioxidant levels in ethanol-induced liver dysfunction.

Key words: Alcohol, Liver disease, Lipid Peroxidation, Mice, Herbal Formulation, Antioxidant enzymes.

INTRODUCTION

Oxidation is a chemical reaction in the body that produces free radicals. These free radicals lead to chain reactions within the body that damage other cells. Commonly the consecutive reduction of oxygen through adding electrons cause the formation of a variety of ROS, which include superoxide (O_2^-), hydroxyl radical (·OH), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2). In general, the harmful effects of ROS comes in the form of DNA damage, lipid preoccupation (oxidation of polyunsaturated fatty acids), protein amino acid oxidation and inactivation of specific enzymes through oxidation of their cofactors.[1-3] These damages can be contributing factor to many general and specific problems and diseases such as Parkinson's, Alzheimer's, asthma, aging, cancer, Rheumatoid Arthritis, Liver disorder etc. Therefore, antioxidant parameters and oxidative stress indices are considered

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potential biomarkers and are frequently used as screening tools to assess the impacts of environmental stress. Important antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione S-transferees (GST) and glutathione peroxidase (GPx). In addition, glutathione, vitamins and carotene also help the organism to mitigate the external pollutants and help the protective enzyme system of the organism.[4-6]

Liver is a major organ attacked by ROS.[7] Parenchymal cells are primary cells subjected to oxidative stress induced injury in the liver. When the ROS is excessive, the homeostasis will be disturbed, resulting in oxidative stress, which plays a critical role in liver diseases and other chronic and degenerative disorders.[8] The oxidative stress not only triggers hepatic damage by inducing irretrievable alteration of lipids, proteins and DNA contents and more importantly, modulating pathways that control normal biological functions. Since these pathways regulate genes transcription, protein expression, cell apoptosis and hepatic stellate cell activation; oxidative stress is regarded as one of the pathological mechanisms that results in initiation and progression of various liver diseases, such as chronic viral hepatitis, alcoholic liver diseases and non-alcoholic steatohepatitis.[9-11] Moreover, systemic oxidative stress arising during liver disease can cause damage to extra-hepatic organs, such as brain impairment and kidney failure.[12]

Worldwide, alcoholic liver disease (ALD) is a major cause of illness and mortality. ALD, a common effect of prolonged and heavy alcohol intake, is one of the leading health problems after cancer and cardiovascular diseases. In the modern way of life, intake of alcoholic beverages is a common characteristic and nowadays alcoholism ranks as a major health problem.[13,14] Experimental and epidemiologic studies confirmed that the duration and the degree of alcohol consumption promote the progression and genesis of liver damage. The liver is the major site of ethanol metabolism. Liver executes several important mechanisms which play crucial roles in digestion, storage, assimilation and detoxification.[15] Various study reports illustrate that ethanol causes the accumulation of reactive oxygen species (ROS) such as hydroxyl radical, superoxide radical and hydrogen peroxide in the hepatocytes that leads to the oxidation of DNA, protein and cellular membranes, resulting in the depletion of reduced glutathione and liver damage.[16,17]

Antioxidant a molecule that inhibits or stops the oxidation of other molecules in the body. So, antioxidant protect the body from cell damage. The potent sources of natural antioxidants are medicinal herbs and spices. Phenolic components in herbs and spices have been reported to be effective as natural antioxidants.[18] Intake of alcohol is associated with increase susceptibility of membranes to peroxidation and an increased requirement of antioxidant. The flavonoids are a large group of naturally occurring compounds that are found in plants and are frequently consumed as part of the human diet. Flavonoids are receiving much attention now a day for their potential pharmacological properties.[19] The antioxidant activity of flavonoids has been demonstrated by their ability to inhibit enzymes such as lipoxygenase, cyclooxygenase, along with chelating metal ions and scavenging free radicals.[20,21]

Based on previous reports, we developed a multi herbal formulation (MHF) containing nine Indian medicinal plants out of which six were medicinal plants and three were medicinal spices. Therefore, the present study was undertaken to evaluate the oxidative stress induction and tissues injury in liver of adult mice followed exposure to ethanol at different concentrations and its deleterious recovery by the application of multi herbal formulation.

MATERIALS AND METHODS

Chemicals

Disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and trichloroacetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents used were of analytical grade. 2-thiobarbituric acid (TBA) was obtained from Fluka Chemie (Buchs SG, Switzerland).

Collection and Authentication of the Herbs

All raw medicinal plants were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by taxonomists of Department of Botany, Uluberia College, University of Calcutta, India and kept as voucher specimen. The identification was based on Ayurvedic parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti(shape) and Parimana (size). The plants and plant parts used in preparation of the extract are listed in Table 1.

Preparation of Extract

Plant parts were air dried after cleaning with double distilled water and kept in oven at 80°Cfor 10 min and 60°C for 30 min. Then they were ground by a blade mill to fine powder. Subsequently, the extraction of the polar fraction was performed according to the method of Taamalli *et al.* (2015) with some modifications.[22] 5 gm. of dry plant parts were dissolved using 10 ml of methanol, sonicated at room temperature for 30 min

using an ultrasonic bath, centrifuged at 3000 rpm for 15 min and finally the supernatant was removed. This procedure was repeated four times, collecting all the supernatants, which were finally evaporated in rotary evaporator under reduced pressure at 35°C. Finally, the residue was re-constituted in 3 ml of methanol, filtered using what man filter papers (GE Healthcare and Life Sciences, MA, USA) and kept at 4°C for further use.

Animals

In the present study 40 male mature Swiss albino mice (25-30 g) were obtained from the CPCSEA approved animal house of Jadavpur University, Kolkata. The animals were maintained at 12 h light/dark cycle, at constant temperature (20±2°C) and humidity (50±5%). Mice were feed standard pellet diet (Purchase from Hind liver India Limited, Mumbai) containing 19.4% protein, 5.5% fiber, 11.1% water, 54.6% carbohydrates, 6.7% essential mineral mixture and 2.6% by weight of lipids and water *ad libitum*. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any pathogenic infection. All the experimental procedure were carried out according to the guidelines of CPCSEA, Govt. of India, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC).

Experimental Design

The mice were divided into four groups (10 per each). The studied groups were as follows: group I as control, group II as sham treated with multi herbal formulation (MHF) (300mg/kg orally, daily), group III as liver damage control and group IV as ethanol treated with multi herbal formulation (MHF) (300mg/kg orally, daily) after induce liver damage, respectively (Table 2). Liver damage was induced in the 3rd and 4th groups by ethanol administration (50% v/v).

Blood Collection

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Serum was separated and used for further analyses.

Preparation of Tissue Homogenate

Prior to biochemical analysis, liver samples (100 mg/mL) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10000 rpm for 15 mins[23] and the supernatant obtained was collected and used for further studies. Protein concentrations of

Table 1: Composition of ingredient(s) present in novel multi herbal formulation (MHF).

Sl. No.	Botanical Name	Common Name	Part Used	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Stem	20 mg
2.	*Terminalia chebula*	Haritaki	Fruit	20 mg
3.	*Azadirachta indica*	Neem	Leaves	50 mg
4.	*Andrographis paniculata*	Kalmegh	Leaves & Steam	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Leaves & Steam	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Rhizome	20 mg
7.	*Trigonella foenum-graecum*	Methi	Seed	10 mg
8.	*Piper nigrum*	Black pepper	Seed	10 mg
9.	*Elettaria cardamomum*	Cardamom	Seed	10 mg

* Amount required for preparation of 5 ml extract.

Table 2: Experimental Design.

Groups	Treatment
I	Normal control received only the normal drinking water for 60 days.
II	Sham treated control received multi herbal formulation (300mg/kg-bw/day) for 60 days.
III	Animals treated with ethanol (50% v/v, single dose/day) for 60 days.
IV	Treatment with ethanol (50% v/v) for 30 days and then pre-treated with multi herbal formulation - MHF (300 mg/kg-bw/day) for next 30 days.

liver supernatant were determined using commercially available kit (Span Diagnostics Ltd, India) following procedure prescribed by manufacturer.

Levels of Malonedialdehyde (MDA)

Lipid peroxidation was estimated in the serum samples by measuring the Malondialdehyde (MDA) formation using the thiobarbituric acid method.[24] Briefly, 100 µL of animals was mixed with 500 µL of 150 mM Tris-HCl and 1.5 mL of 0.375% TBA and vortexed for 10 sec. The reaction mixture was then incubated at 100°C for 45 min in a water bath. At the end of incubation, the samples were centrifuged at 1000×g for 10 min. The MDA content was calculated from the absorbance measurement at 532 nm and using a Shimadzu spectrophotometer (Tokyo, Japan) an absorption coefficient = $1.56 \times 105\ cm^{-1}\ M^{-1}$.

Determination of ROS activity

Amount of ROS in blood was measured using 2', 7'-dichlrofluorescein diacetate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides (including hydrogen peroxide). The assay was performed as described by Socci *et al*. 20 Fluorescence was determined at 488 nm excitation and 525 nm[25] emission using a fluorescence plate reader (Tecan Spectra Fluor Plus).

Determination of Glutathione (GSH) and GPx Content

Reduced glutathione (GSH) was determined from liver homogenate spectrophotometrically according to Ellman's method. GPx activity was assayed using a modified method of Lawrence and Burk.[26]

Determination of Superoxide Dismutase (SOD)

The activity of SOD was measured according to a reported method.[27] In brief, 2.8 mL reagent solution (xanthine 0.3 mM, EDTA 0.67 mM, 150 µM NBT), sodium carbonate 0.4 M and bovine albumin (30 mg/30 mL) was added to 0.1 mL sample and 50 µL xanthine oxidase (10 µL in 2 M ammonium sulphate), incubated at 25°C for 20 min and mixed with, 0.1 mL 8 M copper chloride. The color reaction was measured at 560 nm.

Determination Catalase (CAT)

Catalase activity was measured according to the method of Maehly (1955).[28] Briefly, after addition of 5µL liver homogenate to 0.995 mL 30 mM H_2O_2 solution in potassium phosphate buffer (pH 7.0), change in absorbance at 240 nm was monitored for 1 min to determine catalase activity. The enzyme activity was expressed as U/mg protein.

Statistical analysis

All quantitative data are expressed as mean ± standard deviation (SD) unless otherwise stated. One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison *t* tests were executed for comparison of different parameters between the groups using a computer program GraphPad Prism (version 5.00 for Windows), Graph Pad Software, California, USA. P and amp; lt; 0.05 was considered significant.

RESULTS

Determination of serum and liver lipid peroxidation

In the present study we developed a novel multi herbal formulation (MHF) containing six Indian medicinal herbs and three medicinal spices (Figure 1) enrich with high flavonoids and polyphenols. The medicinal effect of multi herbal formulation (MHF) on serum and liver MDA level of mice was determined. The levels of MDA in serum and liver are shown in Figure 2. The level of serum MDA in the untreated liver damage mice was significantly (87%) higher ($P<0.001$) than that of control animals. The level of MDA in the serum of ethanol treated liver dysfunction mice with multi herbal formulation (MHF) was very low, similar to the level (92% recovery) found in the control animals. The treatment of hepatic damage animal with MHF could significantly (92%) inhibit the elevation of MDA in comparison with the untreated experimental animals. The level of liver MDA in the untreated hepatic damage animal was significantly (84%) higher than that of control animals. The treatment of liver damage animal with multi herbal formulation (90%) inhibit the

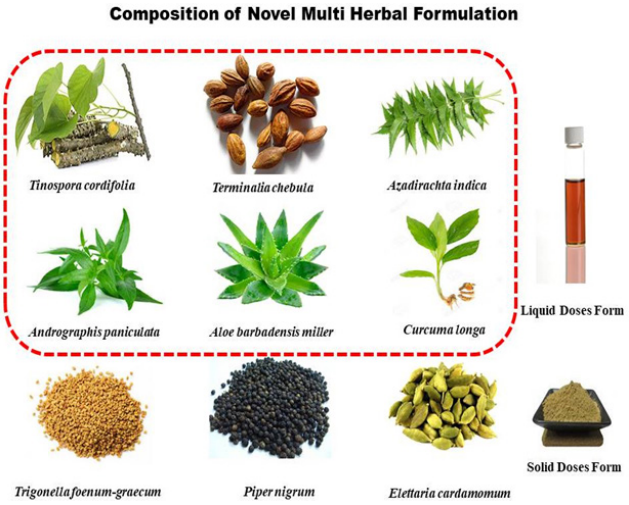

Figure 1: Ingredients present in the novel multi herbal formulation containing six medicinal herbs and three medicinal spices.

increasing of MDA (P<0.001) in comparison with the untreated hepatic damage animals. The level of MDA in the serum and liver of sham mice treated with multi herbal formulation were low, similar to the level found in the control animals.

Determination of serum and liver ROS content

Beneficial effect of multi herbal formulation (MHF) on serum and liver ROS level of mice was determined. The levels of ROS in serum and liver are shown in Figure 2. The level of serum ROS in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of ROS in comparison with the untreated experimental animals. The level of liver ROS in the untreated liver damage mice was significantly (87%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (93%) in ROS level compared with the untreated control animals.

Determination of serum and liver SOD and CAT content

Beneficial effect of multi herbal formulation (MHF) on serum and liver SOD and CAT level of mice was determined. The levels of SOD and CAT in serum and liver are shown in Figure 3. The level of serum SOD and CAT in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of SOD and CAT in comparison with the untreated experimental animals. The level of liver SOD and CAT in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in SOD and CAT level compared with the untreated control animals.

Determination of serum and liver GSH and GPx content

Effect of multi herbal formulation (MHF) on serum and liver GSH and GPx level of mice was determined. The levels of GSH and GPx in serum and liver are shown in Figure 4. The level of serum GSH and GPx in the untreated liver damage mice was significantly (86%) lower (P<0.001) than that of control animals. The treatment of hepatic damage animal with MHF could slightly increase of GSH and GPx in comparison with the untreated experimental animals. The level of liver GSH and GPx in the untreated liver damage mice was significantly (89%) lower (P<0.001) than that of control animals. The multi herbal formulation (MHF) treated ethanol induced liver damage animals showed significantly (P<0.001) elevation (96%) in GSH and GPx level compared with the untreated control animals.

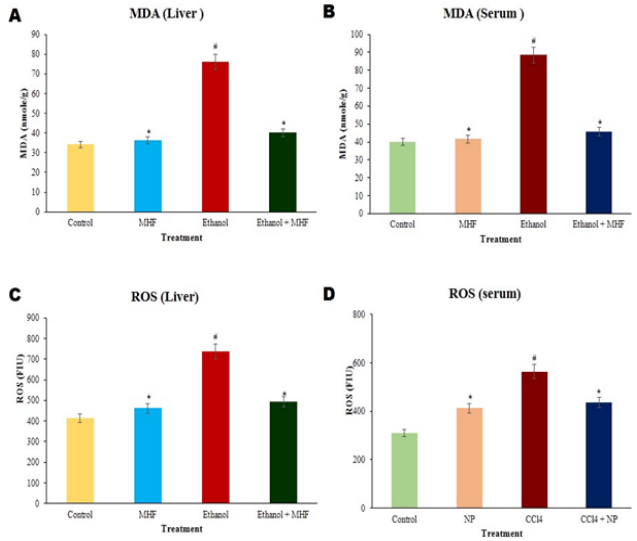

Figure 2: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) MDA content in serum B) MDA content in liver C) Level of ROS in serum D) Level of ROS in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol * (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

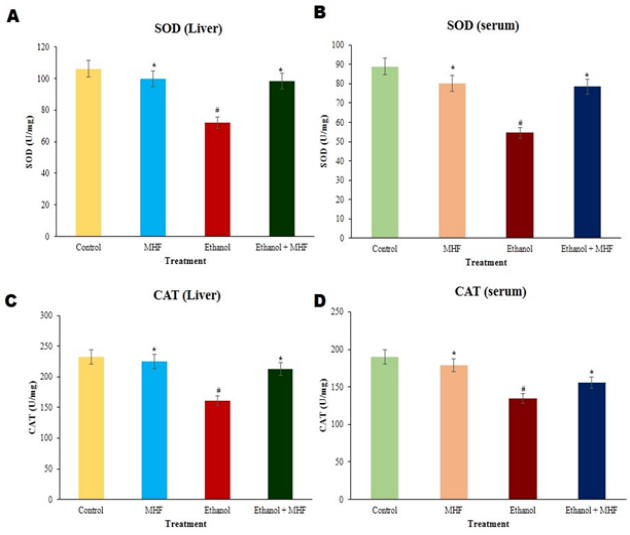

Figure 3: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) SOD content in serum B) SOD content in liver C) Level of CAT in serum D) Level of CAT in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # (p<0.001) and significantly different from Ethanol * (p<0.001) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

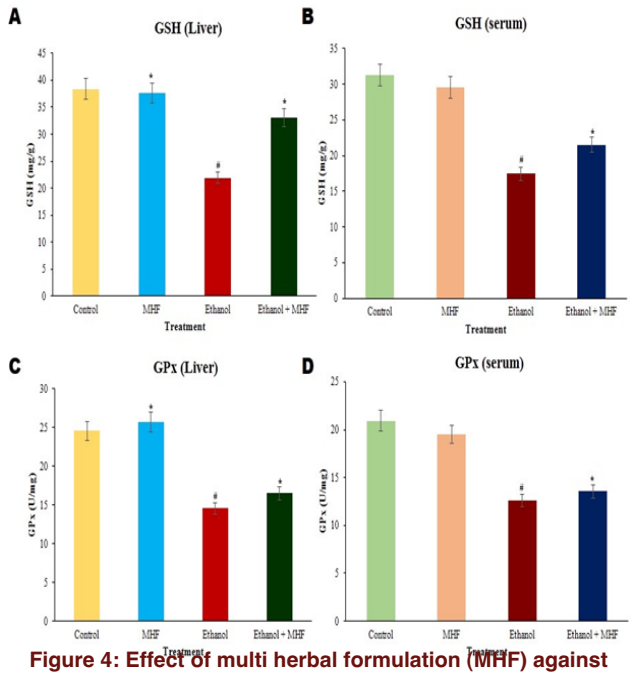

Figure 4: Effect of multi herbal formulation (MHF) against ethanol induced oxidative stress in mice. A) GSH content in serum B) GSH content in liver C) Level of GPx in serum D) Level of GPx in liver. Values are expressed as Mean ± SD (n = 10 per group). *Significantly different from control # ($p<0.001$) and significantly different from Ethanol * ($p<0.001$) using analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test.

DISCUSSION

In the past decades, considerable evidence has established the role of oxidative stress in the pathogenesis of liver complications. Indeed, several studies have reported that ethanol induced liver damage contribute to the accumulation of ROS and antioxidants deficiency (e.g., SOD and GSH) in both experimental animals and patients. This study showed that multi herbal formulation (MHF) increased serum and liver antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) and decreased lipid peroxidation in ethanol induced experimental mice. The current work showed a significant increase in the level of lipid peroxidation (LPO) in serum and liver tissue after 60 days of oral administration of ethanol (50% v/v). LPO is an auto catalytic process leading to oxidative degradation of lipids causing demolition of cell membranes and subsequently cell damage.[29] It is mainly generated by the effect of various reactive oxygen species (ROS) such as hydrogen peroxide, superoxide's and hydroxyl radical.[30] LPO is a chain reaction initiated by the hydrogen abstraction from the side chain of polyunsaturated fatty acids resulting in cell membranes deterioration.[31] Decomposition of latest compounds lead to production of several products particularly malondialdehyde (MDA).[32] This tending to diminish the fluidity of cell membrane which plays a vital role in cell functioning. The elevated level of MDA observed in the current work, which is an indicator of LPO, denotes cell membrane damage in serum and liver of ethanol treated animals. Treatment with multi herbal formulation (MHF) significantly inhibited increasing of serum and liver lipid peroxidation in comparison with the ethanol treated animals.

Glutathione is a non-enzymatic cellular antioxidant which plays a crucial role in scavenging damaging free radicals. GSH can function as a co-substrate for peroxide detoxification by glutathione peroxidases.[33] GSH also catalyses' the reduction of hydrogen peroxide to water.[34] Depletion of cellular glutathione content may be one of the reasons for the increase in cell vulnerability to oxidative stress.[35-37] In this study decline in GSH level in this investigation could be caused by ethanol intoxication. Treatment with multi herbal formulation (MHF) significantly inhibited decreasing of serum and liver glutathione in comparison with the ethanol treated animals.

Study showed that there was a significant decrease in SOD and CAT activities in liver and renal tissues following oral administration of ethanol. SOD is an enzyme that repairs cells and decreases their damage through conversion of endogenous cytotoxic superoxide radicals to hydrogen peroxide and ordinary molecular oxygen, which have harmful effects on proteins and polyunsaturated fatty acids.[38] CAT is an important enzyme in protecting the cell from oxidative damage by catalyzing the decomposition of hydrogen peroxide to water and oxygen.[32] In the presence of insufficient activity of CAT to decompose hydrogen peroxide, more of it could be converted to toxic hydroxyl radicals that might contribute to oxidative stress after intoxication with ethanol. The significantly suppressed catalase activities after intoxicated with ethanol were recovered by the administration of multi herbal formulation (MHF). The endogenous scavenger, SOD, which removes the superoxide anion radicals by converting them into hydrogen peroxide (H_2O_2) and O_2, was significantly increased in the MHF experimental groups. The observed increase in liver SOD enzyme activity after administration of the multi herbal formulation (MFH) may be a consequence of oxidative activation of enzyme protein or increased of their synthesis. Therefore, the increase in the activity of SOD in liver tissues of treated mice might indicate a reduce accumulation of superoxide anion radical with oxidative stress, contributing decrease liver toxicity.[39]

GPx has a role in defending cells against oxidative stress and this in turn involves GSH as a cofactor. GPx catalyzes the oxidation of GSH to GSSG at the cost of H_2O_2. Decreased GPx activity was observed in the alcohol exposure group. This reduced activity may be involved in either free radical-dependent inactivation of enzyme or depletion of its co-substrate (i.e., GSH) or NADPH on ethanol treatment. Administration of multi herbal formulation (MHF) significant increased reduced GPx level activity after alcohol exposure.

CONCLUSION

In summary, the exposure of male Swiss albino mice to alcohol revealed signs of toxicity that were evidenced by a reduction in antioxidant defense system. Moreover, the activities of SOD, CAT, GPx and the concentration of MDA and GSH in the liver and kidney clearly indicate that our developed multi herbal formulation (MHF) is able to inhibit the oxidative stress during the co-exposure with ethanol, but its effect depends on the dose and time of exposure.

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CONFLICT OF INTEREST

Authors disclose no conflicts of interest for publication of the manuscript.

ABBREVIATIONS

SOD: Superoxide dismutase; **CAT:** Catalase; **GSH:** Glutathione; **GPx:** Glutathione peroxidase; **MDA:** Malondialdehyde; **ROS:** Reactive Oxygen Species; **GST:** Glutathione, S-transferase; **DNA:** deoxyribonucleic acid; **ALD:** Alcoholic liver disease; **MHF:** Multi herbal formulation; **TBA:** Thiobarbituric acid; **IAEC:** Institutional Animal Ethics Committee; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **EDTA:** Edetate disodium; **DCF-DA:** Dichlrofluorescein diacetate; **NADPH:** Nicotinamide adenine dinucleotide phosphate.

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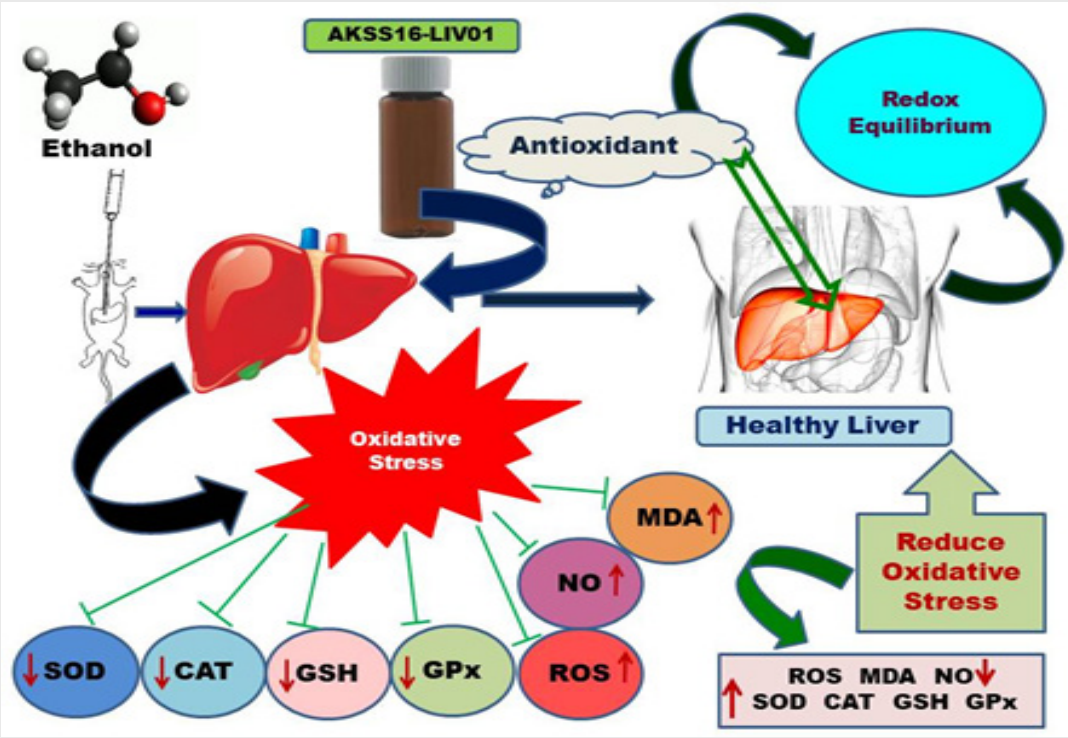
PICTORIAL ABSTRACT



SUMMARY

Life nearly in all its aspects is intricately linked to the intracellular redox homeostasis and its modulation. Intracellular redox potential is dependent on an intricate balance between the intrinsic and extrinsic reactive oxygen species (ROS). Worldwide, alcoholic liver disease (ALD) is a major cause of illness and mortality. Prolonged and heavy alcohol intake, is one of the leading health problems after cancer and cardiovascular diseases. Regular intake of alcohol impaired the cellular redox balance which produces oxidative stress and disrupts cells integrity. Liver is the main target organ of alcohol that inhibits normal hepatic function creates lots of cellular complications. Scientist from all over the world are engaged for a safe and symptomatic medication which mitigates ethanol induced liver dysfunctions. We developed a novel multi herbal composition containing six medicinal herbs and three medicinal spices. Dose dependent application of the herbal composite gradually inhibits ethanol induced hepatic abnormalities. Our *in vivo* experimental results depict that administration of this novel formulation upon mice normalized the hepatic oxidative stress caused by ethanol. So, the formulation maintained redox equilibrium and supplied antioxidants towards the cell which maintains cellular homeostasis.

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Original Article

Preliminary Assessment of Acute and 28-Day Repeated Dose Oral Toxicity of a Newly Developed Herbal Mixture on Experimental Animal

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ABSTRACT

Objectives: Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. The present study was designed to evaluate the acute oral toxicity study and 28 days repeated toxicity study of Herbal Mixture (HM) according to OECD guidelines. **Materials and Methods:** In acute oral toxicity study, Herbal mixture was administered at 2000mg/kg orally and animals were observed for toxic signs at 30 min, 1, 2 and 4 hr and thereafter once a day for the next 14 days. In repeated dose-28-day toxicity study, the animals were divided into four groups of 6 animals each. Group-1 animals served as a control. Group II Animals received low dose of test drug 100 mg/kg (orally). Group III animals received middle dose of test drug 200 mg/kg (orally) once daily for 28 days respectively. Group IV animals received high dose of test drug 400 mg/kg (orally) once daily for 28 days respectively. **Results:** The study results showed that neither the acute toxicity study of herbal mixture at the dose level of 2000mg/kg nor the repeated dose study did not produce any toxic sign or mortality during study. In repeated dose toxicity study, no significant changes were observed in the haematological and biochemical parameters, relative organ weight, gross necropsy and histopathological examination with herbal mixture treatment. **Conclusion:** The results of the present study suggest that LD_{50} of newly developed Herbal Mixture (HM) > 2000mg/kg and the mixture is completely safe and non-toxic for therapy.

Key words: Acute oral toxicity, Sub acute toxicity, Herbal mixture, Haematology, Liver function test, Histology.

INTRODUCTION

Medicinal plants have burgeoned in recent times due to increased efficiency of drugs derived from plants, growing interest in natural products and raising concerns about the side effects of conventional medicine.[1] Herbal drug combinations have shown that they possess better efficacy and reduced side-effects in comparison with single herbal drugs. The World Health Organization (WHO) estimates that 80% of the world's population relies on these "alternative" plant-based medicines as their primary medical intervention especially in the developing and in the developed countries where modern medicines are predominantly used.[2] Over the years, the use of herbs in the treatment of illnesses has been very successful and its historic usage has been useful in drug discovery development. Herbal prescriptions and natural remedies are commonly employed in developing countries for the treatment of various diseases.[3,4]

Toxicology may be defined as the study of harmful/poisonous effects of drugs and other chemicals with emphasis on detection, prevention and treatment of poisonings. After gaining relevant information on the harmful effects of a compound, the levels for its safe usage or the degree of its safety is established, this is known as its

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(compound) Biosafety level.[5] Acute toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effect characteristics of a test substance and its primary purpose is to provide information on potential health hazards that may result from a short term exposure.

Traditional and alternative medicine is extensively practiced in the prevention, diagnosis and treatment of various illnesses. It has attracted increasing public attention over the past 20 years as this type of medicine is easily accessible in some regions.[6] Medicinal plants contribute great importance in daily life by providing wide range of nutrients, vitamins and other compounds which widen in therapeutic arsenal. In general, natural products play a dominant role in the development of novel drug leads for the treatment and prevention of diseases.[7] Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. Determination of efficacy and safety of herbal remedies is necessary because many people using these agents as self-medication. Since, there is limited data available about the safety of the commonly used herbal remedies, therefore, efforts to elucidate health benefits and risks of herbal medicines should be intensified.[1] It is the need of the hour to evaluate acute and chronic toxicities of herbal drugs.[8]

Herbal formulations available with a wide range of indications like protective to liver, appetite and growth promoters, gastrointestinal and hepatic regulator, as treatment for hepatic dysfunction, for hepatic regeneration as well as liver stimulant and tonic. Despite the widespread use, there is a lack of scientific evidence on their efficacy and safety. In fact, there is lack of evidence on quality, safety and efficacy of many herbal preparations. Although many herbal preparations are non-toxic, many plants currently used for medicines have been shown to be highly toxic when given either acutely or sub-chronically.[9,10] The increasing number of plant based medication users around the globe and lack of experimental reports on their safety make it basic to direct toxicological investigation on natural herbal products.[11,12]

Herbal medicines have attained the widespread acceptability as natural therapeutic agents for various diseases like diabetes, arthritis, renal and liver diseases, obesity and cardiovascular disorders. It is proved that herbal combination made up of different herbs which produce maximum therapeutic outcomes than the individual herbs. These combinations are employed for the betterment of various chronic disorders. Currently worldwide there is need to found out the safe, less toxic, cost effective polyherbal remedies that can be effective against various chronic diseases like diabetes, obesity, liver dysfunction. Here we developed a herbal formulation which is made up of six Indian medicinal plants and three medicinal species with minimum quantity and maximum therapeutic potential. We hope the newly developed herbal medicine may be very effective to treat the various chronic diseases. Hence it has become necessary to standardize the preclinical safety and efficacy study on animal model for further therapeutic study to establish the formulation as a drug. So, in the present study, toxic effects of Herbal Mixture (HM) in swiss albino mice were conducted at dose of 2000mg/kg body weight for a period of 14 days for the acute toxicity study (followed OECD 402 guideline); and at dosages of 100, 200 and 400 mg/kg body weight for a period of 28 days for the sub-acute toxicity study (followed OECD 410 guideline).

MATERIALS AND METHODS

Experimental animals

Swiss albino wistar mice (30-40g) were obtained from the animal house of Jadavpur University. The room was well ventilated and maintained on light for 12 hr and 12 hr darkness. Temperatures were maintained at 27-30°C. The mice were provided with the standard pellets and clean water *ad libitum*. The experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee's (IAEC) rules and regulation of this institute and the experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) having IAEC No. IAEC/JU/s/8/2018.

Composition of herbal formulation

The composition of each 5ml of Herbal mixture compose of *Azadirachtaindica* (Neem)50mg; *Curcuma longa* (Turmeric) 20mg; *Terminalia chebula* (*Chebulic myrobalan*) 20mg; *Aloe barbadensis* Miller (Aloe Vera) 20mg; *Tinospora cordifolia* (guduchi) 20mg; *Citrus limon* (Lemon)10mg; *Trigonella foenum graecum* (Methi) 10mg; *Piper nigrum* (Black pepper) 10mg; *Elettaria cardamomum* (cardamom) 10mg (Figure 1).

Assessment of Acute toxicity test

Acute toxicity study was performed in healthy swiss albino mice (30-40gm) as per guidelines (AOT 425) suggested by the Organization for Economical Co-operation and Development (OECD). The animals were randomly assigned into two groups of 5 mice each and kept overnight fasting prior to extract administration. Group 1 served as the control and the mice were orally administered with 2ml distilled water. Single concentrations of the polyherbal extract 2000 mg/kg body weight

Figure 1: Composition of Herbal Mixture.

was constituted in 5ml distilled water through a mice gavage. Food was withheld for further 3 hr.

The mice were observed after every 30 min post extract administration for the first 2 hr and later once a day up to the 14th for changes in skin and fur, eyes and mucus membranes, behavior pattern, tremors, salivation, diarrhea, sleep, coma, mortality, moribund, ill health or any visible reaction to treatment. Weight recording was done before extract administration, at day 1, day 7 and day 14 using a sensitive balance.

Clinical Observation

The treated animals were observed for mortality (twice daily) and the clinical signs were recorded to note the onset, duration and reversal (if any) of toxic effect at 2, 4, 6 and 8 hr after the administration of last substances and once daily thereafter for 14 days. The routine cage side observation s included changes in skin and fur, eye and mucus membrane, somatomotor activity, general behavior pattern were noted. Clinical symptoms like arching of the back, alopecia, wound, nasal discharge, lacrimation and loose stool were also recorded during the observation.

Body weight

Body weight data of individual animals were recorded following the period of fasting on the day of dosing, weekly thereafter and at termination on day 15. Weekly changes in body weight gain were calculated and recorded.

Repeated dose 28-day oral toxicity study

Sub-acute (Repeated dose 28-day oral toxicity study) was carried out as per OECD guidelines Guideline-407 2. Healthy Swiss albino mice were used for the study. The Animals were divided into four groups of 6 animals each. Group-1 animals served as a control animals. Group II Animals received low dose of test drug (Herbal formulation) 100 mg/kg orally. Group III animals received middle dose of test drug 200 mg/kg orally. Group IV animals received high dose of test drug 400 mg/kg orally. The animals were administrated with the study drug once daily for 28 days. The doses were selected as par various previous studies. All the experimental animals were observed for clinical signs of mortality and morbidity once a day, at the same time each day, till the completion of treatment.

Haematological study

On the last day of dose administration all the animals were kept for overnight fasting (water *ad libitum*). The overnight fasted animals were anaesthetized under general anaesthesia using isoflourane, blood samples were collected using heparinised microhematocrit tubes by retro-orbital puncture into a potassium EDTA containing blood collection tubes (for haematological) and 11% w/v Tri-sodium Citrate (TSC) containing tubes (for biochemical fanalysis). Blood smear was prepared from the EDTA containing blood sample, air dried and stained (Hemacolor rapid staining of blood smear, E.Merck, Mumbai, India) for Differential Leukocyte Count (DLC). Haematological analysis were performed using automated haematology analyser (Model PE 6000 Rapid Diagnostics Pvt Ltd, New Delhi, India), which includes analysis of haemoglobin (HGB), Red Blood Cell count (RBC), White Blood Cell count (WBC), platelet count and Hematocrit (HCT).

Liver function test

The plasma thus collected was analysed for glucose, triglyceride, cholesterol, Alkaline Phosphatase (ALP) Aspartate Transaminase (AST) Alanine Transaminase (ALT) Lactate Dehydrogenase (LDH), total bilirubin creatinine, urea, protein and albumin levels by using biochemical kits (Accurex Biomedical Pvt. Ltd, Thane, India) in semi-automated biochemical analyser (Model: Star 21 Plus, Rapid Diagnostics Pvt Ltd, New Delhi, India).

Statistical analysis

Data were expressed as mean ± standard error mean. Data obtained from repeated dose studies were analysed by Student's *t*-test using GraphPad prism 5.0 to determine significant difference between the means of control and test groups. *p* value 0.05 was considered significant.

RESULTS AND DISCUSSION

Traditional medicine has maintained greater popularity all over developing world and the use is rapidly on the increase. Despite this, the safety of herbal medicine

use has recently been questioned due to reports of illness and fatalities; hepatotoxicity and nephrotoxicity. Although there are many traditional herbal medicines available, only a few have been verified by clinical trials, their efficacy and safety are still questioned by consumers.[13,14]

Observation included the change in skin, fur, eyes and mucus membrane. Appearance of toxicity related to central nervous system, Cardiovascular system and Autonomic nervous system such as tremors, convulsions, sedation, stereotypic behaviour, respiratory distress, cardiovascular collapse, response to sensory stimuli, salivation, diarrhea, pilo erection, Muscular coordination, Muscular grip, posture, gait, limb paralysis, lethargy, sleep, coma and mortality were observed with special attention (Table 1). The results revealed no treatment related death or signs of toxicity in the treated animals in all the doses throughout the study. Body weight gain of both male and female mice was also observed (Table 2) when compared with before and after treatment of the observed groups (Figure 2). Further, there were no gross pathological abnormalities which prove the LD_{50} value was found to be greater than 2000mg/kg b.wt.

There were no treatment-related toxicity signs and mortality observed in mice treated at 100mg, 200mg and 400 mg/kg orally for a period of 28 days and in the satellite group of mice. Bodyweight gain was observed between control and treated groups during the study (Table 1). Food and water consumption of treated groups were found to be insignificant when compared to the control groups (Figure 3 and 4). Since there is no significance decrease in mean body weight and there is considerable increase in mean body weight of control and treatment groups.

Hematological profile such as Packed Cell Volume (PCV), Red Blood Cells (RBC) count, White Blood Cell Count (WBC), Platelet Count, Hemoglobin (Hb), Mean Cell Haemoglobin Concentration (MCHC), Mean Red Cell Volume (MCV), Mean Cell Hemoglobin (MCH), Mean Platelet Volume (MPV), Neutrophils, Eosinophil's, Basophils, Lymphocytes and Monocytes were found to be within the normal physiological limits for rodents and no significant change has been observed in treatment groups when compared with the control groups (Table 3). Hence there are no serious toxicological implications such as destruction of Erythrocytes.

Lipid profiles such as HDL, LDL, VLDL, TGL and Total Cholesterol did not show any significant changes. The main product of protein metabolism is urea and an increased level of urea in the blood is an indicator of renal impairment (Table 4). The present study showed

Table 1: Clinical observations of mice at 2,000 mg/kg dose of Herbal Formulation (HF).

Signs and symptoms	Male	Female	Male	Female	Male	Female
	Day 1	Day 1	Day 7	Day 7	Day 14	Day 14
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Values are mean ± S.D (n = 6).

Table 2: Effect of Herbal Mixture (HM) on the body weight, Food consumption and Necropsy of mice at 2,000 mg/kg dose.

Animals	Body weight (g)			Food consumption (g)			Observed lesions during study
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14	Day 14
Male	25.2±1.02	27.6±1.03	31.7±0.98	4.62±0.06	4.78±0.06	5.01±0.05	Nil
Female	25.4±1.11	27.9±0.94	31.4±1.12	4.81±0.05	4.88±0.08	5.15±0.04	Nil

Values are mean ± S.D (n = 6).

no significant changes pertaining to renal parameters. Serum marker enzymes are biochemical parameters associated with health indices and are of diagnostic significance in routine clinical evaluation of the state of health. Alanine amino Transaminase (ALT) and Aspartate amino transaminase (AST) are largely used in the assessment of liver damage by drugs or any other hepatotoxin (Table 4). So, to elucidate the toxicity produced during liver metabolism of drug, transaminase markers play a vital role. Aspartate Transaminase (AST), Alanine amino Transaminase (ALT) which are the indicators of hepatocellular injury also did not show any significant alterations in the polyherbal formulation treated groups and control groups. Serum MDA level did not show any significant alterations in the HM treated group and control group (Table 5). The histopathological studies revealed no significant weight changes and normal architectural changes in the vital organs such as heart, brain, lungs, spleen, kidneys, liver, stomach, testes and ovary suggesting that the preparationis devoid of serious organ degenerative potential both dose levels but high dose should have slight detrimental kidney, liver effect (Figure 5).

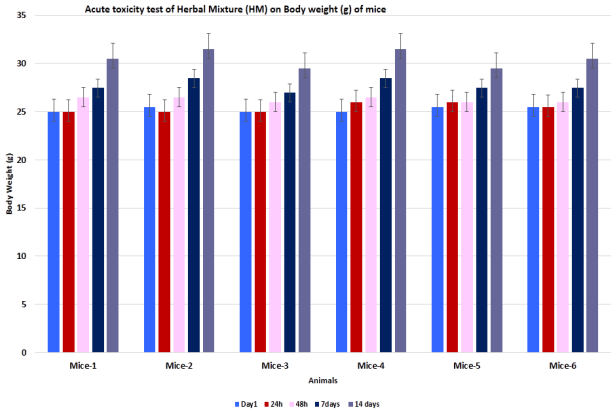

Figure 2: Acute Toxicity Test of HM on Body Weight.

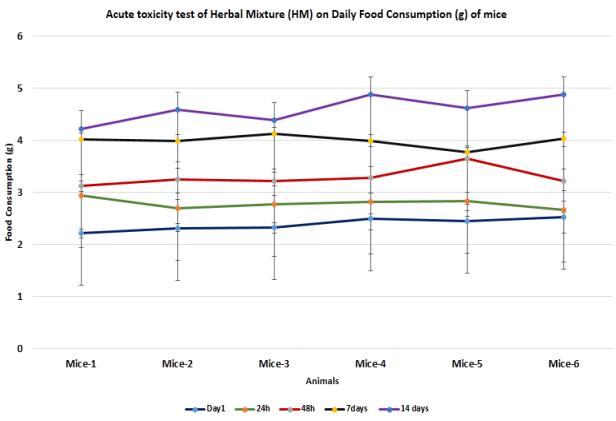

Figure 3: Acute Toxicity Test of HM on Daily Food intake.

World Health Organization estimated that 80% of the word's inhabitants still rely mainly on traditional medicines for their health care. The subcontinent of India is well-known to be one of the major biodiversity centers with about 45,000 plant species. In India, about 15,000 medicinal plants have been recorded, in which the communities used 7,000-7,500 plants for curing different diseases. In *Ayurveda*, single or multiple herbs (polyherbal) are used for the treatment. The '*Ayurvedic Literature Sarangdhar Samhita*' highlighted the concept of polyherbalism to achieve greater therapeutic efficacy. The active phytochemical constituents of individual plants are insufficient to achieve the desirable therapeutic effects. When combining the multiple herbs in a particular ratio, it will give a better therapeutic effect and reduce the toxicity. Here we developed a herbal formulation which is made up of six Indian medicinal plants and three medicinal species with minimum quantity and maximum therapeutic potential. We hope the newly developed herbal medicine may be very effective to treat the various chronic diseases. The developed formulation is very affordable for the common mass for treatment in the common chronic disorders.

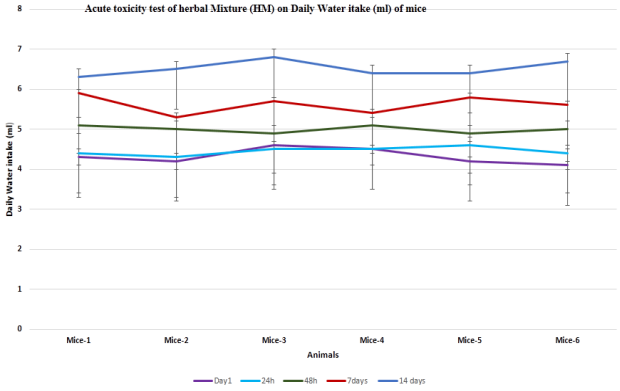

Figure 4: Acute Toxicity Test of HM on Daily Water Consumption.

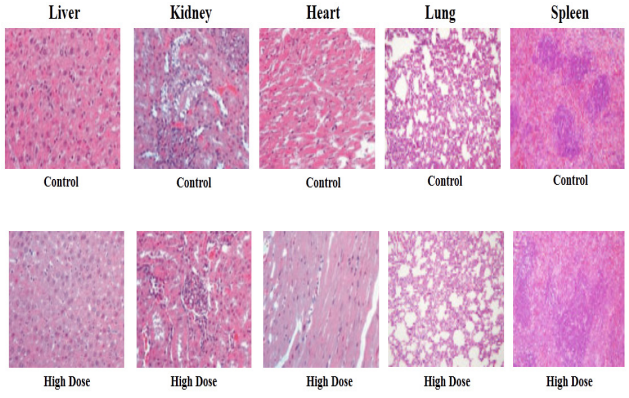

Figure 5: Histological examination of Herbal Mixture in different organs.

Table 3: Haematological Parameters as studied across the group C (Control); LD (Low dose); MD (Middle dose); HD (High dose).

Parameters	Control		HM 100mg/kg		HM 200mg/kg		HM 400mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
Hb (g%)	11.6±2.3	11.2±2.1	12.6±2.0	12.2±1.9	10.8±1.1	10.4±1.5	12.7±3.0	12.5±3.1
RBC (x10^6 cm^2)	10.8±2.3	10.2±1.9	11.5±1.0	11.6±1.1	9.1±1.1	9.0±1.2	10.4±3.2	10.3±2.6
RT (%)	2.8±0.5	2.5±0.3	4.8±0.6	4.5±0.6	3.1±0.9	2.9±0.9	2.5±2.4	2.6±1.9
HCT (%)	34.1±6.2	32.1±5.1	36.4±5.4	36.9±5.9	30.7±3.2	30.1±3.7	38.6±2.6	37.9±2.1
MCV (µm^3)	37.2±1.5	37.1±1.5	31.7±2.6	31.0±2.5	33.7±3.7	33.2±3.2	37.1±2.9	37.1±1.8
MCH (pg)	21.4±2.6	21.4±2.4	21.9±3.3	21.8±3.1	21.5±5.4	21.6±4.9	22.6±2.6	22.5±1.2
MCHC (%)	40.2±6.5	40.5±6.2	36.5±6.2	36.1±4.2	38.2±5.9	38.2±4.1	32.4±5.2	32.5±2.5
Platelets	6.5±1.2	6.2±1.9	5.5±1.1	5.2±1.2	3.9±1.0	3.6±1.5	4.8±1.1	4.2±1.2
WBC (x10^5 cm$^{2)}$)	9.1±2.2	9.4±2.1	9.9±3.0	9.1±2.6	9.5±0.9	9.7±0.8	11.5±1.6	11.1±1.3
L	72±5.1	74±5.2	79±6.5	78±5.6	74±4.9	76±4.1	74±5.5	73±4.5
N	25±2.5	24±2.3	18±2.6	19±2.1	23±3.4	24±3.1	23±2.1	25±1.2

Data are expressed as mean ± standard deviation (*n*=6)
Hb: Haemoglobin; RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCV: Mean corpuscular volume; MCH: Mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration; WBC: White Blood corpuscle

Table 4: Effect of Sub acute toxicity study of Herbal Mixture (HM) on biochemical parameters in mice.

Parameters	Control		HM 100mg/kg		HM 200mg/kg		HM 400mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
(AST (IU/L)	101.3±12.0	105.2±11.2	106.6±12.56	104.1±11.5	100.5±11.27	100.3±12.7	102.4±11.58	101.7±14.5
ALT (IU/L)	19.46±6.87	21.5±5.2	20.56±5.14	19.6±3.8	20.64±4.29	20.7±3.9	21.33±3.58	20.9±4.5
ALP (IU/L)	140.25±14.25	138.7±13.1	138.25±16.11	139.4±15.2	142.57±19.65	142.5±14.6	145.28±17.54	144.2±1.6
Blood sugar (mg/dl)	82.33±12.5	81.0±12.6	79.33±11.29	79.4±12.9	82.5±13.64	80.2±11.4	81.02±11.05	82.4±11.5
BUN (mg/dl)	19.67±6.87	18.9±5.9	19.32±5.14	19.2±4.8	18.96±4.29	18.7±4.1	18.57±3.58	19.6±3.9
Creatinine (mg/dl)	0.785±0.5	0.765±0.4	0.766±0.4	0.745±0.6	0.766±0.3	0.714±0.9	0.754±0.4	0.754±0.5
Cholesterol (mg/dl)	122.7±14.0	121.3±11.9	118.2±14.7	122.5±11.2	115.6±12.5	119.6±11.6	116.3±12.3	115.7±11.9
Triglycerides mg/dl)	74.5±9.65	75.8±8.54	77.5±8.55	76.1±7.8	78.5±5.21	75.6±7.5	74.5±8.47	72.1±6.9
HDL (mg/dl)	59.17±4.21	60.2±4.2	61.6±3.22	59.4±3.2	60.7±6.01	61.3±5.1	60.9±6.05	61.5±4.2
LDL I (mg/dl)	55±6.54	48.2±6.5	41.1±5.97	42.5±3.6	45.2±5.49	49.6±2.8	55.1±3.64	55.2±2.6
VLDL (mg/dl)	14.43±2.51	13.2±2.3	17.32±2.64	14.2±2.4	15.8±1.23	15.1±1.9	16.2±2.11	15.1±1.2

Values are mean ± S.D (*n*=6 per group). Control and treatment groups were compared statistically using one-way ANOVA followed by Dunnett's test.

Table 5: Serum MDA level as studied across the group C (Control); LD (Low dose); MD (Middle dose); HD (High dose).

Groups	Lipid peroxidation (µmoles MDA)	Lipid peroxidation (µmoles MDA)	Lipid peroxidation (µmoles MDA/ g liver)	Lipid peroxidation (µmoles MDA/ g liver)
	Male	Female	Male	Female
C	21.32 ±2.55	20.58 ±2.69	38.74 ±3.25	37.35±3.06
LD	24.15 ±5.64	22.98 ±5.61	39.22 ±2.62	39.41 ±2.11
MD	23.67 ±3.44	23.12 ±2.98	40.54 ±4.01	41.05 ±3.99
HD	32.47 ±2.99	32.05 ±2.92	52.16 ±3.62	50.49 ±3.12

Data are expressed as mean ± standard deviation (*n*=6).

CONCLUSION

The present Acute and sub-acute toxicity results suggest that LD_{50} of developed formulation>2000mg/kg. Further studies on long term toxicity and clinical trials may be rational to substantiate the study results.

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CONFLICT OF INTEREST

Authors disclose no conflicts of interest for publication of the manuscript.

ABBREVIATIONS

HM: Herbal Mixture; **OECD:** The Organisation for Economic Co-operation and Development; **LD_{50}:** Lethal Dose 50; **WHO:** World Health Organization; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **AOT:** Acute Oral Toxicity; **EDTA:** Ethylenediaminetetraacetic Acid; **TSC:** Tri-sodium citrate; **DLC:** differential leukocyte count; **HGB:** Haemoglobin; **RBC:** Red blood cell; **WBC:** White blood cell; **HCT:** Haematocrit; **ALP:** alkaline phosphatase; **AST:** aspartate transaminase; **ALT:** Alanine transaminase; **LDH:** Lactate dehydrogenase; **PCV:** Packed Cell Volume; **MCHC:** Mean cell Haemoglobin Concentration; **MCV:** Mean Red Cell Volume; **MCH:** Mean Cell Hemoglobin; **MPV:** Mean platelet volume; **HDL:** High-density lipoprotein; **LDL:** Low-density lipoprotein; **VLDL:** Very-Low-Density Lipoprotein; **TGL:** Triglycerides.

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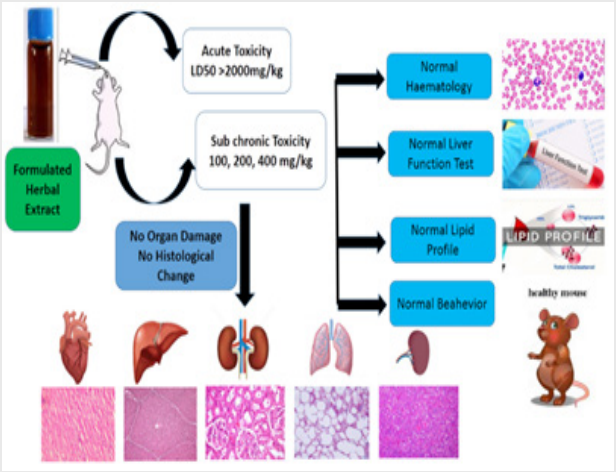
SUMMARY

- Herbal medicines are the most popular form of therapy for most of the world's population. A large number of populations in the developing countries still rely on herbal medicine practitioners to meet their primary healthcare needs. Toxicology constitutes an essential role in the development of herbal medicines. With the advancements of analytical techniques and molecular technology, coupling with the conventional test systems, the '-omic-' technology makes a significant contribution to the predictive and preclinical toxicology of herbal medicine.
- The purpose of this study was to evaluate and assess the potential acute and subacute toxicity (28-day) of Herbal Mixture (HM) administered orally to mice by single and repeated dosing, respectively; and to provide information to assist in selection of doses for future repeated-dose studies.
- Based on our results, we conclude that HM were found to be safe up to a dose of 2000 mg/kg. Hematological, biochemical and histopathological investigations clearly demonstrates that single oral administration upto 2000 mg/kg in acute toxicity study and daily oral administration of the HM for 28 days upto 400 mg/kg in sub-acute toxicity study caused no significant adverse changes in the organs like heart, lungs, liver, spleen and kidney.

PICTORIAL ABSTRACT



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Ameliorative efficacy of novel multi herbal formulation (AKSS16-LIV01) upon Haematological modulations induced by fixed dose combination of tramadol hydrochloride/paracetamol (THP)

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ABSTRACT

Background: Tramadol hydrochloride/paracetamol (THP) a fixed dose combination (FDC) is widely spread analgesic used to treat moderate to moderately severe pain. Over dose or chronic use of this fixed dose combination produce serious adverse effects. An acute Tramadol hydrochloride/paracetamol (THP) overdose can lead to a fatal liver damage.

Objectives: There is a worldwide need to develop a safe and symptomatic medication which controls the different medical complications.

Materials and Methods: Healthy adult swiss albino mice were assigned to four groups of six mice each according to their weights. Group-I serve as control, Group-II received Multi herbal formulation (AKSS16 LIV01) 400 mg/kg/day, Group-III received Tramadol hydrochloride/paracetamol (THP) 1.68 g / 300ml water and Group-IV received THP along with AKSS16-LIV01 (400 mg/kg). Blood samples were collected from the retro orbital plexus of each animal to determine various blood parameters and liver transaminase. **Results:** Administration of THP showed decline body weight, food consumption and water intake in mice whereas treatment with Multi herbal formulation (AKSS16-LIV01) normalized the same as compared with untreated animals. Treatment with THP (Group-III) decline the packed cell volume (PCV), haempglobin (Hb), means cell volume (MCV), means cell hemoglobin (MCH) and greater the white blood cell (WBC) compared with control. Pre-treatment with AKSS16-LIV01 significantly ($p<0.001$) increased the PCV, Hb, MCH, MCH and decreased WBC count in experimental animals. On the other hand elevated liver transaminase enzymes i.e. AST and ALP by THP was restored with administration of Multi herbal formulation (AKSS16-LIV01).

Conclusion: Chronic administration of THP indicated adverse effects on haematologic parameters upon experimental animals. Simultaneous administration with newly developed multi herbal formulation (AKSS16-LIV01) was ameliorate these adverse effects and may be potent drug In the future which controls the blood related medical complications against the toxicants.

Keywords: Tramadol hydrochloride/paracetamol; Fixed dose combination; Multi herbal formulation; Hematologic parameters; Liver transaminase; Swiss albino mice

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INTRODUCTION

Tramadol hydrochloride/paracetamol is a fixed dose combination (FDC) used to treat moderate to moderately severe pain. This fixed dose combination (FDC) contains 37.5 mg of tramadol hydrochloride and 325 mg of paracetamol [1]. Immediate release (IR) formulation orally relief pain within an hour. Tramadol has a central acting mechanism via serotonin receptors and acts by binding μ-opioid receptors and neurons, and it is also a serotonin–norepinephrine reuptake inhibitor (SNRI) [2]. Over dose and chronic consumption of this combination produce constipation, itchiness and nausea [3]. Some times more serious adverse

effects like insomnia, drug dependency and a high risk of serotonin syndrome may occur [4].

Intake of low dose of tramadol hydrochloride/paracetamol can acts as an effective analgesic but at high dosage and over a prolonged period the combination may cause various complications and disrupt body's homeostasis [5, 6]. Recent study showed that application of tramadol hydrochloride/paracetamol (THP) alters normal value of the various haematological parameters in animals [7]. Apart from this prolonged or chronic administration of THP may cause severe thrombocytopenia, leading to failure of the immune system, anemia and a very low erythrocyte count [8, 9].

Multi herbal formulations mean a dosage form consisting of one or more herbs or processed herbs in specified quantities which have potent therapeutic efficacy without adverse effects [10, 11]. Scientific study revealed that this plant based formulation is very effective to cure anaemia and control the blood [12]. Here we developed a multi herbal formulation (AKSS16-LIV01) based on six Indian medicinal plants and three Indian spices. Our previous study established that the formulation is completely safe in various does upon experimental animals [13]. With view of the above, there is need to developed and safe and symptomatic medication that controls all haematological parameters in the body when system exposed with fixed dose combination.

MATERIALS AND METHODS

Chemicals

Tramadol hydrochloride and paracetamol were obtained from Dey's Medical Stores (Mfg.) Ltd., Kolkata as a gift sample. Ethanol, sodium chloride, sodium hydroxide and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. ALT and AST were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

Preparation of plant extract

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. Plants parts were cleaned and dry with normal temperature. The dried plant parts were used for preparation of multi herbal formulation as per standard validated protocol [14]. The plants and plant parts used in preparation of the extract are listed in Table 1.

Table 1: Details of ingredient(s) present in the newly developed multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Quantity used in extract
Indian Medicinal Herbs			
1.	*Tinospora cordifolia*	Guduchi	20 mg
2.	*Terminalia chebula*	Haritaki	20 mg
3.	*Azadirachta indica*	Neem	50 mg
4.	*Andrographis paniculata*	Kalmegh	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	20 mg
Indian Medicinal Spices			
7.	*Trigonella foenum-graecum*	Methi	10 mg
8.	*Piper nigrum*	Black pepper	10 mg
9.	*Elettaria cardamomum*	Cardamom	10 mg

* Amount required for preparation of 5 ml extract.

Animals

Twenty four young, healthy Swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received Multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received Tramadol hydrochloride/paracetamol (THP) daily at dosage of 1.68 g/300 ml of water and Group-IV received THP (1.68 g/300 ml of water) along with AKSS16-LIV01 (400 mg/kg).

Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge

tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

Hematological Parameters

For hematological studies, the blood was collected in heparinized tubes. Blood-cell count was done using blood smears in Sysmax-K1000 Cell Counter. Parameters studied were hemoglobin, total red blood cell, reticulocyte, hematocrit, packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, total white blood cell and differential count.

Determination of biochemical parameters

Liver function enzymes such as AST and ALT were used as biochemical markers for hepatotoxicity and assayed by the standard protocol.

Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Statistical significance was acceptable to a level of $p< 0.05$.

RESULTS

Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in table 2. Administration of Tramadol hydrochloride/paracetamol (THP) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity as compared with Tramadol hydrochloride/paracetamol (THP) treated animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Table-2: Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	Groups			
	Control	AKSS16-LIV01	THP	THP + AKSS16-LIV01
Body weight (g) Initial	25.16±2.57	25.84±2.44	26.01±5.21	25.11±4.85
Body weight (g) Final	36.98±2.55	36.91±2.69	21.22±2.61#	36.67±1.47*
Body weight (g) gain or loss	11.82±0.06	11.07±0.04	4.79±0.006	11.56±0.03
Food consumption (g)	4.81±0.05	4.39±0.07	2.97±0.05#	5.18±0.04*
Water intake (ml)	4.18±0.04	4.27±0.04	2.98±0.02#	4.55±0.04*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from (THP) group values at $p<0.001$

Effect of multi herbal formulation (AKSS16-LIV01) on Haematological parameters

Haematological parameters of control and experimental groups are shown in table 3 and figure 1 to 5. Four weeks treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day did not showed significant differences in PCV, haemoglobin (Hb), WBC, RBC, mean corpuscular haemoglobin concentration (MCHC), mean cell volume (MCV), and mean cell hemoglobin (MCH) compared with the control. Significant reduction in Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p < 0.001$), and MCH ($p <0.001$) was noticed in THP intoxicated mice when compared with the Control (Figure 1-4). The WBC count (Figure 5) was significantly ($p < 0.001$) greater in Group C compared with the control. In contrast, no significant differences were observed in RBC and MCHC between the control and Group C. Administration of multi herbal formulation (AKSS16-LIV01) along with THP significantly increased Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p < 0.001$), and MCH ($p < 0.001$) when compared with the THP intoxicated animals. On the other hand WBC count was significantly reduced in Group D THP + AKSS16-LIV01 intoxicated animals. Others haematological parameters (table 3) like Read Blood corpuscle (RBC); Reticulocyte (RT); Haematocrit (HCT); Mean corpuscular haemoglobin concentration (MCHC) did not show any significant changes upon all the experimental groups.

Table-3: Effect of multi herbal formulation (AKSS16-LIV01) on haematological Parameters

Parameters	Groups			
	Control	AKSS16-LIV01	THP	THP + AKSS16-LIV01
RBC ($x10^6$ μL^{-1})	10.8±4.1	10.2±5.3	10.1±4.2	10.6±5.1
RT (%)	2.8±1.1	2.4±1.6	2.8±2.4	2.9±1.6
HCT (%)	34.8±1.3	32.8±2.1	32.8±2.1	35.1±3.1
MCHC (%)	41.4±7.6	41.7±2.4	40.4±1.4	41.4±1.4
Platelets ($x10^3$ μL^{-1})	6.6±2.0	6.9±1.2	6.3±1.2	6.5±2.6

Data are expressed as mean ± standard deviation (N=6)

RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCHC: Mean corpuscular haemoglobin concentration;

Effect of multi herbal formulation (AKSS16-LIV01) on Biochemical parameters

Table 4 shows the mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in control and experimental groups of mice. Data indicate that THP intoxicated mice had significantly greater mean AST and ALT compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day normalized the elevated AST and ALT levels when compared with THP treated mice. Four weeks treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in AST and ALT when compared with control group.

Table-4: Effect of multi herbal formulation (AKSS16-LIV01) on serum biochemical parameters

Groups	AST (Unit/L)	ALT (Unit/L)
Control	55.28±6.34	26.82±4.11
AKSS16-LIV01	54.91±5.81	27.54±4.62
THP	106.28±8.17#	67.59±6.01#
THP + AKSS16-LIV01	62.99±5.44*	31.83±5.18*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at $p<0.001$ and *Significantly different from (THP) group values at $p<0.001$

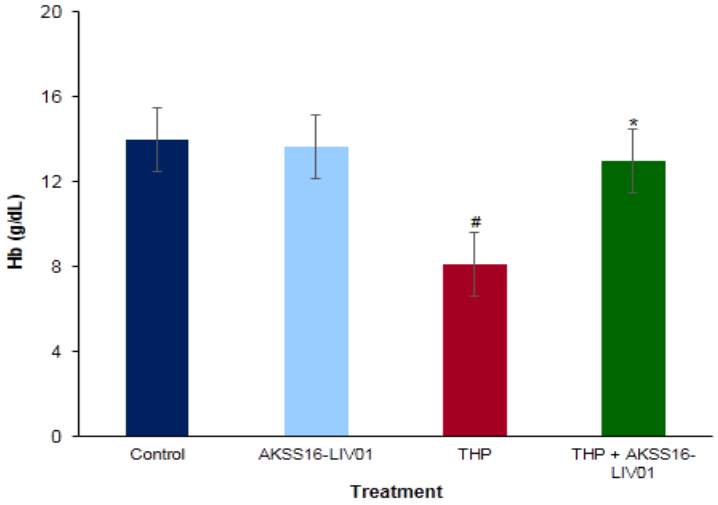

Figure 1: Effect of multi herbal formulation (AKSS16-LIV01) on haemoglobin (Hb) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (THP) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

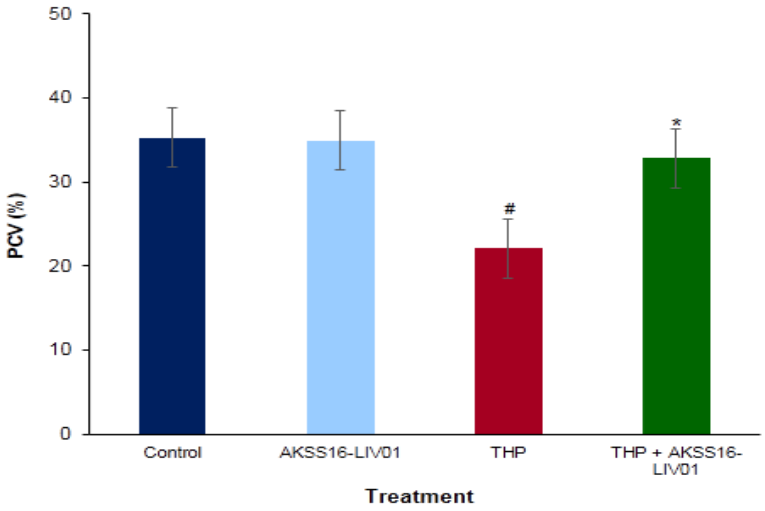

Figure 2: Effect of multi herbal formulation (AKSS16-LIV01) on packed cell volume (PCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at $p<0.001$ and *significantly different from (THP) group values at $p<0.001$. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

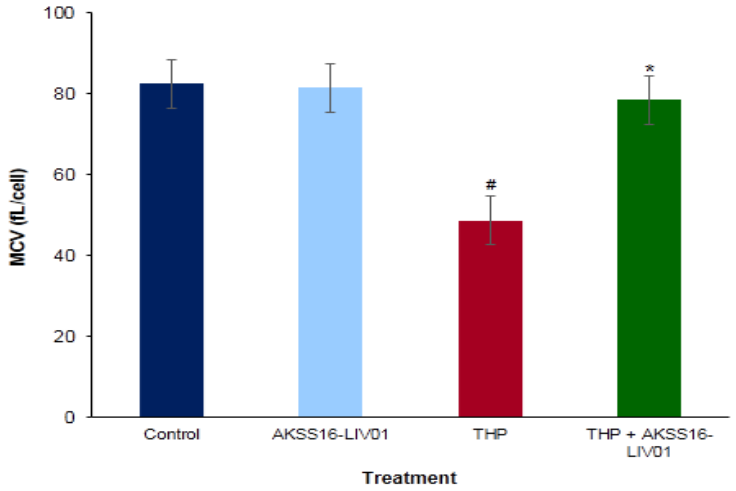

Figure 3: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell volume (MCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

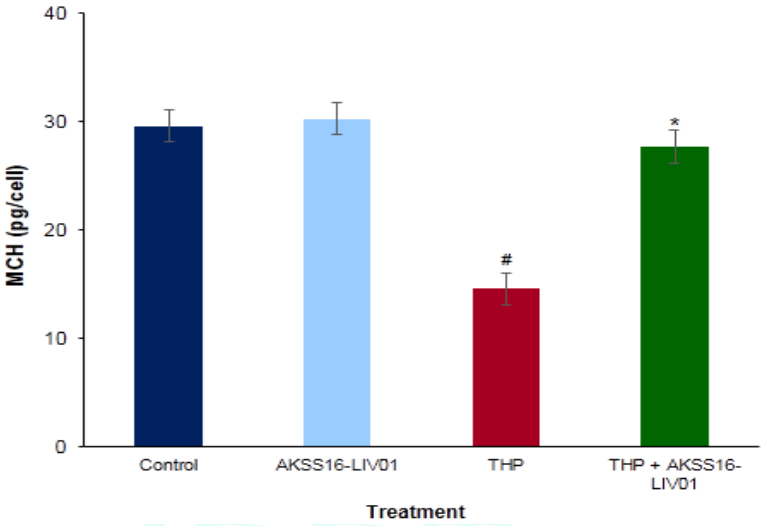

Figure 4: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell hemoglobin (MCH) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

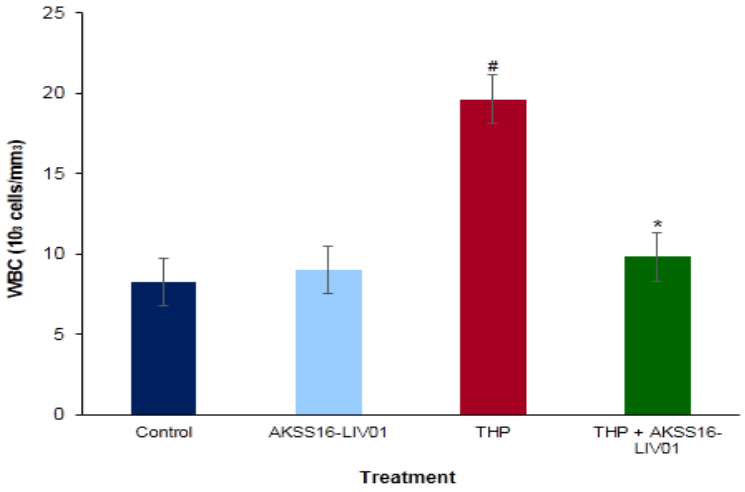

Figure 5: Effect of multi herbal formulation (AKSS16-LIV01) on white blood cell (WBC) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (THP) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

DISCUSSION

Analgesics as fixed dose combination are very useful for fast pain relief. Tramadol hydrochloride/paracetamol (THP) is a fixed dose combination consists of two analgesics tramadol and paracetamol used for treats moderate to severe pain [15]. It is well established that overdose or chronic use of analgesics specially fixed dosses form developed mild to severe adverse effects and sometimes damage various organs like liver, kidney and brain [16]. In very recent study confirm that administration of THP upon animal model severely disturbed hematological and biochemical parameters [17]. To prevent these deleterious effects we simultaneously administered our newly developed multi herbal formulation (AKSS1-LIV01) in mice. It is reported that treated with THP at a dose of 1.68 g/300 ml of water on mice reduced the haemoglobin (Hb), packed cell volume (PCV), and mean corpuscular volume (MCV) values. Another report depict that lower haemoglobin (Hb) value leads to iron deficiency anaemia which is characterized by a microcytic hypochromic blood picture. In the present study our result also confirm that administration of THP (1.68 g/300 ml of water) decline Hb, PCV, MCH and MCV values could be attributed to disturbed hematopoiesis, destruction of erythrocytes. The low PCV and Hb concentration and the abnormally low values of MCV and MCH are indications of microcytic anaemia. Medicinal plants enrich with various compounds capable to control and maintained the various blood parameters. Pre-treatment with newly developed multi herbal formulation (AKSS16-LIV01) along with THP elevate Hb, PCV, MCH and MCV values may indirectly protect the body from the anaemia.

Elevated aspartate transaminase (AST) and alanine transaminase (ALT) levels are strong indicators of inflammatory conditions and injury to the liver, while increased white blood cells (WBC) level is generally recognized as an inflammatory response [18, 19]. Inflammatory conditions may induce malnutrition in the body[20]. It is reported that inflammatory conditions can interfere with the body's ability to use stored iron and absorb iron from the diet[21]. Our result clearly showed that treatment with THP abruptly increased serum aspartate transaminase (AST) and alanine transaminase (ALT) levels as well as elevate white blood cells (WBC) count indicate THP produce inflammatory response and affects liver cell, disturbed homeostasis. On the other hand administration with newly developed multi herbal formulation (AKSS16-LIV01) along with THP decline the AST, ALT value and WBC count protect the liver against THP induced inflammation. Thus our developed multi herbal formulation composed with six medicinal plants and three medicinal spices may be able to protect haematological disturbance caused by THP.

CONCLUSION

This investigation shows that multi herbal formulation (AKSS16-LIV01) has the ability to protect the haematopoietic cells from the damaging effects of exposure to Tramadol hydrochloride/paracetamol (THP) and this protection might be attributed to the anti-oxidative power of multi herbal formulation (AKSS16-LIV01). Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of haematological dysfunction.

Acknowledgement

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Authors' Contribution

Soumendra Darbar and Atiskumar Chattapadhyay conceived and designed the experiment. Soumendra Darbar and Srimoyee Saha conducted the animal and biochemical experiments. Soumendra Darbar , Atiskumar Chattapadhyay and Kaushikisankar Pramanik wrote and revised the manuscript.

Conflicts of Interest

All authors report no conflicts of interest regarding this manuscript.

Authors' Funding

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ORIGINAL ARTICLE **OPEN ACCESS**

Therapeutic Application of Novel Multi Herbal Formulation (AKSS16LIV01) against the inductive influence of Carbon Tetrachloride (CCl_4) upon Tissue and serum protein in Experimental animals

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ABSTRACT
Serum proteins are an important indicator of the nutritional status. Disturbance in normal protein levels indicates vital organs dysfunctions which may be fatal for life. The main aim of the study was to determine the serum and tissue protein levels against CCl_4 intoxication and its mitigation by newly developed novel herbal medicine (AKSS16-LIV-01). Healthy adult swiss albino mice were assigned to four groups of six mice each according to their weights. Group-I serve as control, Group-II received Multi herbal formulation(AKSS16-LIV01)400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4(1 ml/kg-bw) along withAKSS16-LIV01 (400 mg/kg).Administration of carbon tetrachloride (CCl_4) showed decline body weight, food consumption and water intake in mice whereas treatment with Multi herbal formulation(AKSS16-LIV01)normalized the same as compared with untreated animals. Treatment with CCl_4 (Group-III) decline the total protein, albumin and globulin levels in serum, liver and kidney compared with control. Pre-treatment with AKSS16-LIV01 significantly ($p<0.001$) increased thetotal protein, albumin and globulin levels in serum, liver and kidney compared CCl_4 intoxicated animals. On the other hand higher level of albumin/globulin ratio clearly indicate that liver and kidney might be affected by CCl_4 treatment. Multi herbal formulation (AKSS16-LIV01) protect the liver and kidney by maintaining the albumin/globulin ratio. So, in conclusion it may be predicted that phyto constituents and antioxidant enrich this novel formulation maintain normal protein pattern and protects the body from various dysfunctions.
Keywords:*Multi herbal formulation;serum protein; albumin; globulin; Swiss albino mice*

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INTRODUCTION

The body needs *protein* to function and survive. Alteration of serum total protein leads to various complications and sometimes damage vital organs [1]. The major components of the serum protein are albumin and globulin, represents the nutritional status of the body which maintain the colloidal osmotic pressure in blood [2-4]. These two proteins also maintain body's immune function, prevent infection [5]. Scientific literature revelled that albumin-to-globulinratio (AGR) is a prognostic factor of various diseases and medical complications [6,7]. Clinical study indicate that decline albumin levels showed poor nutrition status, sometimes very fatal to survive [8,9].

Carbon tetrachloride (CCl_4) is a major industrial pollutant associated with production of free radicals which creates various organ dysfunction like liver and kidney [10]. It is established that metabolic activation ofCCl_4 by cytochrome P450 produced trichloromethyl radical ($\cdot CCl_3$) and peroxytrichloromethylradical ($\cdot OOCCl_3$) which initiates lipid peroxidation, responsible for membrane disruption leads to liver and kidney injury[11]. Long-time exposure of CCl_4 alter the normal protein level in the body which creates various type of organ dysfunctions [12]. Animal study showed that

Administration of CCl_4 decrease normal food and water intake, produce nutrition deficiency syndrome[13].

Long term safe and symptomatic medication without side effects is one of the main approach ofalternative system of medicine comprising herbal products [14] . The plant based formulation is enrich With various essential phytochemicals and enormous antioxidants, serrates to prevent diseases With view this concept, we developed a novel, low cost herbal formulation composed of six Indian medicinal plants and three Indian spices. Our previous study upon animals showed that this formulation does not produce any toxic effects upon animals and safe for therapeutic medication[17,18]toHere we try apply this traditional medicine for maintaining the essential protein levels caused by CCl_4.

MATERIAL AND METHODS

Chemicals

Carbon tetrachloride (CCl_4) and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. total protein, albumin and globulin were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

Preparation of plant extract

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. Plants parts were cleaned and dry with normal temperature. The dried plant parts were used for preparation of multi herbal formulation as per standard validated protocol[19]. The plants and plant parts used in preparation of the extract are listed in Table 1.

Animals

Twenty four young, healthy swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ºC and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II received Multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg).

Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinkingbottle from the initial water given to the animals.

Blood Collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. after centrifugation clear light yellow colour serum was separated and used for further analysis.

Preparation of tissue homogenate

A small portion of the liver and kidney tissues was homogenized in ice-cold 0.9% w/v saline using a homogenizer to obtain 20% homogenate. Aliquots of the liver homogenate were stored at 4^0C prior to biochemical analysis.

Determination of serum, liver and kidney protein

Serum and tissue homogenate were used for the determination of total protein, albumin and globulin. Total protein, albumin and globulin were determined according to the standard biochemical protocol with slight modification using colorimetric kit obtained from Thermo Scientific, USA.

Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

RESULTS

Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in table 2. Administration of carbon tetrachloride (CCl_4) (1 ml/kg-bw) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity as compared with control animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney total protein

Figure 1 shows the mean serum, liver and kidney total protein (TP) levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney total protein compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day significantly increased the decline total protein levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows any significant differences in serum, liver and kidney protein levels when compared with control group.

Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin

Figure 2 shows the mean serum, liver and kidney albumin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney albumin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16- LIV01) at a dose of 400 mg/kg/day significantly increased the decline albumin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16- LIV01) at a dose of 400 mg/kg/day alone did not shows any significant differences in serum, liver and kidneyalbumin levels when compared with control group.

Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney globulin

Figure 3 shows the mean serum, liver and kidney globulin levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly lower mean serum liver and kidney globulin compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16- LIV01) at a dose of 400 mg/kg/day significantly increased the decline globulin levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16- LIV01) at a dose of 400 mg/kg/day alone did not shows any significant differences in serum, liver and kidney globulin levels when compared with control group.

Effect of multi herbal formulation (AKSS16-LIV01) on serum, liver and kidney albumin/globulin (AGR) ratio

Table 3 shows the mean serum, liver and kidney albumin/globulin ratio in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly higher mean serum liver and kidney albumin/globulin ratio (AGR) compared with the control ($p<0.001$). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day normalized the decline albumin/globulin ratio (AGR) levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in serum, liver and kidney albumin/globulin ratio (AGR) levels when compared with control group.

Table 1 Details ofingredient(s) present in the newly developed multi herbal formulation

Sl. No.	Botanical Name	Common Name	Family	Quantity used in extract
1.	*Tinosporacordifolia*	Guduchi	Menispermaceae	20 mg
2.	*Terminaliachebula*	Haritaki	Combretaceae	20 mg
3.	*Azadirachtaindica*	Neem	Meliaceae	50 mg
4.	*Andrographispaniculata*	Kalmegh	Acanthaceae	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	20 mg
7.	*Trigonellafoenum-graecum*	Methi	Fabaceae	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	10 mg
9.	*Elettariacardamomum*		Zingiberaceae	10 mg

* Amount required for preparation of 5 ml extract.

Table-2: Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake

Parameters	Mice			
	Group-I	Group-II	Group-III	Group-IV
Body weight (g) Initial	26.35±1.91	26.51±2.35	26.71±4.2	26.68±5.1
Body weight (g) Final	37.84±2.03	36.94±1.69	21.81±2.41#	36.97±1.67*
Body weight (g) gain or loss	11.49±0.06	10.43±0.04	4.90±0.006	10.29±0.03
Food consumption (g)	4.52±0.05	4.37±0.07	2.94±0.06#	5.11±0.04*
Water intake (ml)	4.01±0.04	4.25±0.04	3.01±0.02#	4.31±0.06*

All data were expressed as means± SE (n=6/group). Data comparison was performed using two way ANOVA followed by Tukey's Multiple Comparison Test. #Significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001

Table 3: Effect of AKSS16-LIV01 on serum, liver and kidney albumin/globulin (AGR) in CCl_4 induced toxicity

Groups	Albumin/Globulin ratio		
	Serum	Liver	Kidney
Control	1.32±0.12	1.45±0.14	1.48±0.11
AKSS16-LIV01	1.31±0.11	1.37±0.16	1.41±0.12
CCl_4	1.76±0.16	1.58±0.14	1.57±0.15
CCl_4 + AKSS16-LIV01	1.26±0.13	1.43±0.19	1.50±0.18

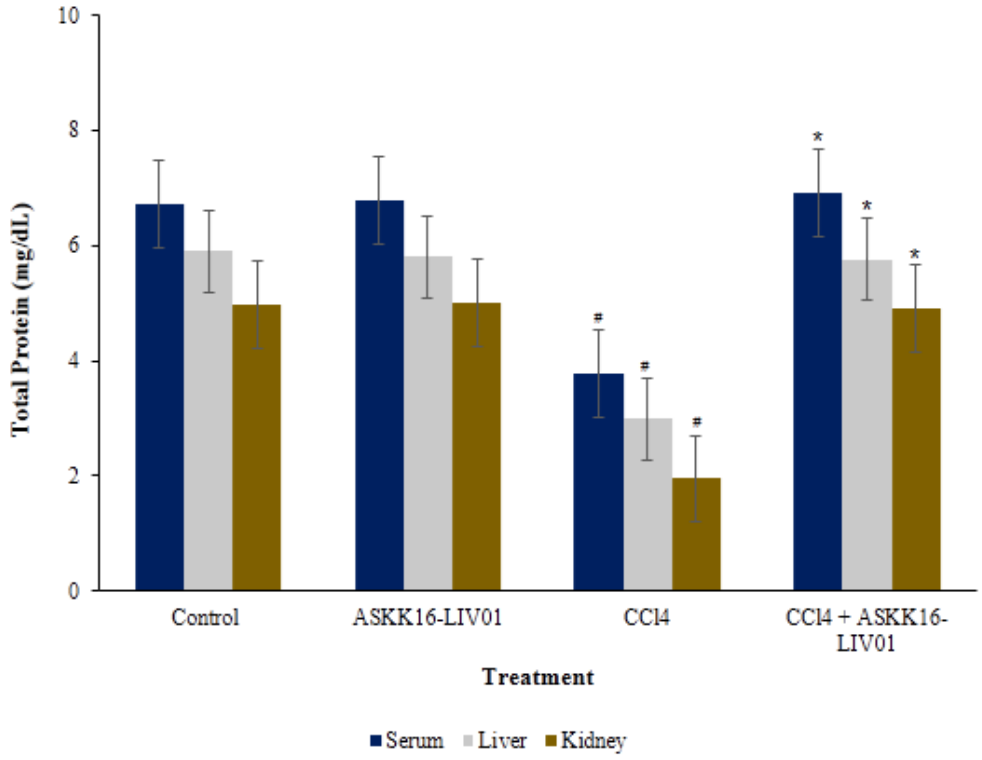

Figure 1: Effect of multi herbal formulation (AKSS16-LIV01) on Total protein levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

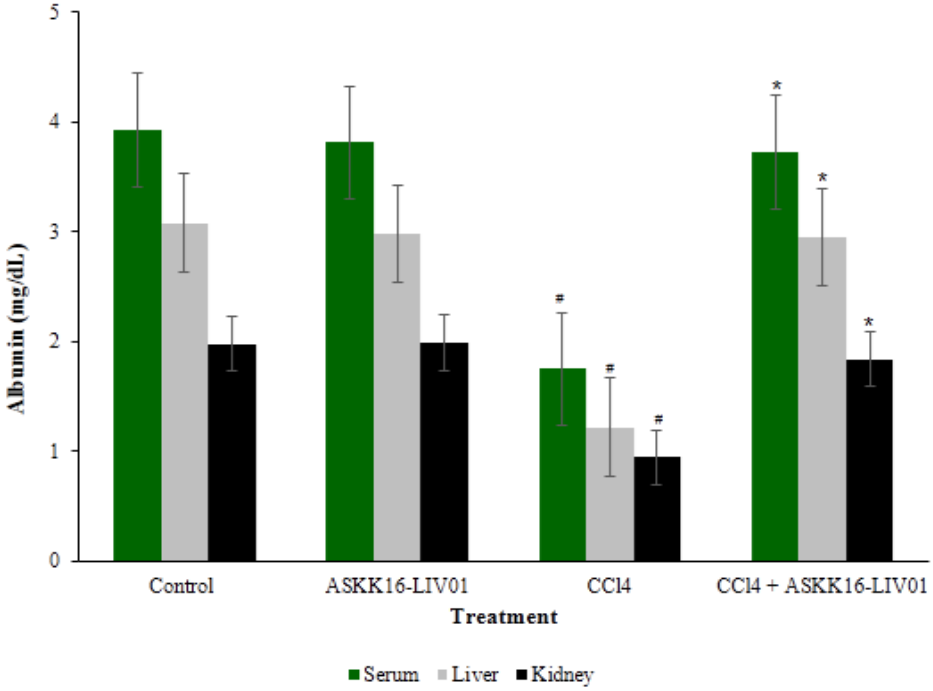

Figure 2: Effect of multi herbal formulation (AKSS16-LIV01) on albumin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

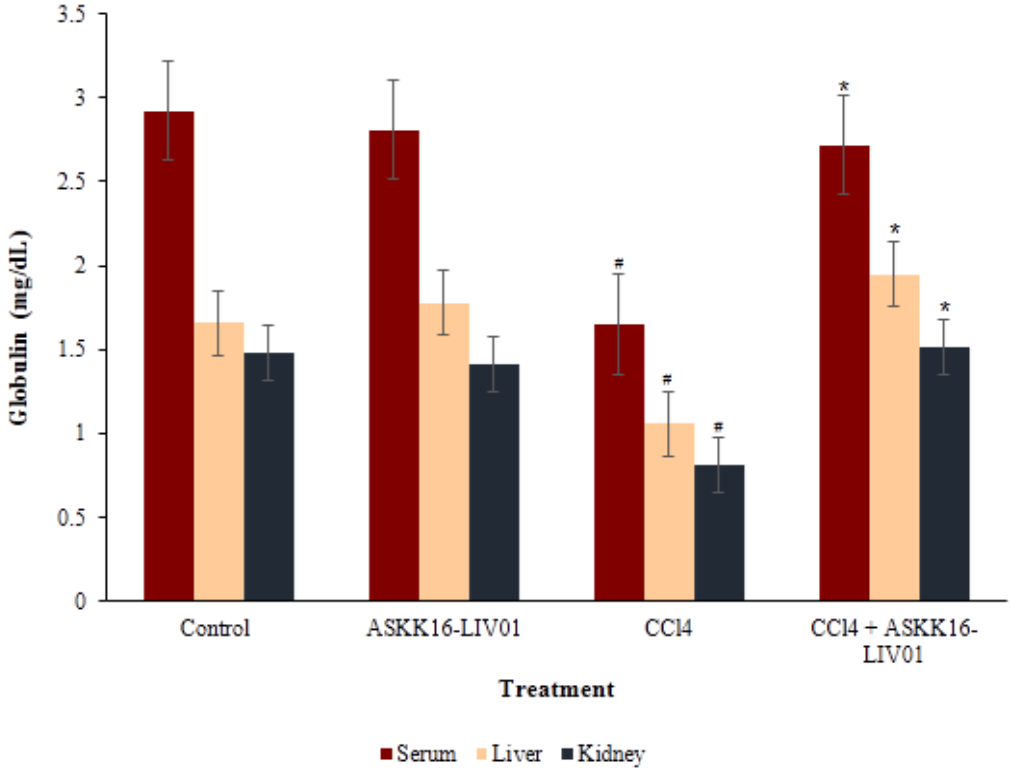

Figure 3: Effect of multi herbal formulation (AKSS16-LIV01) on globulin levels in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

DISCUSSION

Various secondary metabolites of the medicinal plants are mainly responsible for therapeutic effects[21]. Poly herbal drug are very useful for treatment of various diseases due to the synergistic effects of different plants [22]. Phenolic compounds and flavonoids present in the aromatic plants are mainly responsible for pharmacological functions and prevent oxidative stress [23]. Our study showed that administration of carbon tetra chloride (CCl_4) inhibit normal body growth, food consumption and water intake. Co administration of our developed formulation retained the body weight, food consumption and water intake. Protein is responsible for normal body growth and development. Abnormal protein level inhibit the body growth which may be occur when subject exposed with environmental toxin [24,25]. Total serum protein is an indicator in liver and kidney damage [26]. In the present study we observed that carbon tetra chloride (CCl_4) significantly decreased the serum, liver and kidney protein levels. Co administration with AKSS16-LIV01 maintained the normal serum, liver and kidney protein levels.

Albumin play a crucial role to maintain physiological activities of human body [27,28]. It is one of the liver biomarker as it generates from the liver cells. Low level of albumin is responsible for poor nutrition [29-32]. In this study we observed that chronic administration of CCl_4 decline normal albumin levels in serum, liver and kidney which was recovered when animals pre-treated with novel multi herbal formulation (AKSS16-LIV01). The result clearly indicate that AKSS16-LIV01 capable to maintain the normal albumin level against the environmental toxicant like CCl_4. On the other hand scientific study revealed that serum globulin is involved in chronic inflammation. Recent study showed that carbon tetra chloride (CCl_4) alter the serum, liver and kidney globulin and disrupt normal homeostasis. Our study also confirm that application of CCl_4 decreased normal globulin levels in serum, liver and kidney. Treatment with the developed formulation (AKSS16-LIV01) normalized the globulin level in experimental animals. Albumin/globulin ratio also confirm the protein alteration.

CONCLUSION

Chronic administration of carbon tetrachloride (CCl_4) suppressed the normal body growth and reduced normal food and water intake capacity in mice. This environmental toxin reduced the total protein, albumin and globulin levels both in serum and tissues. Our developed novel multi herbal formulation might be able to maintain the normal essential protein values and prevent the CCl_4 induced deleterious effects in mice. Thus, we believe that the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future.

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CONFLICT OF INTEREST

All authors report no conflicts of interest regarding this manuscript.

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CITATION OF THIS ARTICLE

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In vitro phytochemical constituents, antimicrobial and antifungal activity of a low cost novel multi herbal formulation (AKSS16-LIV01)

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Abstract- In the present study a multi herbal formulation was developed combining six Indian medicinal herbs and three. The low cost formulation has great commercial value to combat various diseases and organ related medical complications. Plant secondary metabolites in the developed multi-herbal formulation (MHF) (AKSS16-LIV01) were detected through quantitative analysis. Anti-bacterial and anti-fungal activity of the AKSS16-LIV01 was also determined. The phytochemicals like triterpenes, flavonoids, saponins, and tannins were abundantly found in the AKSS16-LIV01 extract. Aqueous extract of the formulation showed optimum in vitro DPPH, hydroxyl, nitric oxide (NO) and superoxide free radical scavenging activity. Inhibitory action of AKSS16-LIV01 was dose dependent and significant anti-bacterial activity was seen in the case of *Escherichia coli* followed by *Streptococcus aureus* and *Klebsiella pneumonia* respectively. The AKSS16-LIV01 also showed antifungal activity, the activity was maximum in case of *C. albicans* and was least for *A.niger.* Conclusion: The multi-herbal formulation AKSS16-LIV01 showed promising results *in-vitro* studies and further studies are required to explore its efficacy in animals and humans. This low-cost formulation can improve health as well as economy of the developing countries.

Keywords – Multi herbal formulation; Phytoconstituents; Antimicrobial activity; Antifungal activity; Plant metabolites

I. INTRODUCTION

Therapy by herbal medicine extensively used from the ancient age and have less adverse effects from compared with synthetic medicines [1]. As per world health organization's (WHO) report 25% of the crude modern drugs used in last decade are derived from plants, out of which approximately 5-15% have been explored for bioactive compounds [2]. It is noted that last few decades use of multi-herbal remedies extensively increase due to their therapeutic effects upon various diseases. In the traditional herbal medical system India is the pioneer country from the ancient time. From the time of Charaka and Susutra it is proved that this medicinal plants used prevent various life threatening human diseases [3]. Alkaloids, flavonoids, tannins, polyphenols, terpenoids, resins, steroids etc. are the major key phytochemicals found in medicinal plants responsible for preventing the various chronic diseases without any adverse side effects. Theses phytochemicals have various antimicrobial and antifungal properties. Bacteria including, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Proteus vulgaris* and *Bacillus subtilis,* were mostly responsible for infections [4,5] . These phytochemicals have capable to prevent various infectious diseases as either suppression of bacterial growth destroy the bacterial membranous lipids [6].

Now a day's approximately 85.5% people throughout the globe depend upon herbal drugs for the medications of lots of infectious & non infectious diseases, wound healing activity, inflammations, etc. Mostly 95% medicinal plants those are used in ayurvedic medicines are less toxic, cost effective and produce optimum sanative effects in compared to allopathic modern medicine [7,8]. So, over the globe scientist and researchers are engaged to formulate some novel herbal medicine those are potent for various diseases.

We developed novel, low cost herbal formulation (AKSS16-LIV01) containing nine Indian medicinal plants out of which six were medicinal plants and three were medicinal spices. The present highlighted to screen the in-vitro phytochemicals analysis and antimicrobial and antifungal study of the developed formulation.

II. METHODOLOGY

2.1 Collection and Authentication of herbs –

All raw medicinal plants were collected from registered local herbal suppliers and authenticated by pharmacognosist. They were further identified by an expert taxonomist of the Jadavpur University, and kept as a voucher specimen. The identification was based on Ayurvedic parameters such as Varna (color), Gandha (odor), Ruchi (taste), Akriti (shape) and Parimana (size). The plants and plant parts used in the preparation of the extract are depicted in Table 1.

Table 1: Details ingredient(s) present in the newly developed multi herbal formulation (AKSS16-LIV01)

Sl. No.	Botanical Name	Common Name	Family	Quantity used in extract
1.	*Tinospora cordifolia*	Guduchi	Menispermaceae	20 mg
2.	*Terminalia chebula*	Haritaki	Combretaceae	20 mg
3.	*Azadirachta indica*	Neem	Meliaceae	50 mg
4.	*Andrographis paniculata*	Kalmegh	Acanthaceae	50 mg
5.	*Aloe barbadensis miller*	Aloe vera	Liliaceae	50 mg
6.	*Curcuma longa*	Curcuma, Haldi	Zingiberales	20 mg
7.	*Trigonella foenum-graecum*	Methi	Fabaceae	10 mg
8.	*Piper nigrum*	Black pepper	Piperaceae	10 mg
9.	*Elettaria cardamomum*	Cardamom	Zingiberaceae	10 mg

* Amount required for preparation of 5 ml extract.

2.2. Preparation of Extract –

Fresh parts of the medicinal plants were first air-dried after cleaning with double distilled water and kept in an oven at 80ºC for 10 min and 60ºC for 30 min and grounded by a blade mill to a fine powder. After that the polar fraction was extracted by modified method of Adhikari et al. (2018) [9].

2.3. Quality control analysis –

Wt. per ml, pH, homogeneity, total ash, LOD and bacterial limit tests were carried out according to the standard pharmaceutical protocol (IP-2014).

2.4. Phytohemical screening –

Various essential plant secondary metabolites such as sterols and triterpenes, Mg^{2+} turning test of flavonoids, alkaloids, saponins, glycosides, tannins, phenolic content, total flavonoids content in the developed multi-herbal formulation (AKSS16-LIV01) were detected through quantitative analysis with slight modification as described by Evans and Gueverra [10].

2.5. In vitro study –

2.5.1 DPPH activity–

Free radical scavenging activity of AKSS16-LIV01 was determined by using DPPH method.

$$\text{Percentage Inhibition} = \text{AbsControl-AbsTestAbsControl} \times 100\%$$

Effective 50% the concentration value that scavenged 50% of the DPPH radicals and antiradical power (ARP or AE) is the reciprocal of it (AE=1/EC50). Quercetin and ascorbic acid were used as reference standard.

2.5.2 Hydroxyl radical scavenging activity–

Hydroxyl radical scavenging activity was carried out according to the standard established method of Erica et. al. [12].. Intensity of the developed yellow color formed was measured at 412 nm.

2.5.3 Superoxide scavenging activity–
Superoxide scavenging activity was determined according to standard protocol with slight modification [13]. This activity measured the removal rate of xanthine/xanthine oxidase-generation from the substances.

2.5.4 Nitric oxide scavenging activity–
Established standard method was used for determination of Nitric oxide (NO) scavenging activity [14]. In brief at first we prepared 2.0 ml of 10 mM sodium nitroprusside in 0.1 M phosphate buffer, pH 7.4 and were taken in a conical flask. Then 0.15 ml plant extract of different concentration was added carefully. At room temperature the solution were incubate for two hours. The solution was placed for cooling and then gently add 5 ml Griess reagent. The absorbance of chromophore was measured at 546 nm.

2.5.5 Reducing power assay–
Reducing power activity of AKSS16-LIV01 was carried out by the method of Abdullahi with slight modification [15]. Reaction was initiated by adding 2.5 ml of the extract, 2.5 ml of phosphate buffer and 1% potassium ferricyanide followed by gentle shaking. It was then placed in a water bath for 20 min at 50°C for initiation to complete. The solution was cooled and 2.5 ml of 10% trichloroacetic acid (TCA)was added subsequently. It was then centrifuged at 3,000 rpm for 10 min..5 ml of distilled water was mixed with 5.0 ml fraction from the supernatant and 1ml of 1% ferric chloride was added to it and resulting solution was placed for incubation at room temperature for 10 min. The absorbance was noted at 700nm.

2.6 Antimicrobial activity of AKSS16-LIV01–
Antimicrobial activities of developed formulation AKSS16-LIV01 were determined according to the standard method with slight modification. Five bacterial pathogens namely *Staphylococcus pyogenes*, *Escherichia coli, Streptococcus aureus*, *Staphylococcus typhi*, and *Klebsiella pneumonia* were chosen for this study [16]. Tetracycline was used as a positive control. Nutrient broth was used for culturing the microbes and then placed for incubation at 37 ° C for 24hours and seeded in Mueller-Hinton sterile agar plates. The plates were left undisturbed for about 10 minutes to enhance the culture. A set of four dilutions (25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml) of the herbal extract AKSS16-LIV01 was used for measured the activity. The whole procedure was repeated for three times for obtaining the precise result.

2.7 Antifungal activity of AKSS16-LIV01–
To determine the in-vitro antifungal activity of AKSS16-LIV01 we used three fungal pathogens namely A. niger, Aspergillus oryzae, and Candida albicans [17]. The plates were prepared with Potato dextrose agar (PDA) media and inoculated carefully with the fungal pathogens after the solidification of PDA. Five wells of size 5 mm were cut out on the agar plates. A set of four dilutions such as 25 µg/ml, 50 µg/ml , 75 µg/ml and 100 µg/ml of the newly prepared plant extract AKSS16-LIV01 and antifungal agent (positive control) ketoconazole (20 mg/ml) was introduced in well. At room temperature, the plates were placed for 3 to 4 days incubation. After 3 days, the zone of inhibition obtained was measured.
.

III. RESULTS

The composition of the polyherbal preparation AKSS16-LIV01 is given in Table 1. We have extracted the plant materials in seven well-known solvents and evaluated various properties including extraction efficiency, the quantity of various phytochemicals extracted and antioxidant activity to choose the best one. The highest yield was obtained with AQE while with EA the yield was the lowest.

3.1 Quality control analysis–
The developed multi herbal formulation (AKSS16-LIV01) showed a clear brown color liquid extract with a characteristic odour (Table 2). Results depict that its Wt. per ml is 1.189 g and pH 6.88. The developed extract is uniform in nature. Total ash content is <5% w/w and LOD is 46 within the IP limit. 243 cfu/ml found in the bacterial Limit Test showed less bioburden and less pathological load. This formulation complies with the entire relevant quality control test as per Indian Pharmacopoeia limit.

Table 2: Routine Quality Control analysis of multi herbal formulation (AKSS16-LIV01)

Sl. No.	Test	Results
1.	Description	A brown colour liquid
2.	Wt. per ml	1.189 g
3.	pH	6.88
4.	Order	Characteristic
5.	Homogeneity	Uniform
6.	Total ash	<5% w/w
7.	LOD	46
8.	Bacterial Limit Test	243 cfu/ml

3.2 Phytochemical constituents–

From the qualitative analysis (Table 3) of the plant secondary metabolites of AKSS16-LIV01 multi extract, it is observed that sterols are present in trace amounts, alkaloids and glycosides are in moderate amount, but triterpenes, flavonoids, saponins, and tannins are abundantly available. In the dichloromethane (DM) extract, sterols were abundant flavonoids, alkaloids, and glycosides were moderately present and saponins were detected in trace amount. in the ethyl alcohol (EA) extract triterpenes were not detected. In the aqueous extract (AQ) all the above phytochemical constituents were detected abundantly. We can conclude that more polar secondary metabolites were extracted with the solvents used compared to non-polar metabolites.

Table 3: Qualitative analysis of the phytochemical constituents of multi herbal formulation (AKSS16-LIV01)

Phytochemicals	DM	EA	AQ	ET	ME	AQM	AQE
Alkaloids	(++)	(+)	(++)	(+)	(+)	(+)	(+)
Flavonoids	(++)	(++)	(+++)	(++)	(++)	(+)	(+)
Glycosides	(+)	(-)	(++)	(++)	(++)	(-)	(-)
Tannins	(+)	(+)	(+++)	(+)	(+)	(+)	(+)
Triterpenes	(++)	(-)	(+++)	(-)	(+)	(-)	(-)
Sterols	(+)	(+)	(++)	(++)	(+)	(+)	(+)
Saponins	(+)	(++)	(++)	(+)	(-)	(+)	(+)

(+) = traces, (++) = moderate, (+++) = sufficient, (-) = absence of various constituents

DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

3.3Total polyphenol and flavonoids content –

Flavonoids and total phenolic compounds of the various solvent extract were measured spectrophotometrically. The concentrations of total phenolic were higher compared to that of total flavonoids. Table 4 represents the flavonoids and total phenolic content of the various solvent extracts of AKSS16-LIV01. The flavonoid and total phenolic content of the five different solvent extract of AKSS16-LIV01 were significantly ($P<0.05$) varied from each other's. The total phenolic content in EA (2.29±0.41) gGAE/100g of raw material and in AQ was highest (8.59±0.45) gGAE/100g of raw material. The total flavonoids content was lowest in EA (0.45±0.03) gQE/100g of raw material but highest in AQ (1.48±0.05) gQE/100g of raw material.

Table 4: Flavonoids and total polyphenol content of multi herbal formulation (AKSS16-LIV01)

Fraction	Total Polyphenols (gGAE/100g raw material)	Total flavonoids (gQE/100g raw material)
DM	2.42 ± 0.73^{a}	0.84 ± 0.04^{b}
EA	2.32 ± 0.37^{a}	0.48 ± 0.02^{a}
AQ	8.64 ± 0.47^{d}	1.21 ± 0.03^{d}
ET	4.51 ± 0.51^{b}	1.52 ± 0.06^{e}
ME	5.70 ± 0.53^{c}	1.10 ± 0.04^{c}
AQM	4.11 ± 0.44^{c}	0.91 ± 0.05^{b}
AQE	5.89 ± 0.51^{c}	0.97 ± 0.04^{c}

DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%).

3.4 In vitro study–

The purpose of the study was to confirm the high antioxidant content of the multi herbal extracts. For this, we evaluated DPPH, hydroxyl, superoxide and nitric oxide scavenging activity of the multi herbal formulation. The results are depicted in Fig 1-5. In organic fractions of the extract, it was observed that increasing polarity of the solvent increased the DPPH radical scavenging capacities and higher DPPH scavenging activities was seen in all aqueous fractions which had a positive correlation with total phenolic component (TPC). In case of AQE fraction, the observed EC50 and ARP (or AE) values (EC50=0.065 mg/mg DPPH; AE=15.4) found to be comparable to quercetin (EC50=0.0652 mg/mg DPPH; AE=16.4) and even better than Trolox (EC50=0.0958 mg/mg DPPH; AE=10.6), the two well-known standards frequently used to compare antioxidant efficacy. In aqueous fractions higher superoxide scavenging activity was observed in comparison to organic fractions. The neutralization of O_2- radicals via hydrogen donation and inhibition of xanthine oxidase by various phenols present are responsible for the O_2- scavenging properties of the extracts. Significant higher activity was found in AQE fractions ($p<0.05$ compared to other solvents) in terms of hydroxyl free radical and nitric oxide scavenging.

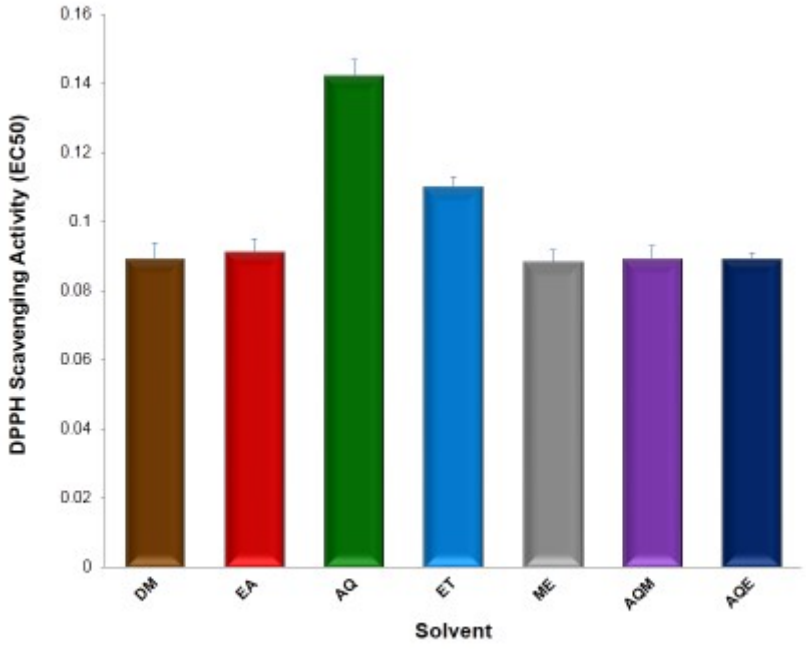

Figure 1 DPPH Assay DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

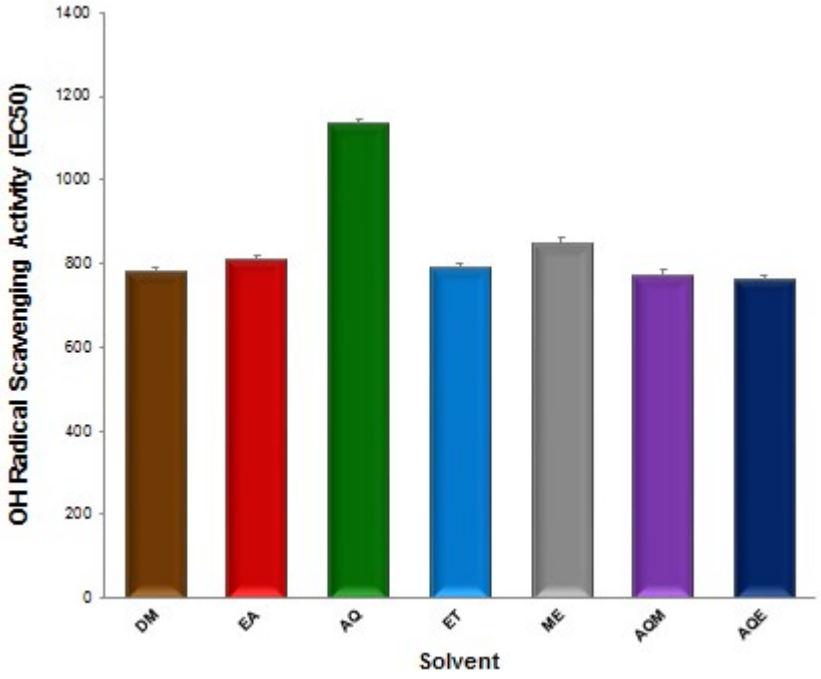

Figure 2. Observation of OH radical activity. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

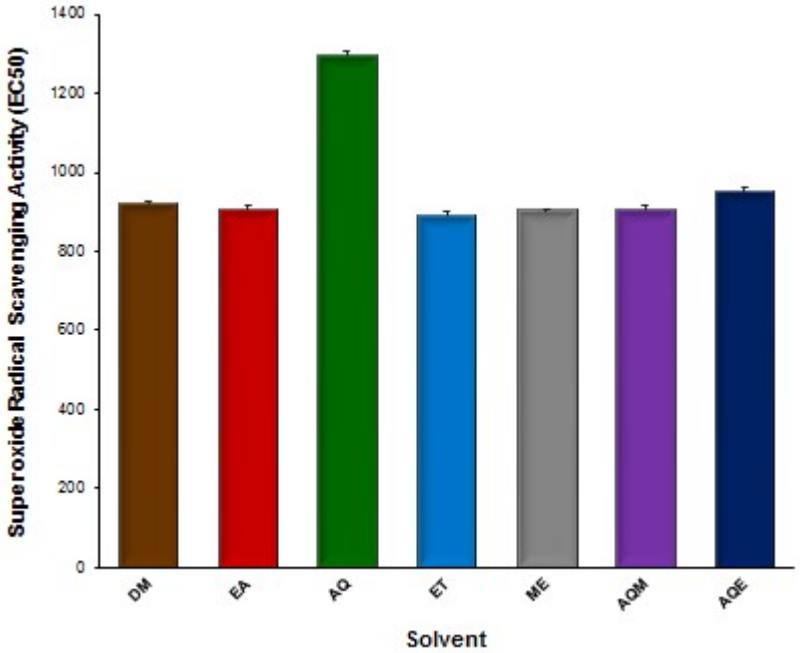

Figure 3. Superoxide radical scavenging activityDM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

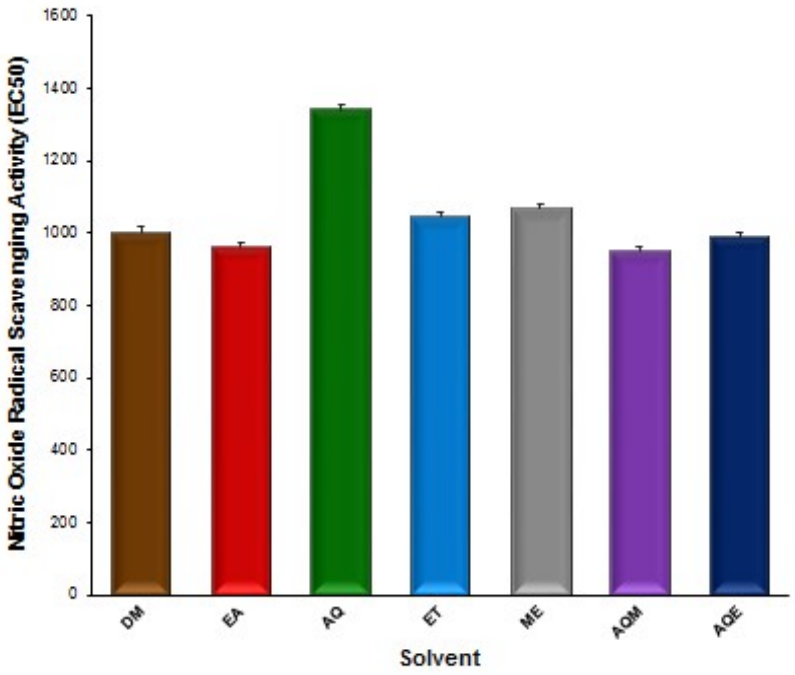

Figure 4.Nitric oxide radical assay. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

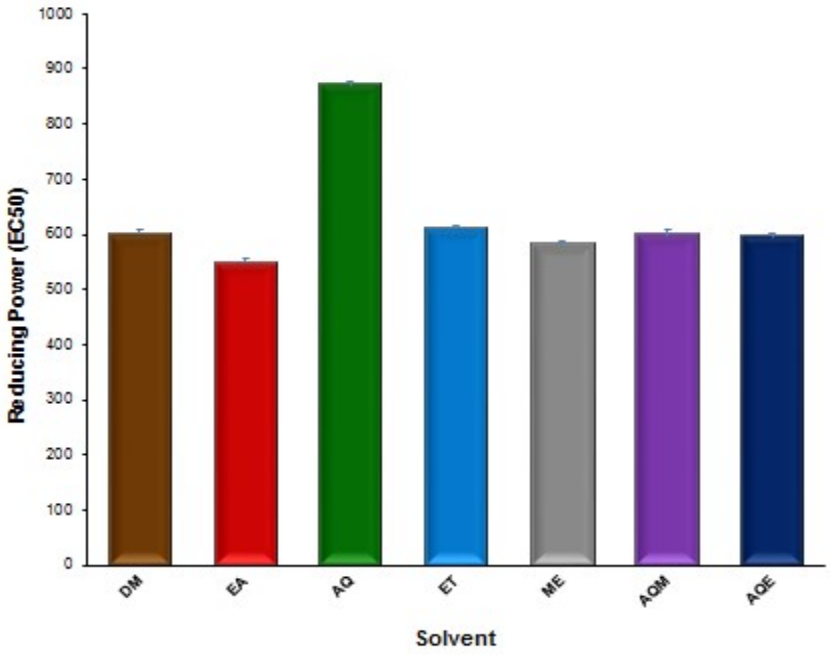

Figure 5.. In-vitro Reducing power activity of AKSS16-LIV01. DM: Dichloromethane, EA: Ethyl Alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%)

3.5 Antibacterial and Antifungal activity–

The data from in vitro antibacterial study revealed that the inhibitory action of herbal extract AKSS16-LIV01 was dependent on the dose, increasing with an increase in concentration. The inhibitory action on different pathogens was also variable. Significant activity was seen in the case of *Escherichia coli* which were followed by *Streptococcus aureus* and *Klebsiella pneumonia*, *Staphylococcus pyogenes* and *Staphylococcus typhi* showed less inhibition as seen from Fig 6. The Fig 6 reveals that the different concentrations of herbal extract AKSS16-LIV01

showed efficient antifungal activity for three fungal pathogens taken. The antifungal activity was more for C. albicans and was least for *A.niger*. We can conclude that the herbal extract possesses the antimicrobial property.

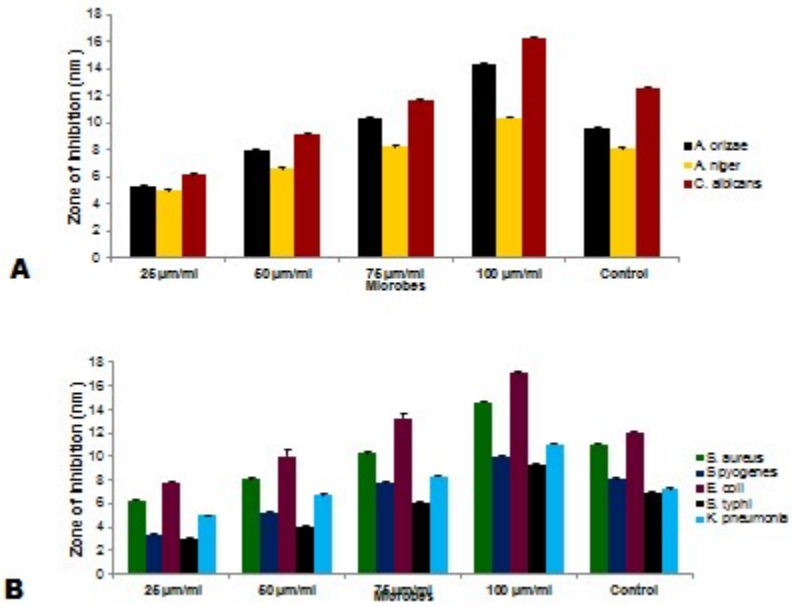

Figure 6. A. Antifungal activity of AKSS16-LIV01 upon respiratory disease effected subjects. Data represent mean ± standard deviation. B. Antibacterial activity of AKSS16-LIV01 upon respiratory disease effected subjects. Data represent mean ± standard deviation.

IV. DISCUSSION

Scientific reports [18,19] confirmed that secondary metabolites of the plants were associated with various bioactivities and showed inhibitory action against microorganisms and pathogens. Upon these different metabolites, alkaloids have extensive antimicrobial and antiviral activities [20]. On the other hand research established that other metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols have potent anti-pathogenic activity [21,22]

In-vitro study showed that our newly developed multi herbal combination (AKSS16-LIV01) have different plant secondary metabolites upon seven solvents, namely, DM: Dichloromethane, EA: Ethyl alcohol, AQ: Water, ET: Ethanol, ME: Methanol, AQM: Aqueous methanol (80%), AQE: Aqueous ethanol (80%). In this experiment aqueous (AQ) extracts of the multi herbal extract have optimum secondary metabolites like flavonoids, glycosides, saponins, triterpenes, tannins and sterols. Other solvent extracts like DM, EA, ET and ME showed moderate secondary metabolites. Sterols and glycosides were completely absent in AQM (80%), and AQE(80%) solvent extract. The stated phyto constituents present in the aqueous (AQ) extracts have significant bioactivity against microorganisms and pathogens.

Scientific study reports that aqueous extract of the medicinal plants have enormous flavonoids and phenolic compounds are responsible for antioxidant activity [23]. Hydroxyl constituents and their aromatic structure are mainly responsible for antioxidant activity as free radicals scavengers [24]. It is established that biological activities mainly depends upon the flavonoid content of the plants which improve the extracts quality [25]. It is evident from Fig.5 the phenolic and flavonoids compounds are chief components of AKSS16-LIV01and are responsible for antioxidant properties. Data of the study represents that flavonoids and phenolic contents are remarkably higher in the aqueous extract compared to others solvent extracts [26]. The routine Quality Control analysis of multi herbal formulation (AKSS16-LIV01) indicates that the developed drug complies with all the relevant parameters as per IP 2014 specification. Medicines from plant sources have gained global importance because of medicinal and economic importance [27]. The widespread sale of adulterated products and misleading health claims of herbal products require stringent regulations. [28]. Generally, most of the plants have more than one key ingredient which carries

antimicrobial property. Herbs containing in AKSS16-LIV01also have some antimicrobial activity which shows its medicinal values against diseases. AKSS16-LIV01at concentration of 75 µg/ml and 100 µg/ml showed higher antimicrobial activity than the standard drug Tetracycline in the inhibiting the growth of Klebsiella pneumonia, Salmonella typhimurium, Staphylococcus aureus, Escherichia coli and Streptococcus pyogenes. Thus this superior antimicrobial activity was because the various active ingredients released form medicinal plants penetrated and disrupted the cell membrane of bacteria [29].

V.CONCLUSION

We can conclude from this preliminary study that the multi-herbal formulation AKSS16-LIV01showed promising results in in-vitro studies and further studies are required to explore its efficacy in humans. The developed novel formulation may be a low cost, safe and symptomatic medication in future.

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Authors' Contribution
Soumendra Darbar and Atiskumar Chattapadhyay conceived and designed the experiment. Soumendra Darbar and Srimoyee Saha conducted the animal and biochemical experiments. The manuscript was written by Soumendra Darbar , Atiskumar Chattapadhyay and Kaushikisankar Pramanik.

Conflicts of Interest
The authors declare that there is no conflict of interest.

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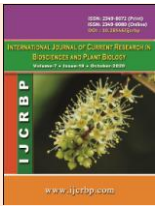

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Role of newly developed novel multi herbal formulation (AKSS16-LIV01) in ameliorating carbon tetrachloride induced haemato-toxicity in Swiss albino mice

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ABSTRACT

Haematological disorders and related complications are very common phenomenon against hazardous chemicals. Alteration of hematologic parameters disrupts the body's normal homeostasis. There is a worldwide need to develop a safe and symptomatic medication which controls the haematological complications. Healthy adult Swiss albino mice were assigned to four groups of six mice each according to their weights. Group-I serve as control, Group-II received multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg). Blood samples were collected from the retro orbital plexus of each animal to determine various blood parameters and liver transaminase. Administration of carbon tetrachloride (CCl_4) showed decline body weight, food consumption and water intake in mice whereas treatment with multi herbal formulation (AKSS16-LIV01) normalized the same as compared with untreated animals. Treatment with CCl_4 (Group-III) decline the packed cell volume (PCV), haemoglobin (Hb), means cell volume (MCV), means cell hemoglobin (MCH) and greater the white blood cell (WBC) compared with control. Pre-treatment with AKSS16-LIV01 significantly ($p<0.001$) increased the PCV, Hb, MCH, MCH and decreased WBC count in experimental animals as compared with CCl_4 treated group. On the other hand elevated liver transaminase enzymes i.e. AST and ALP by CCl_4 was restored with administration of multi herbal formulation (AKSS16-LIV01). Chronic administration of CCl_4 indicated adverse effects on haematologic parameters upon experimental animals. Simultaneous administration with newly developed novel multi herbal formulation (AKSS16-LIV01) was able to ameliorate these adverse effects and may be a potent drug in future which controls the blood related medical complications against the toxicants.

Introduction

Last few decades in various industrial processes carbon tetrachloride (CCl_4) is extensively used as a solvent (Arindkar et al., 2012). Due to its solvent property, this hazardous chemical used as refrigerator fluids, as a propellant for aerosol cans, as a dry-cleaning agent in industry, as a household spot remover, as grain fumigant and as intermediate in the synthesis of chlorofluorocarbons. As a result CCl_4 can easily found in the water bodies and contaminant the ground and surface water. Exposure and consumption of excessive CCl_4 disrupt body's homeostasis and make liver and kidney damage (Essawy et al., 2010; Gupta et al., 2004). Within the body CCl_4 can generate reactive oxygen species (ROS) like peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen caused oxidative damage. Hepatotoxicity is very common when people exposed with CCl_4 (Mandal et al., 1998).

Adverse effect of carbon tetrachloride (CCl_4) in blood is well established. A recent study depicted that administration of CCl_4 reduced red blood cell (RBC), packed cell volume (PCV) and Haemoglobin (Hb) that disturbed the haematopoiesis (Travlos et al., 1996; Uchechukwu et al., 2018).

Various ultra-structural abnormalities in the leukocytes in the blood were visible under electron microscopy of mice those treated with CCl_4, clearly demonstrated that this notorious chemical makes the structural deformities in blood (Parasuraman et al., 2014).

Multi herbal formulations mean a dosage form consisting of one or more herbs or processed herbs in specified quantities which have potent therapeutic efficacy without adverse effects (Hasan et al., 2009; Srivastava et al., 2012). Scientific study revealed that this plant based formulation is very effective to cure anaemia and control the blood (Darbar et al., 2020; Thyagarajan et al., 2002).

Here we developed a multi herbal formulation (AKSS16-LIV01) based on six Indian medicinal plants and three Indian spices. Our previous study established that the formulation is completely safe in various doses upon experimental animals (Abdel- Wahhab and Aly, 2005; Adhikari et al., 2018). With view of the above, there is need to developed and safe and symptomatic medication that controls all haematological parameters in the body when system exposed with toxicant.

Materials and methods

Chemicals

Carbon tetrachloride (CCl_4) and TRIS buffer were obtained from Merck, India. PBS pH 7.4 was procured from Sigma-Aldrich. Biochemical determination kits i.e. ALT and AST were procured from Thermo Scientific, USA. All others reagents used in this study are laboratory grade.

Preparation of plant extract

All the medicinal plant and spice ingredients were collected from registered local herbal suppliers and authenticated by pharmacognosist. Plants parts were cleaned and dry with normal temperature. The dried plant parts were used for preparation of multi herbal formulation as per standard validated protocol (Adhikari et al., 2018). The plants and plant parts used in preparation of the extract are listed in Table 1.

Animals

Twenty four young, healthy Swiss albino mice weighing 25g ± 5g have been randomly included for the study. The animals have been housed in healthy atmospheric conditions (12 h light and dark cycles, at 25±2 ^{0}C and 50-60% humidity), normal feeding, drinking, and medical care based on the CPCSEA guidelines. Mice were kept under observation for one week before the onset of the experiment for acclimatization and to exclude any unsercurrent infection. The experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. 261/JU/s/IAEC/Pharma/2018).

Experimental procedure

The mice were randomly assigned to four major groups of six mice each according to their body weights such that each group was made up of mice within the close range of body weight. The groups are as follows: Group-I serve as control, Group-II

received multi herbal formulation (AKSS16-LIV01) 400 mg/kg/day, Group-III received carbon tetrachloride (CCl_4) 1 ml/kg-bw and Group-IV received CCl_4 along with AKSS16-LIV01 (400 mg/kg).

Body weight, food consumption and water intake

Body weights were measured on weekly basis from the initial day to the final day of experiment to calculate body weight alteration. Feed intake was determined by measuring feed residue on weekly basis since the beginning of the experiment. Feed conversion was obtained by dividing total feed intake by body weight gain. Water intake was determined by subtracts the remaining of water found in the drinking bottle from the initial water given to the animals.

Blood collection and serum preparation

At the end of the respective fasting period, blood was collected from each mouse by retro orbital venous puncture. 200 µL of blood sample were collected into micro-centrifuge tubes with and without EDTA (2%). Collected bloods were placed in slanting position at room temperature for 2 hrs. Then, they were centrifuged at 3500 g for 10 min. Clear light yellow colour serum was separated and used for further analyses.

Evaluation of haematological parameters

Complete blood count includes hemoglobin (Hb), packed cell volume (PCV), total red blood corpuscles (TCRBC), total count of white blood cells (TCWBC), differential count (DC), platelets count, RBC indices such as mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were analysed by Sysmex KX-21, TRANSASIA, a fully automated 3-part differential hematology analyzer.

Determination of biochemical parameters

Liver function enzymes such as AST and ALT were used as biochemical markers for hepatotoxicity and assayed by the standard (14).

Statistical analysis

Data are presented as mean ±SE. Statistical analysis of the data was carried out using two way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Statistical significance was acceptable to a level of $p< 0.05$.

Results

Effect of multi herbal formulation (AKSS16-LIV01) on Body weight, Food Consumption and Water Intake

Gross body weights and relative changes, food consumption and water intake was presented in table 2. Administration of carbon tetrachloride (CCl_4) (1 ml/kg-bw) significantly reduced ($p<0.001$) the body weight, food intake and water intake capacity as compared with control animals. Treatment with multi herbal formulation (AKSS16-LIV01) 400mg/kg/day normalized the body weight, daily food intake and water intake capacity and reduced the liver weight as compared with control animals. Administration of AKSS16-LIV01 did not show any abnormal changes as compared with control animals.

Effect of multi herbal formulation (AKSS16-LIV01) on Haematological parameters

Haematological parameters of control and experimental groups are shown in Table 2 as well as in figures (Fig. 1 to Fig. 5). 28days treatment with newly developed novel multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day did not showed significant differences in PCV, haemoglobin (Hb), WBC, mean cell volume (MCV), and mean cell hemoglobin (MCH) compared with the control (figure 1-5). Significant reduction in Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p = 0.001$), and MCH ($p< 0.001$) was noticed in CCl_4 intoxicated mice when compared with the control. The WBC count was significantly ($p< 0.001$) greater in Group C compared with the control. In contrast, no significant differences were observed in RBC and MCHC between the control and Group C. Administration of multi herbal formulation (AKSS16-LIV01) along with CCl_4 significantly increased Hb ($p<0.001$), PCV ($p<0.001$), MCV ($p< 0.001$), and MCH ($p< 0.001$) when compared with the CCl_4 intoxicated animals.

On the other hand WBC count was significantly reduced in Group D CCl_4 intoxicated animals. Others haematological parameters (table 3) like Read Blood corpuscle (RBC); Reticulocyte (RT); Haematocrit (HCT); Mean corpuscular haemoglobin concentration (MCHC) did not show any significant changes upon all the experimental groups.

Table 1. Details of ingredient(s) present in the newly developed novel multi herbal formulation (AKSS16-LIV01).

Botanical name	Common name	Family	Part used	Quantity used in extract
Tinospora cordifolia	Guduchi	Menispermaceae	Stem	20 mg
Terminalia chebula	Haritaki	Combretaceae	Fruit	20 mg
Azadirachta indica	Neem	Meliaceae	Leaves	50 mg
Andrographis paniculata	Kalmegh	Acanthaceae	Leaves & Steam	50 mg
Aloe barbadensis miller	Aloe vera	Liliaceae	Leaves & Steam	50 mg
Curcuma longa	Curcuma, Haldi	Zingiberales	Rhizome	20 mg
Trigonella foenum-graecum	Methi	Fabaceae	Seed	10 mg
Piper nigrum	Black pepper	Piperaceae	Seed	10 mg
Elettaria cardamomum	Cardamom	Zingiberaceae	Seed	10 mg

* Amount required for preparation of 5 ml extract.

Table 2. Effect of multi herbal formulation (AKSS16-LIV01) on body weight, food consumption and water intake.

Parameters	Mice			
	Group-I	Group-II	Group-III	Group-IV
Body weight (g) Initial	26.35±1.91	26.51±2.35	26.71±4.2	26.68±5.1
Body weight (g) Final	37.84±2.03	36.94±1.69	21.81±2.41$^{\#}$	36.97±1.67*
Body weight (g) gain or loss	11.49±0.06	10.43±0.04	4.90±0.006	10.29±0.03
Food consumption (g)	4.52±0.05	4.37±0.07	2.94±0.06$^{\#}$	5.11±0.04*
Water intake (ml)	4.01±0.04	4.25±0.04	3.01±0.02$^{\#}$	4.31±0.06*

Table 3. Effect of novel multi herbal formulation (AKSS16-LIV01) on haematological parameters.

Parameters	Mice			
	Group-I	Group-II	Group-III	Group-IV
RBC (x10^{6} µL^{-1})	10.8±4.1	10.2±5.3	10.1±4.2	10.6±5.1
RT (%)	2.8±1.1	2.4±1.6	2.8±2.4	2.9±1.6
HCT (%)	34.8±1.3	32.8±2.1	32.8±2.1	35.1±3.1
MCHC (%)	41.4±7.6	41.7±2.4	40.4±1.4	41.4±1.4
Platelets (x10^{3} µL^{-1})	6.6±2.0	6.9±1.2	6.3±1.2	6.5±2.6
Lymphocyte (%)	76±6.3	72±3.3	73±5.4	73±3.4
Neutrophil (%)	25±6.2	22±4.3	21±5.1	25±6.9
Monocyte (%)	2.3±0.01	2.6±0.01	1.1±0.02	2.4±0.01
Eosinophil (%)	9.6±2.6	9.3±4.1	9.4±2.5	9.2±3.6
Basophil (%)	1.2±0.05	1.5±0.02	1.4±0.02	1.2±0.04

Data are expressed as mean ± standard deviation (N=6); RBC: Read Blood corpuscle; RT: Reticulocyte; HCT: Haematocrit; MCHC: Mean corpuscular haemoglobin concentration.

Table 4. Effect of novel multi herbal formulation (AKSS16-LIV01) on serum biochemical parameters

Groups	AST (Unit/L)	ALT (Unit/L)
Group-I	54.25±6.31	27.88±4.58
Group-II	56.92±7.06	29.58±4.64
Group-III	108.95±9.17$^{\#}$	68.57±7.91$^{\#}$
Group-IV	61.28±5.21*	31.09±5.45*

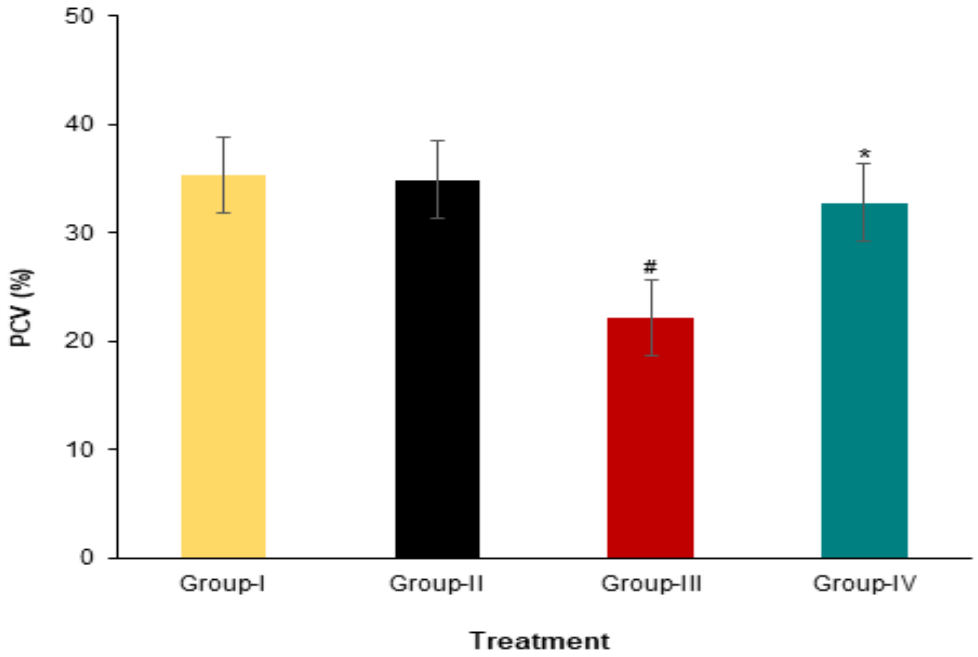

Fig. 1: Effect of multi herbal formulation (AKSS16-LIV01) on packed cell volume (PCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

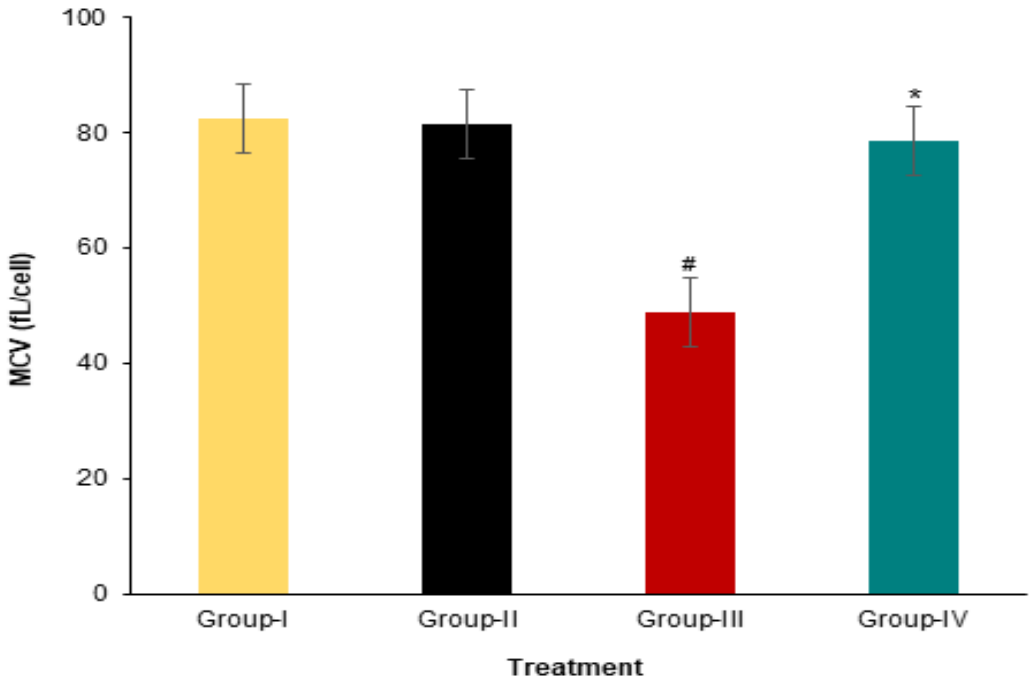

Fig. 2: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell volume (MCV) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

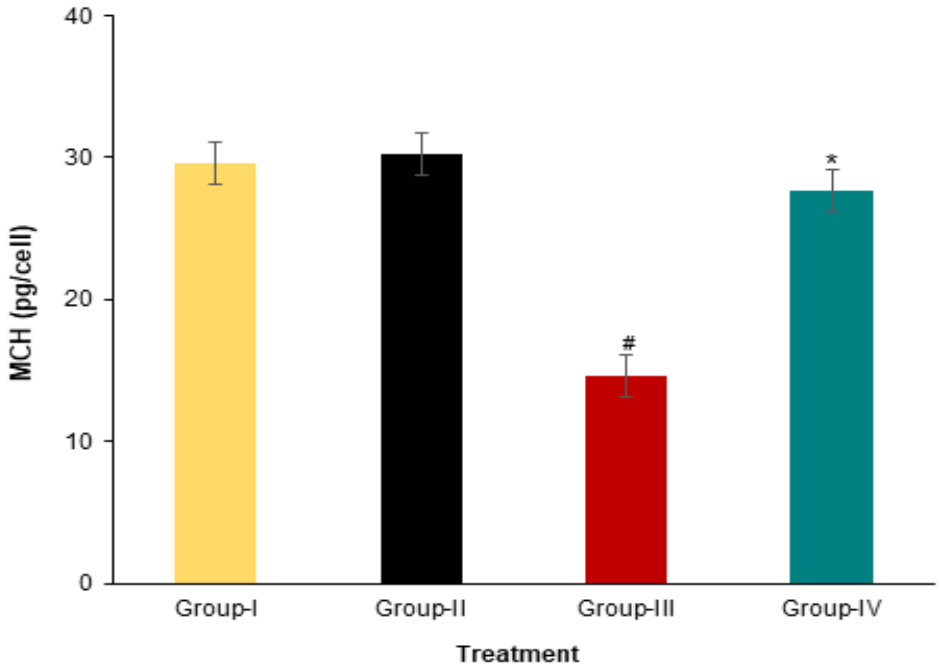

Fig. 3: Effect of multi herbal formulation (AKSS16-LIV01) on mean cell hemoglobin (MCH) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

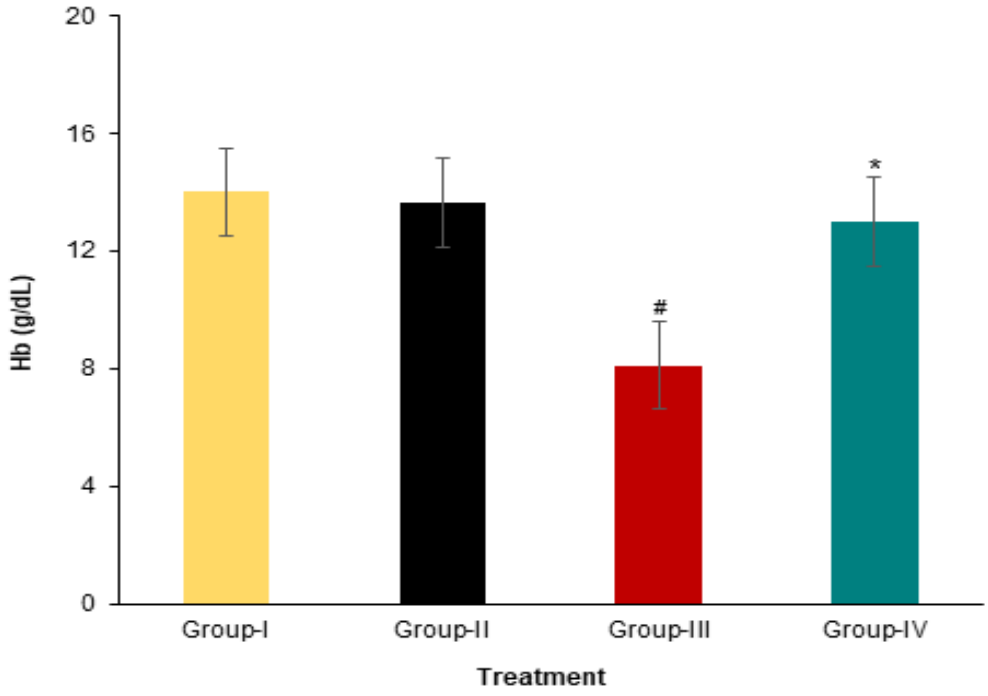

Fig. 4: Effect of multi herbal formulation (AKSS16-LIV01) on haemoglobin (Hb) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

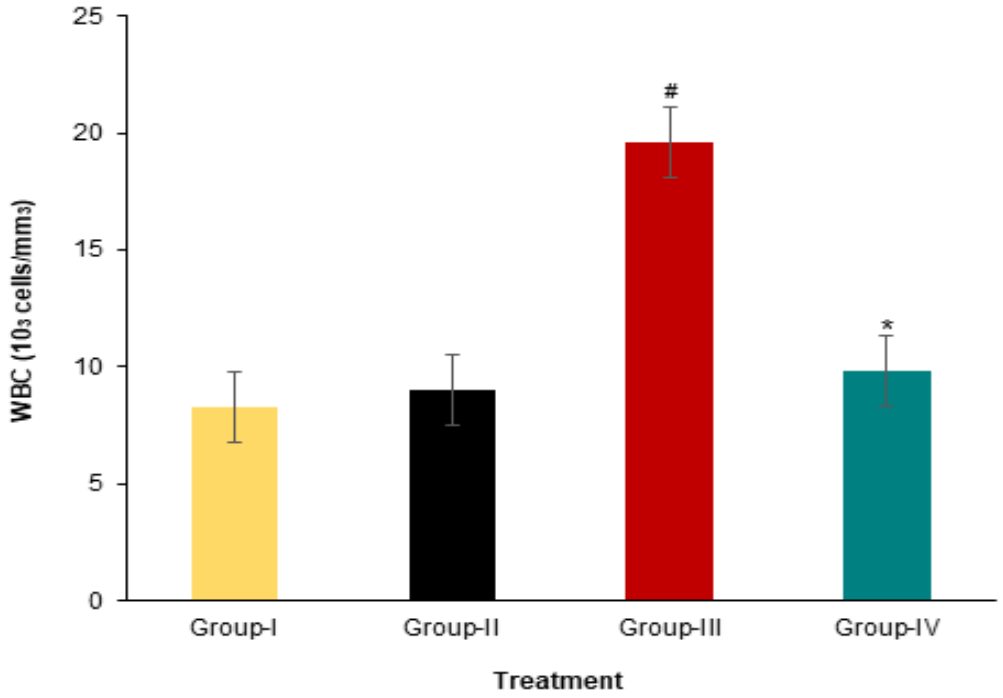

Fig. 5: Effect of multi herbal formulation (AKSS16-LIV01) on white blood cell (WBC) in mice. All data were expressed as means± SE (n=6/group). #significantly different from the control group at p<0.001 and *Significantly different from (CCl_4) group values at p<0.001. Data comparison was performed using one way ANOVA followed by Tukey's Multiple Comparison Test.

Effect of multi herbal formulation (AKSS16-LIV01) on Biochemical parameters

Table 4 shows the mean aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in control and experimental groups of mice. Data indicate that CCl_4 intoxicated mice had significantly greater mean AST and ALT compared with the control (p<0.001). Pre-treatment with multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day normalized the elevated AST and ALT levels when compared with CCl_4 treated mice. 28days treatment with newly developed multi herbal formulation (AKSS16-LIV01) at a dose of 400 mg/kg/day alone did not shows significant differences in AST and ALT when compared with control group.

Discussion

Hazardous toxicants and chemicals leads to various haematological parameters and developed medical complications. Carbon tetrachloride (CCl_4) one of the very common solvent used in various industrial processes traded as an environmental pollutant (Manthorpe et al., 1977). It is reported that treated with CCl_4 at a dose of 0.05 ml on mice reduced the haemoglobin (Hb), packed cell volume (PCV), and mean corpuscular volume (MCV) values (Tung, Cook et al., 1975). Another report depict that lower haemoglobin (Hb) value leads to iron deficiency anaemia which is characterized by a microcytic hypochromic blood picture (Thapa and Walia, 2007). In the present study our result also confirm that administration of CCl_4 (1 ml per kg body weight) decline Hb, PCV, MCH and MCV values could be attributed to disturbed hematopoiesis, destruction of erythrocytes. The low PCV and Hb concentration and the abnormally low values of MCV and MCH are indications of microcytic anaemia. Medicinal plants enrich with various compounds capable to control and maintained the various blood parameters. Pre-treatment with newly developed multi herbal formulation (AKSS16-LIV01) along with CCl_4 elevate Hb, PCV, MCH and MCV values may indirectly protect the body from the anaemia.

Elevated aspartate transaminase (AST) and alanine transaminase (ALT) levels are strong indicators of inflammatory conditions and injury to the liver (Singh, 2013), while increased white blood cells (WBC) level is generally recognized as an inflammatory response. Inflammatory conditions may induce malnutrition in the body. It is reported that inflammatory conditions can interfere with the body's ability to use stored iron and absorb iron from the diet (Gkamprela and Pectasides, 2017; Gonzalez-Casas and Moreno-Otero, 2009). The results of the present study clearly showed that treatment with CCl_4 (1 ml per kg body weight) abruptly increased serum aspartate transaminase (AST) and alanine transaminase (ALT) levels as well as elevate white blood cells (WBC) count indicate CCl_4 produce inflammatory response and affects liver cell, disturbed homeostasis. On the other hand administration with newly developed multi herbal formulation (AKSS16-LIV01) along with CCl_4 decline the AST, ALT value and WBC count protect the liver against CCl_4 induced inflammation. Thus our developed multi herbal formulation composed with six medicinal plants and three medicinal spices may be able to protect haematological disturbance caused by CCl_4.

Conclusion

This investigation shows that the developed novel multi herbal formulation (AKSS16-LIV01) has the ability to protect the haematopoietic cells from the damaging effects of exposure to CCl_4 and this protection might be attributed to the anti-oxidative power of multi herbal formulation (AKSS16-LIV01). Thus, the developed formulation composed of medicinal herbs and medicinal spices might be a therapeutic medicine in future for the prevention of haematological dysfunction.

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Authors' contribution

Soumendra Darbar and Atiskumar Chattapadhyay conceived and designed the experiment. Soumendra Darbar and Srimoyee Saha conducted the animal and biochemical experiments. Soumendra Darbar, Atiskumar Chattapadhyay and Kaushikisankar Pramanik wrote and revised the manuscript.

Conflicts of Interest

All authors report no conflicts of interest regarding this manuscript.

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Toxicological Assessment of Silver Nanoparticles Synthesized through Green Route using *Andrographis paniculata*

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ABSTRACT

Nanomedicine is a combination of nanotechnology and medicine. It provides new direction in medical diagnosis, monitoring and treatment at the level of single molecules or molecular assemblies at the "nano" scale. Additionally, continuing improvement in the pharmacological and therapeutic properties of drugs is driving the revolution in novel drug delivery systems. Nanomedicine is widely explored nowadays for treatment of life threatening diseases, yet comes with various challenges and questions. The present study encapsulates the acute toxicological aspects of the *Andrographis paniculata* coupled with silver nanoparticles *(*AP-Ag NP). For acute toxicity studies according to OCED (Organization for Economic Cooperation and Development) guidelines Swiss Albino male mice (6-7 weeks) were used and were given single intraperitoneal dose of 2000 and 5000 mg/kg body weight of the AP-Ag nanoparticle and were observed for mortality and other side effects for 14 days. The individual components of the formulation, viz. silver oxide in surface modified nano form and at low dose, and *Andrographis paniculata* are both biocompatible materials. No changes were found for general appearance, behavior and body weight, thus concluding that the nanocomposite formulation does not have a single dose toxicity.

1. Introduction

Nanomedicine is the medical application of nanotechnology. The applications of nanomedicine has been far and widespread. The size of nanomaterials is similar to that of most biological molecules and structures; therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy applications, and drug delivery vehicles. Nanomedicine seeks to deliver a valuable set of research tools and clinically useful devices in the near future [1, 2].

In recent years silver nanoparticles are widely applied in consumer products, food technology and textiles/fabrics due to its unique chemical and biological properties [3]. Silver nanoparticles are the first and most widely commercialized nanomaterial in medical and healthcare sectors. AgNPs has been used in a range of biomedical applications, owing to their antibacterial activity [4-6], antifungal properties and antiviral properties [8-10].

Green synthesis is defined as the use of environmentally compatible materials such as bacteria, fungi and plants in the synthesis of nanoparticles [11]. Alternatively, synthesis from biologically derived extracts offers several advantages such as rapid synthesis, high yields and importantly, the lack of costly downstream processing required to produce the particles [12-14]. Hence, nanoparticle synthesis from plant extracts tentatively offers a route for large scale production of commercially attractive nanoparticles.

Toxicity testing is essential in the examination of newly developed drugs before it can be used on humans. It is the limitation of potential hazards a test substance may probably produce and the description of its action, most of the toxicity testing is done on experimental animals [15]. Toxicity studies are divided into, acute toxicity, sub-acute toxicity and chronic toxicity studies. Between the different types of toxicological studies, acute toxicity studies supply input about the overall profile of magnitude of a drug toxicity, its activity and overall effects [16]. One of the basic steps in toxicological estimation of a new substance is the account of its toxicity after a single exposure of that substance. So, current problem for nanomedicine involve understanding the issues related to toxicity and environmental impact of nanoscale materials.

Considering the vast potentiality of plants as sources this work aims to apply a biological green technique for the synthesis of silver nanoparticles as an alternative to conventional methods. In this regard, leaf extract of *Andrographis paniculata* (commonly known as Kalmegh) a species of family Acanthaceae was used for bioconversion of silver ions to nanoparticles. In this study, silver nanoparticles can be produced at low concentration of leaf extract without using any additional harmful chemical/physical methods. The effect of concentration of metal ions and concentration of leaf extract quantity were also evaluated to optimize route to synthesis silver nanoparticle. The method applied here is simple, cost effective, easy to perform and sustainable. Optical properties of the synthesized NPs were measured using UV-visible (UV-Vis) spectroscopy. Morphology of the prepared samples was analyzed by high resolution transmission electron microscopy (HRTEM), X-Ray Diffraction (XRD) were used to study the crystallinity of the sample. The present work evaluates the toxicity of the prepared samples and ensures their safety upon experimental mice model and also analyzes their behavioural changes if any upon their in vivo application.

2. Experimental Methods

Andrographis paniculata was collected from Jadavpur University campus. Swiss albino mice (6-7 days old) weighing 32±5 g were procured from a CPCSEA registered animal house to conduct the toxicity study of the aforesaid nanoparticles.

2.1 Synthesis of AP Conjugated Silver Nanoparticles (NPs)

2.1.1 Plant Extract Preparation

About 1 g of AP leaves were washed thoroughly with plenty of distilled water and both surface of leaves were sterilized using alcohol by gently rubbing. These leaves were heated for 15 min in 50 mL of distilled water at 50 °C. Then the extract was filtrated with Whatman filter paper no 1 and

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further filtered using vacuum filter with pore size of 0.2 µm. The final filtrate was stored in cool dry place for further use.

2.1.2 Green Synthesis of Silver Nanoparticles

Initially, 50 mM of silver nitrate ($AgNO_3$) solution was prepared. Then plant extract was added drop wise to it under stirring. The reaction mixture became yellowish to brownish and precipitate of silver was formed. The reaction mixture was left for 30 min for complete reduction to silver nitrate (Fig. 1). Then the precipitate was collected by centrifugation at 15000 rpm for 10 min at 4 °C. The precipitate was vacuum dried at 30 °C and the sample, i.e AP conjugated silver nanoparticle (AP-Ag NP) was stored for further studies.

Fig. 1 Synthesis of AP-AgNPs

2.2 Experimental Animals

Swiss albino male mice of 6-7 weeks old and weighing approximately (32±2.0 g) were procured from a CPCSEA approved animal house (Registration No. 50/CPCSEA/1999) and randomly divided into three groups of six mice each (n=6) and all of them received standard laboratory diet (Hindustan Lever, Kolkata) and water *ad libitum*. The animals were housed in large clean polypropylene cages in a temperature-controlled room (20±2 °C) with relative humidity (45–60%) under 12 h light and dark cycles during the whole study period. Prior to experimentation, acclimatization was done for 7 days. The animals were maintained according to the guidelines recommended by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee (IAEC) (Approval No. IAEC/PHARM/1503/03/2015 dated 30.11.15). All procedures complied with the Declaration of Helsinki, as revised in 1996.

2.3 Characterization of Synthesized AP-Ag NP

UV-Vis spectra of the aqueous solutions of synthesized Ag NPs were recorded in λ25 spectrophotometer (Perkin Elmer, Germany) within the range of 200 to 800 nm. XRD patterns of the synthesized materials were analyzed in the range of 2θ from 25° to 80° using powder diffractometer, Model D8, BRUKER AXS, using Cu Kα radiation (α = 0.15425 nm). For this purpose, solution of Ag NPs was repeatedly drop casted on glass slides to make a thick coating of Ag NPs. Surface morphology and shape distribution was studies with the help of HRTEM.

2.4 Appraisal of Acute Toxicity

Acute toxicity studies of the nanoconjugate was carried out in mice by using Organisation for Economic Co-operation and Development (OECD) guideline 425 [9]. Before administration of a single dose of the test samples, the mice were deprived of food for 3 h. Doses of 2000 and 5000 mg/kg of the test samples were given to Group II and Group III respectively, whereas Group I served as vehicle control and were orally administered 2 mL of distilled water. The respective doses were suspended in water and were administered orally.

The mice were observed after every 30 min post nanocomposite treatment for 2 hrs. Once daily cage side observations included changes in skin, fur, eyes, mucous membrane (nasal), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence, and defecation) and central nervous system (drowsiness, gait, tremors and convulsion) changes. Mortality, if any, was determined over a period of 2 weeks. After completion of the treatment, the animals were sacrificed by cervical dislocation and necropsied to facilitate gross pathological examination of organs. Surface morphology and shape distribution was studies with the help of HRTEM.

2.5 Clinical Observation

The treated animals were observed for mortality (twice daily) and the clinical signs were recorded to note the onset, duration and reversal (if any) of toxic effect at 2, 4, 6 and 8 hours after the administration of last substances and once daily thereafter for 14 days. The routine cage side observation s included changes in skin and fur, eye and mucus membrane, somatomotor activity, general behavior pattern were noted. Miscellaneous sings like arching of the back, alopecia, wound, nasal discharge, lacrimation and loose stool were also recorded during the observation.

3. Results and Discussion

3.1 Characterization of NPs

The UV-Vis spectra of both chemically synthesized and green synthesized nanomaterials (Fig. 2) showed absorption band–between wavelength range 200-800 nm, with a peak at 360 nm, conforming presence of Ag NPs in the solution. In case of AP conjugated AgNPs (AP-AgNps) the absorption spectra remained almost same with a slight blue shift in the absorption maxima. The XRD pattern of the synthesized nanomaterial (Fig. 2b) showed diffraction peaks at 2θ = 36.2°, 40.6°, 62.6° and 71.3°, which respectively corresponded to (111), (200), (220) and (311) planes of pure silver. All peaks were duly assigned by using JCPDS file no. 04-0783 pertaining to pure silver. HRTEM micrograph confirmed well distribution spherical particles of the synthesized NP.

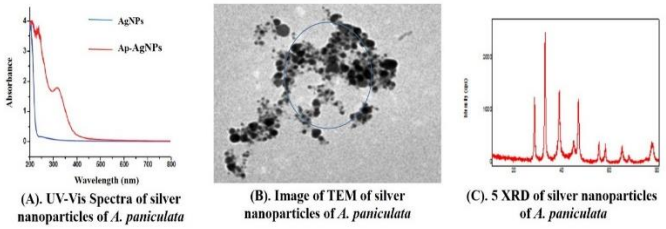

Fig. 2 Characterization of silver nanoparticles (AgNPs)

3.2 Behavioural Observations and General Appearance

The behavioral parameters of animals were observed 2,4 ,6 and 8 h after the administration of the nanocomposite according to the standard protocol. No marked changes were observed in the wellness parameters observed. There were no mortality and all the mice survived the 14 day study period.

3.3 Body Weight, Daily Food Consumption and Daily Water Intake

Tables 1 and 2 shows the change observed before and after administration of the nanocomposite, there were very slight increase in body weight of the animals but were statistically insignificant (Fig. 3). Daily food consumption and daily water intake pattern were almost normal in comparison to normal control animals.

Table 1 Effect of AP-AgNPs on the body weight of mice at 2,000 mg/kg dose

Animals (Mice)	0 Day	7th day	14th day
Control	24.3±2.66	27.5±2.07	30.2±0.99
2000 mg/kg body wt	24.4±1.35	25.4±2.24	27.9±1.22
5000 mg/kg body wt.	26.7±0.89	25.9±1.34	27.6±1.54

Table 2 Effect of AP-AgNPs on the body weight of mice at 5,000 mg/kg dose

Animals (Mice)	0 Day	7th day	14th day
Control	24.2±2.15	27.1±1.65	30.6±1.02
2000 mg/kg body wt	24.5±1.55	26.5±1.93	28.2±1.27
5000 mg/kg body wt.	24.1±0.98	25.8±1.05	27.1±1.06

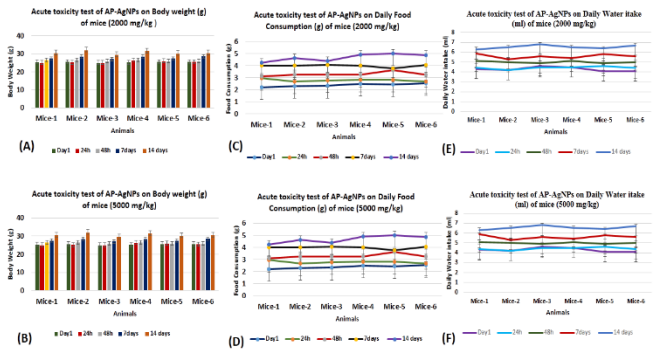

Fig. 3 Effect of AP-AgNPs on body weight, food consumption and water intake

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3.4 Behavioral Observations and General Appearance

In this study the behavioral parameters and appearance of animals after drug administration is indicator of the toxicity of the test drug. The behavioral patterns of animals were observed in 2 h, 4 h, 6 h and 8 h interval and followed by 14 h after the administration. The behavioral parameters and appearance was observed according to the standard protocol. No significant changes were observed in wellness parameters used for evaluation of toxicity. Skin, fur, eyes, mucous membrane, behavioral pattern, salivation and sleep pattern parameters of the treated animals were found to be normal (Tables 3 and 4). No toxic symptom or mortality was observed in any mice. All treated mice lived up to 14 days after the administration of NPs.

Table 3 Clinical observations of mice at 2,000 mg/kg dose of AP-AgNPs

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes and mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Table 4 Clinical observations of mice at 5,000 mg/kg dose of AP-AgNPs

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes and mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Table 5 Effect of AP-AgNPs on the Necropsy of mice at 2,000 mg/kg dose

Experimental Animals	Observed lesions during study (2000 mg/kg)	Observed lesions during study (5000 mg/kg)
1.	Nil	Nil
2.	Nil	Nil
3.	Nil	Nil
4.	Nil	Nil
5.	Nil	Nil
6.	Nil	Nil

3.4 Necropsy

All the animals were sacrificed on day 14 and necropsied. Body cavities (cranial, thoracic, abdominal and pelvic) did not reveal any lesions nor any inflammation or any abnormalities (Table 5).

4. Conclusion

There was no change in the general health of the animals throughout the study. The results indicate that the nanocomposite did not exhibit any toxicity. These findings may facilitate the development of safe and efficient Ag-AP nanoconjugate as an effective therapy against various health disorders.

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Unprecedented redox scavenging signature along with antioxidant action of silver nanoparticle coupled with *Andrographis paniculata* (AP-Ag NP) against carbon tetrachloride (CCl_4) induced toxicity in mice

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ABSTRACT: Nano technology possesses several branches including nanomedicine, which is the most promising field in the future medicine and is a probable therapeutic agent in prevention and medication of life threatening diseases through ROS inhibition. Therapeutic potential and antioxidant activity of Silver Nanoparticle coupled with *Andrographis paniculata* (AP-Ag NP) was assessed against CCl_4 induced oxidative stress at tissue level. The main aim and objective of the study is to find out the comparative efficacy of AP-Ag NP against carbon tetrachloride (CCl_4) induced oxidative stress model. Carbon tetrachloride (CCl_4) was administered upon Swiss albino mice (male) for 28 days concurrently with AP-Ag NP (50 mg/kg body weight) orally to evaluate the therapeutic effects on hepatic oxidative injury, antioxidant potential and heme synthesis pathway. Serum ROS level was significantly elevated and blood and liver superoxide dismutase (SOD), catalase (CAT) activity and GSH level also significantly decreased after exposure of carbon tetrachloride (CCl_4). Treatment with AP-Ag NP, as nano-antioxidant significantly increased SOD, CAT activity and GSH levels which indicate the recovery of oxidative injury and indicates restoring inhibited aminolevulinate dehydratase (ALAD) activity. In conclusion our results suggest that Silver Nanoparticle synthesized using *Andrographis paniculata* (AP-Ag NP) have the potential antioxidant effect in experimental animals.

1 INTRODUCTION

Application of nanotechnology in medicine is needed to threat various disease. Synthesis of nanomaterials through green route can be useful for both in vivo and in vitro in biomedical research. Chronic diseases such as diabetes, cardiovascular disease and liver fibrosis increase oxidants and decrease antioxidants in patients 2,3. Clinical and experimental studies have shown that disturbing the balance of the oxidant–antioxidant system can contribute to the pathogenesis of liver and kidney fibrosis 4-6.

Green route synthesis promotes the use of biologically active microorganisms and plants for the development of novel nanoparticles with therapeutic benefits 7. Andrographis paniculata leaf extract was used in this study as a reducing agent and the concentration of the plant extract upon silver ions for the synthesis of the nanoparticle were also assayed 8.

Radical scavengers kidnap hydrogen atoms from the activated biomaterials under variable physiological parameters and play a vital defending role by ROS (Reactive oxygen species) degradation along with prohibition of oxidative damage 9, 10. The need of the hour is to formulate a novel drug that can combat against ROS and augment redox sanation.

The present study was aimed at investigating the efficacy of Silver Nanoparticle Coupled with Andrographis paniculata (AP-Ag NP) against CCl 4 induced oxidative injury. Liver & Kidney was selected as the major organ for investigation as these are the primary target organs for free radical scavenging activity progressing to hyper lipid peroxoidation activity.

2 MATERIALS AND METHODS

The study initiated by the formation of silver nanoparticles using AP as a reducing agent. Chemical route was taken for the formation of 50 mM (AgNO3) solution. The plant extract was added in a drop wise manner followed by continuous stirring. The change of color from yellow to brown along with a brownish precipitate of silver indicated formation of nanoparticles. The setup was left untouched for half an hour for complete degradation of silver nitrate. Nanoparticles were obtained finally when the precipitate was centrifuged at 15000 rpm for 10min at a temperature of 4°C. Characterization of synthesized AP-Ag NP was carried out by UV-VIS spectra, XRD patterns and FTIR. In the in vivo experiment we developed liver and kidney fibrosis in animal model using CCl_4.

2.1 *Experimental design*

Table 1. Study design.

Groups	Treatment
Control	Normal Saline for 28 days
CCl_4 treated	Received 1:1 (v/v) CCl4 in olive oil for 28 days
CCl_4 + AP-AgNP	Received 1:1 (v/v) CCl4 in olive oil along with 0.50 g/kg/day for 28 days
AP-AgNP	0.50 g/kg/day for 28 days

After the experimental period liver and kidney function enzymes like AST, ALT, ALP, GGT BUN, creatinine and uric acid were measured using biochemical kits. Liver MDA content and different antioxidant enzymes like SOD, CAT, GPx and GSH were measured. Histology of the liver was done as per standard method. H&;E Masson's trichrome (MT) and Sirius red (SR) staining procedures were undertaken to estimate the extent of fibrotic degeneration in liver. Microscopic examinations revealed (Olympus BX51) histopathological modulations.

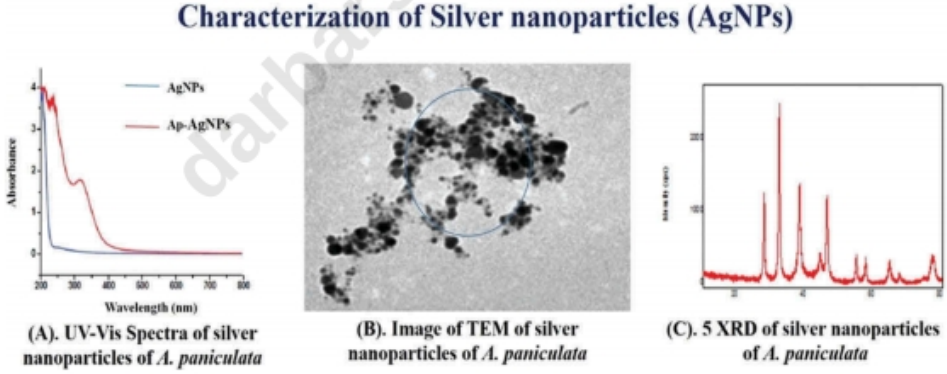

Figure 1. Characterization of Ag nanoparticle.

3 RESULTS & DISCUSSION

Leaf extract of Andrographis paniculata was used as a novel bio-reductant to synthesize silver nanoparticle (AgNPs). The whole process is eco-friendly and cheap, thus can be used for the large scale synthesis of AgNPs in nanotechnology processing industries. AgNPs synthesized from *Andrographis paniculata* leaf extract possesses unprecedented antioxidant activity which can be utilised for a novel drug synthesis in biomedical industries.

Table 2. Effect of AP-AgNP on body weight changes on CCl_4 Exposure in mice.

Groups	BW (initial) (g)	BW (final) (g)	BW gain or loss (g)
Control	25.14 ± 0.13	29.37 ± 0.11	4.23 ± 0.042
CCl_4	24.98 ± 0.14	26.01 ± 0.14	1.03 ± 0.051
CCl_4+AP-AgNP	25.22 ± 0.11	28.99 ± 0.12	3.77 ± 0.039
AP-AgNP	25.31 ± 0.12	29.48 ± 0.11	4.17 ± 0.044

Values are means ± SEM (n = 6)

It was observed that AP-AgNP significantly attenuate CCl_4 induced alteration in the activities of liver function enzymes more effectively. A significant recovery of liver function enzymes was observed after co-administration of AP-Ag NP. Administration of CCl_4 significantly decrease the essential antioxidant effect in the liver. A significant recovery in GSH, CAT and SOD was observed after co-administration of AP-Ag NP. Histology strongly proved that AP-Ag NP attenuate CCl_4 induced redox healing in the in-vivo model.

During the Cellular oxidative stress elevation of cellular ROS level occurred followed by reduced glutathione level along with increased lipid peroxidation and impaired antioxidant defense status. Our study reported elevated ROS levels in CCl_4-treated group, suggesting free radical generation leading to oxidative stress conditions. Increased lipid peroxidation like elevated TBARS and reduced GSH, further signifies oxidative stress condition. Concomitant administration of Ag nanoparticle coupled with *Andrographis paniculata* led to pronounced recovery, suggesting it become a more effective scavenger of free radicals.

Table 3. Effect of AP-AgNP on blood biochemical and antioxidant variables on CCl_4 Exposure in mice.

Blood	Control	CCl_4	CCl_4 + AP-AgNP	AP-AgNP
ROS (FIU)	412.59 ± 14.3	736.13 ± 17.8$^{\#}$	492.81 ± 11.24**	462.14 ± 12.19**
SOD (U/mg)	106.14 ± 8.1	72.05 ± 6.9$^{\#}$	98.34 ± 7.1**	100.02 ± 6.2**
CAT (U/mg)	232.62 ± 21.3	161.1 ± 19.4$^{\#}$	212.4 ± 16.2**	224.9 ± 13.1**
GSH (mg/g)	38.39 ± 0.49	21.92 ± 0.51$^{\#}$	33.12 ± 0.81**	33.66 ± 0.41**
MDA (nmol/g)	34.16 ± 2.14	76.39 ± 3.02$^{\#}$	40.12 ± 3.44**	36.13 ± 2.91**

Values are means SEM (n=6), P<0.05 significant change with respect to control group, P<0.001 significant change with respect to CCl_4 group.

ROS generation proceeds to damaged cellular antioxidant immune system. GSH levels decreased after CCl_4 treatment, possibly to its increased utilization in neutralizing free radicals. As reported by several prominent researchers Glutathione is considered to be the major form of cellular glutathione which validate our results regarding the safety profile of the synthesized nanoparticle. Damage of biological molecules such as lipids are implicated by elevated ROS generation, which are altered by peroxidation. Under the oxidative stress elevation in TBARS is an indicator of lipid peroxidation. We observed a significant elevation in TBARS level following CCl_4 exposure and back to normal level after NP administration.

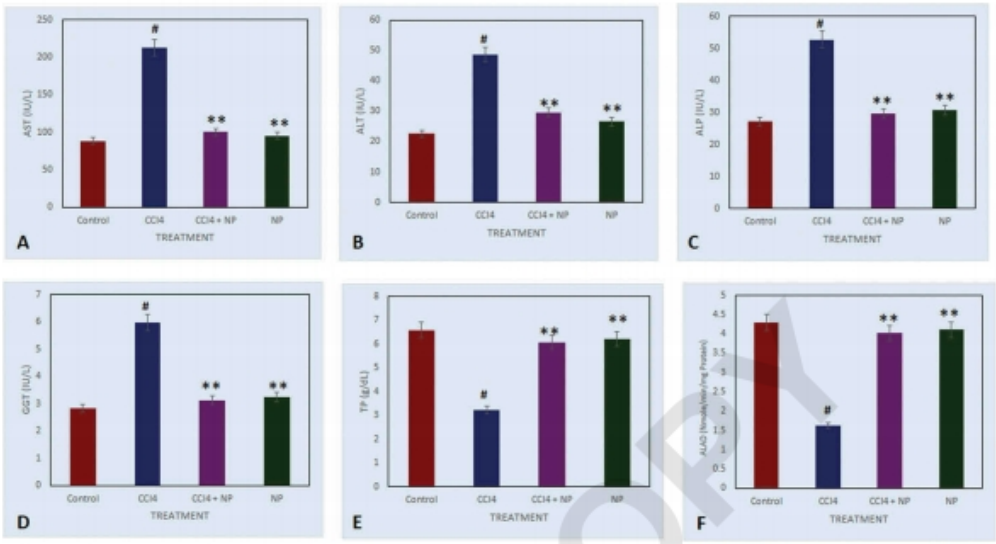

Figure 2. Effect of AP-AgNP on Liver biochemistry on CCl_4 exposed mice. Values are means SEM (n = 6), P<0.05 significant change with respect to control group, P<0.001 significant change with respect to CCl_4 group.

Interestingly the level of plasma AST, ALT, ALP and GGT were restored to normal in the animals co-exposed to AP-Ag NP.

Antioxidant profile was determined to detect the antioxidant status. The detrimental signatures of the superoxide ion was prohibited by SOD which transformed them into less toxic hydrogen peroxides which consequently breaks down into nontoxic water and oxygen molecule by catalase action.Catalase is another major antioxidant enzyme whose activity decreases during oxidative stress, leading to H_2O_2 accumulation and finally peroxidation of lipids. We observed decreased hepatic SOD activity in our study. Intrinsic antioxidant defense systems are regulated by various components like SOD is culpable for dissemination of Superoxide radicals. Concurrently in times of oxidative stress the body uses its defense mechanism to nullify the activity of lipid peroxidation by using the antioxidant enzymes such as SOD, therefore, the activity of this enzyme become higher in initiation of damage, but if the insult prolongs, the

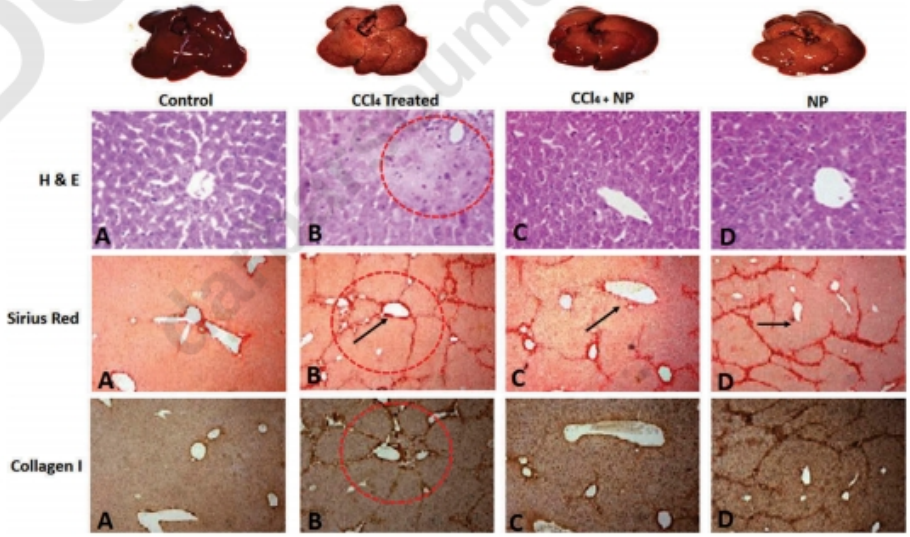

Figure 3. Effect of AP-AgNP Histopathology of Liver on CCl_4 exposed mice.

Table 4. Effect of AP-AgNP on renal function test variables on CCl_4 Exposure in mice.

Renal Function Test	Control	CCl_4	CCl_4+ AP-AgNP	AP-AgNP
Urea (mg/dl)	32.52 ± 2.5	64.16 ± 3.1$^{\#}$	38.06 ± 2.9**	39.16 ± 3.4**
Uric acid (mg/dl)	1.410 ± .24	2.920 ± 0.22$^{\#}$	1.850 ± 0.14**	1.510 ± 0.31**
Creatinine (mg/dl)	0.510 ± 0.05	0.84 ± 0.04$^{\#}$	0.56 ± 0.04**	0.59 ± 0.06**
BUN (mg/dl)	15.2 ± 1.8	35.14 ± 2.3$^{\#}$	17.03 ± 2.1**	16.22 ± 1.8**

Values are means SEM (n = 6), $P < 0.05$ significant change with respect to control group, $P < 0.001$ significant change with respect to CCl_4 group.

enzyme load become depleted which refers to the fact that in advance stages of per-oxidation the action of SOD is diminished. We noticed a cogent increase in liver SOD and Catalase activity on NP exposure which responded favourably to the co-administration of antioxidants.

4 CONCLUSION

The AgNPs were synthesized successfully using the leaf extract of *Andrographis paniculata* following inexpensive and better bioavailable green route technique.It can be summarized from this study, that the synthesized green nanohybrid (AP-Ag NPs), a prospective nanomedicine, ameliorates oxidative stress in mice most effectively. The synthesis process is not only facile but cost and time effective as well.

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PRELIMINARY ACUTE ORAL TOXICITY STUDY OF A NEWLY DEVELOPED HERBAL FORMULATION

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ABSTRACT

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. Acute toxicity studies are conducted to evaluate the effects of a single substances on animal model. The present study was designed to evaluate the acute oral toxicity study of herbal formulation (HF) according to OECD guidelines. In the present study, a single administration of the poly herbal extract at a dose of 2000 mg/kg, respectively, was given to the mice. Mice were observed for general appearance, behavior, body weight, adverse effects, mortality and necropsy up to 14 days post-treatment. In this study we could not find any mortalities during the experimental period. No changes in general appearance and mortality was observed. HF was found to be safe at dose of 2000mg/kg. In the conclusion these results demonstrate that the extract may not have any single dose toxicity.

KEYWORDS: Acute toxicity; Herbal formulation; OECD guidelines; Necropsy.

INTRODUCTION

Toxicology may be defined as the study of harmful /poisonous effects of drugs and other chemicals with emphasis on detection, prevention and treatment of poisonings. After gaining relevant information on the harmful effects of a compound, the levels for its safe usage or the degree of its safety is established, this is known as its (compound) Biosafety level.[1] Acute

toxicity testing in animals is typically the initial step in the assessment and evaluation of the health effect characteristics of a test substance, and its primary purpose is to provide information on potential health hazards that may result from a short term exposure.

Traditional and alternative medicine is extensively practiced in the prevention, diagnosis, and treatment of various illnesse. It has attracted increasing public attention over the past 20 years as these type of medicines are easily accessible in some regions.[2] Medicinal plants contribute great importance in daily life by providing wide range of nutrients, vitamins and other compounds which widen the therapeutic arsenal. In general, natural products play a dominant role in the development of novel drug which leads for the treatment and prevention of diseases.[3] Medicinal plants behave as authentic medicines because the chemical substances of which they are formed can have a biological activity in humans. Determination of efficacy and safety of herbal remedies is necessary because many people use these agents as self-medication. Since, there is limited data available about the safety of the commonly used herbal remedies, therefore, efforts to elucidate health benefits and risks of herbal medicines should be intensified. It is the need of the hour to evaluate acute and chronic toxicities of herbal drugs.[4]

Herbal formulations available with a wide range of indications like protective to liver, appetite and growth promoters, gastrointestinal and hepatic regulator, as treatment for hepatic dysfunction, for hepatic regeneration as well as liver stimulant and tonic. Despite the widespread use, there is a lack of scientific evidence on their efficacy and safety. In fact, there is lack of evidence on quality, safety and efficacy of many herbal preparations. Although many herbal preparations are non-toxic, many plants currently used for medicines have been shown to be highly toxic when given either acutely or sub-chronically.[5,6] The increasing number of plant based medication users around the globe and lack of experimental reports on their safety make it basic to direct toxicological investigation on natural herbal products.[7,8]

There is now growing evidence that many herbal medicines do cause serious toxicity to their users. Therefore, much more scientific attention is now being given to assess the potential toxicity of herbal medicines than before.

The present study aims to determine the toxicity of newly developed herbal formulation (HF) using an acute oral toxicity test in animal models.[9] The acute oral toxicity testing was

carried out on both sexes of animals under the Organization for Economic Cooperation and Development (OECD) guidelines.[10]

MATERIAL AND METHOD

Experimental animals

Swiss albino mice (30– 40 g) were obtained from the animal house. The room was well ventilated and maintained on light for 12 hours and 12 hour darkness. Temperatures were maintained at 27– 30 °C. The mice were provided with the standard pellets and clean water *ad libitum*. The experimental procedures were carried out in strict compliance with the Institutional Animal Ethics Committee's (IAEC) rules and regulation of this institute and the experiments were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Composition of herbal formulation

The composition of each 5ml of Herbal Composition compose of *Azadirachta indica* (Neem) 50mg; *Curcuma longa* (Turmeric) 20mg; *Terminalia chebula* (Chebulic Myrobalan) 20mg; *Aloe Barbadensis Miller*(Aloe Vera) 20mg; *Tinospora cordifolia* (guduchi) 20mg; *Citrus limon* (Lemon) 10mg; *Trigonella foenum-graecum* (Methi) 10mg; *Piper nigrum* (Black pepper) 10mg; *Elettaria cardamomum* (cardamom) 10mg.

Assessment of Acute toxicity test

Acute toxicity study was performed in healthy swiss albino mice (30-40gm) as per guidelines (AOT 425) suggested by the Organization for Economical Co-operation and Development (OECD). The animals were randomly assigned into two groups of 6 mice each and kept 3h fasting prior to extract administration. Group 1 served as the control and the mice were orally administered with 2ml distilled water (Group 2).

Mode of Administration

Single concentrations of the polyherbal extract 2000 mg/kg body weight was constituted in 5ml distilled water through a mice gavage. Animals were fasted 3h prior to dosing (only food was withheld for 3h but not water) and 3 hours further after drug administration.

The mice were observed after every 30 minutes post extract administration for the first 2 hours and latter once a day up to the 14 th for changes in skin and fur, eyes and mucus membranes, behavior pattern, tremors, salivation, diarrhea, sleep, coma, mortality, moribund,

ill health or any visible reaction to treatment. Weight recording was done before combination extract administration, a t 24 hours, 48 hours, day 7 and day 14 using a sensitive balance.

Clinical Observation

The treated animals were observed for mortality (twice daily) and the clinical signs were recorded to note the onset, duration and reversal (if any) of toxic effect at 2, 4, 6 and 8 hours after the administration of last substances and once daily thereafter for 14 days. The routine cage side observation s included changes in skin and fur, eye and mucus membrane, somatomotor activity, general behavior pattern were noted. Miscellaneous sings like arching of the back, alopecia, wound, nasal discharge, lacrimation and loose stool were also recorded during the observation.

Body weight and food intake

Body weight data of individual animals were recorded following the period of fasting on the day of dosing, weekly thereafter and at termination on day 15. Weekly changes in body weight gain were calculated and recorded.

RESULTS AND DISCUSSION

Herbal remedies positioned themselves in various forms such as dietary supplements, mono or polyherbal drugs, dietary ingredients, etc., and have become famous and safe commercial commodities. However, the herbal preparations, irrespective of the popular belief that they are safe based on ancient literature, required to be confirmed for their non-toxic/relatively less toxic effects compared to the chemical therapeutic counterparts.[11]

Behavioral Observations and General appearance

In this study the behavioral parameters and appearance of animals after drug administration is indicator of the toxicity of the test drug.[12,13] The behavioral patterns of animals were observed in 2h, 4h, 6h and 8h interval and followed by 14 h after the administration. The behavioral parameters and appearance was observed according to the standard protocol.[14] No significant changes were observed in wellness parameters used for evaluation of toxicity. Skin, fur, eyes, mucous membrane, behavioral pattern, salivation and sleep pattern parameters of the treated animals were found to be normal (table 2). No toxic symptom or mortality was observed in any mice. All treated mice lived up to 14 days after the administration of herbal formulation (HF).

Body Weights and food intake

An increase in body weight of the animal after test drug administration is indicator of its toxic effect.[15] Figure 1 showed the change observed before and after the administration of the HF. Although, the body weights of all the mice were increased after the oral administration of HF. But, the changes of the body weights were found to be statistically insignificant. Insignificant increase in body weight of test animals indicates that the administration of the HF had no toxic effect on animals. Intake of food during the study was normal (table 1).

Necropsy

All limit test animals were euthanized at study termination (day 14) and necropsied. Body cavities (cranial, thoracic, abdominal and pelvic) were opened and examined. No lesions were observed in all mice (table 3).

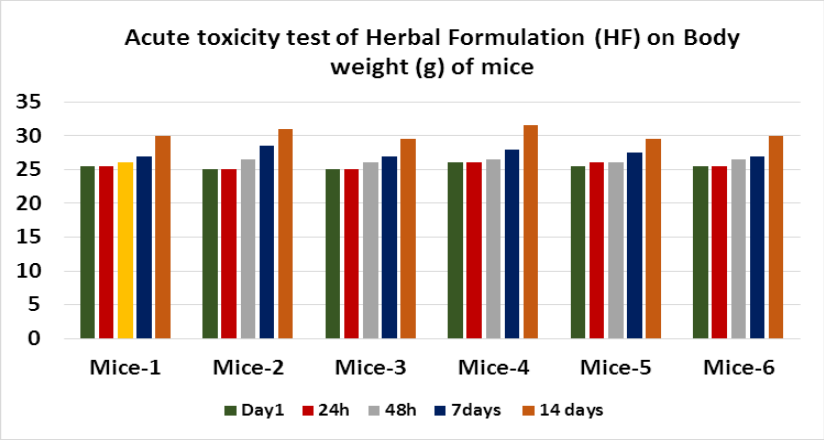

Fig. 1: Effect of Herbal Formulation (HF) on the body weight (g) of mice at 2,000 mg/kg dose.

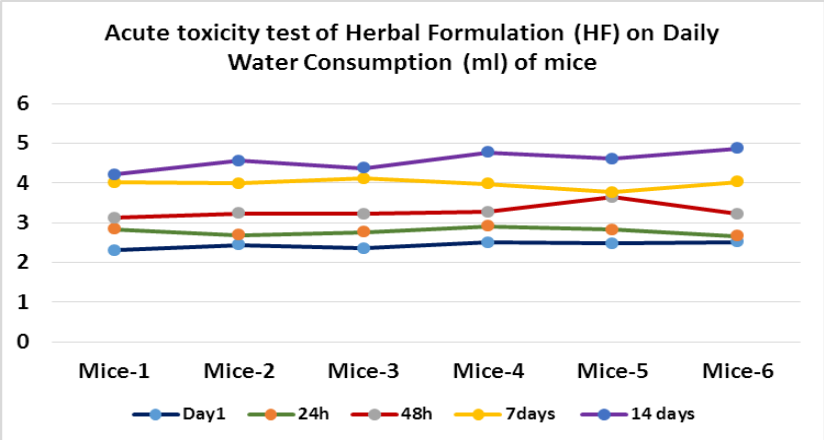

Fig. 1: Effect of Herbal Formulation (HF) on the Water consumption (ml) of mice at 2,000 mg/kg dose.

Table 1: Effect of Herbal Formulation (HF) on the food consumption of mice at 2,000 mg/kg dose.

Swiss Albino Mice	Weight in grams		
	Day 1	Day 7	Day 14
1.	5.13	5.88	5.90
2.	5.22	5.47	6.01
3.	5.28	5.63	5.83
4.	5.28	5.77	6.24
5.	5.02	5.48	6.11
6.	4.96	5.61	6.08

Table 2: Clinical observations of mice at 2,000 mg/kg dose of Herbal Formulation (HF).

Signs and symptoms	Mice 1	Mice 2	Mice 3	Mice 4	Mice 5	Mice 6
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes And mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/ convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Table 3: Effect of Herbal Formulation (HF) on the Necropsy of mice at 2,000 mg/kg dose.

Experimental Animals	Observed lesions during study
1.	Nil
2.	Nil
3.	Nil
4.	Nil
5.	Nil
6.	Nil

SUMMARY AND CONCLUSION

Therefore, it is concluded that the administration of the newly developed herbal formulation is safest & has no adverse effect on animals. All the animals survived by the end of the study; Clinical signs symptoms and gross necropsy did not reveal any major findings. Hence it may be concluded (Category 5 as per OECD guidelines 420, 423 & 425 for acute Toxicity Studies) that the developed HF is practically nontoxic, safe and has no adverse effect.

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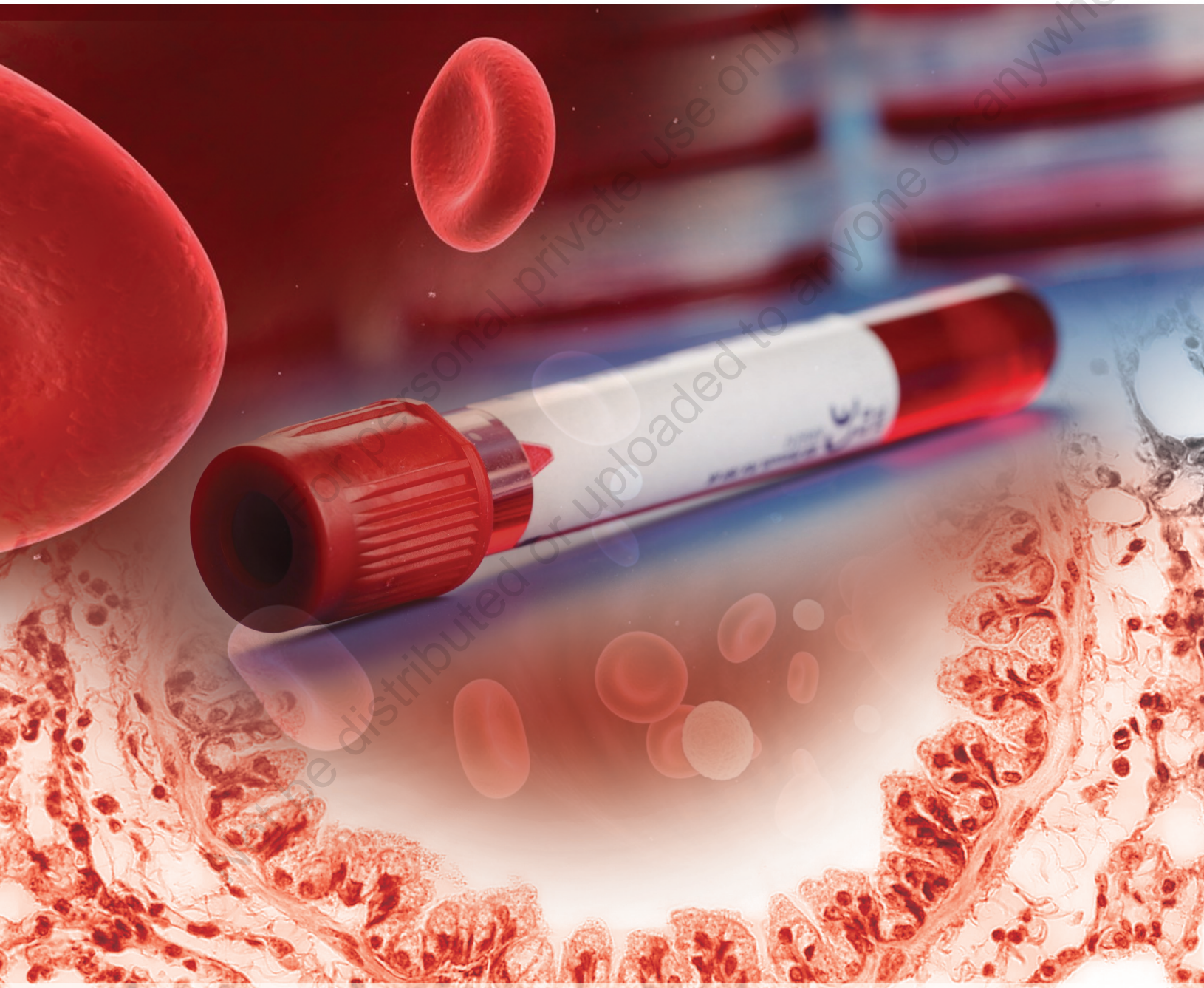
(Hematology)



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CHAPTER 5

Haematological Modulations by Fixed Dose Combination (FDC) of Tramadol Hydrochloride/Paracetamol (THP)

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Abstract: Analgesics as fixed-dose combination are very useful for fast pain relief. Overdose or chronic use of analgesics, especially fixed doses form, develop mild to severe adverse effects and sometimes damage various organs like the liver, kidney and brain. Tramadol hydrochloride/paracetamol (THP) is a fixed-dose combination (37.5 mg of Tramadol hydrochloride and 325 mg of paracetamol) extensively used for the treatment of moderate to severe pain. Administration of THP upon animal model severely disturbed hepatic and renal biochemical parameters, which leads to altering normal cellular homeostasis. In this context, our recent study established that the application of Tramadol hydrochloride/paracetamol produced deleterious effects on haematological parameters in the experimental murine model. 1.12 g/300 ml and 1.68 g/300 ml chronic administration of Tramadol hydrochloride/paracetamol (THP) decrease the packed cell volume (PCV), haemoglobin (Hb), mean cell volume (MCV), means cell haemoglobin (MCH) and increase the mean corpuscular haemoglobin concentration (MCHC) and white blood cell (WBC) in an experimental animal model. Other haematological parameters likes red blood corpuscle (RBC), reticulocyte (RT), haematocrit (HCT) did not show any significant changes upon animals. Increased MCHC inhibits the oxidation process and energy balance, whereas elevated WBC levels indicate immune system damage. Decreased haemoglobin and PCV indicate that Tramadol hydrochloride/paracetamol (THP) is an indirect cause of anaemia. It may be concluded that prolonged or chronic administration of THP may cause severe thrombocytopenia, leading to the failure of the immune system, anemia, and a very low erythrocyte count. These side effects increase according to the dosage and duration of

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Atta-ur-Rahman (Ed.)

Original Article

Effects of Alprazolam Administration on the Vital Organs of Adult Wister Albino Rats, Biochemical and Toxicological Studies

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ABSTRACT

Objective: Effect of Benzodiazepine group of drugs Alprazolam is investigated on different vital organs of Wister albino rat. **Materials and Methods:** The rats (n = 6) were treated with normal doses of Alprazolam for two months along with a placebo group (n = 6). After the treatment blood samples were collected and then the rats were sacrificed to collect the tissues of heart, liver and kidney. **Results:** The levels of enzymes ALT/SGPT, AST/SGOT and the signaling molecule NO were estimated. It was seen that the levels of all these three parameters were increased in all the treated rats. NO is a very important chemical messenger and cellular signaling molecule. Increased level of NO in the tissues of vital organs can modulate the normal physiological and cellular processes and homeostasis of the body in many ways. The NO mediated macrophage activation may also be influenced by the Benzodiazepine (BDZ) group of drugs. So, it's a very important finding from the clinical point of view. Prolonged use of Alprazolam shows different contraindications and affects different vital organs and systems of the body. To know how the drug affects heart, liver and kidney the levels of two clinically important enzymes like ALT/ SGPT and AST/ SGOT were measured in serum and tissue extracts of rat. These two enzymes are very good indicator of cardiac and hepatic health. Results of the experiments show that levels of both the enzymes were increased in serum and tissue extracts, particularly in the tissue extract of heart. **Conclusion:** So, from our above studies it can be concluded that there is a relation between increase of NO and the increase of levels of clinically important enzymes. As a result, before frequent and prolonged administration of the drug Alprazolam, further detailed study is needed regarding its effect on different vital organs and cellular and physiological processes.

Key words: Alprazolam (Alp), Cell death, Necrosis, Biochemical enzymes, Nitric Oxide (NO).

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INTRODUCTION

In the recent decade, there has been increasing concern on the hazardous effects of drugs on different species. Benzodiazepines (BDZ) comprise a large group of psychoactive drugs that are massively used in human pharmaco-therapy for their anxiolytic and hypnotic anticonvulsant properties.[1] Alprazolam (Alp) is a member of BDZs group of drugs, widely used as antipsychotic and anxiolytic agent. They are used for the acute and sub-chronic treatment of insomnia and agitated psychosis.[2] Once it was considered as a safe drug altogether. But for a decade or long reports are coming that Alprazolam can damage the vital organ like heart, liver, kidney etc.

A vast amount of studies have shown that alprazolam in doses of 0.5 mg and higher induce changes in brain oxidative metabolism and impairs a variety of cognitive and psychomotor skills such as memory impairment, speed of responses, tracking

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performance and a considerable potential to induce dependence and abuse.[3-5] Moreover, scientific study established that Alp produce conformational changes in hemoglobin[6] and it also intercalates into the DNA.[7] Tanaka and its co workers reported that some popular drinks may exert cytotoxic effects in combination with BDZs, for example, Alp and ethanol administration in together had toxicity in liver microsomes *in vitro*.[8]

Research revealed that Intravenous administration of Alp caused an acute significant decrease in brain activation as well as in the whole-brain cerebral blood flow of 25% to 30 %.[9] Verster *et al.* concluded that driving is unsafe after administration of Alp because of it produces adverse effects such as drowsiness and sedation, in addition to its therapeutic effect.[10,11]

In addition, it was found that alprazolam produced severe cytotoxicity in human cell lines as it induced elevated level of reactive oxygen species (ROS) and depletion of antioxidants.[12] Generally, it was reported that all benzodiazepines drugs can cause respiratory depression[13] hepatic dysfunction[14] hypotension and thrombophlebitis, [15,16] brain damage.[17] It is also observed that alprazolam caused cytotoxicity in rat vital organs. Yousif *et. al.* studies on mice stated that Alprazolam at a dose of 0.2 mg/kg body weight observed extensive morphological changes like enlargement of the nuclei and proliferation of Kuffer cells in the liver cells.[18]

Alprazolam has lots of toxic and adverse side effects on persons under long term treatment with this drug. Recent study showed,[19] alprazolam was found to induce a significant increase in neutrophil count and a significant decrease in lymphocytes, anti-SRBC titer and IL-2 level with severe depletion of the splenic, thymal and nodal lymphocytes, accompanied by congestion and eosinophilic vasculitis of all vital organs.

So, objective of the study is to establish the potential toxic effects of alprazolam on the several blood enzymes level like Alanine aminotransferase (ALT)/ serum glutamate pyruvate transaminase (SGPT), Aspartate aminotransferase (AST)/ serum glutamate oxaloacetate transaminase (SGOT) in rat serum and tissue extract of heart, liver and kidney as well as to measure the NO levels.

MATERIALS AND METHODS

Drugs and Chemicals

Alprazolam powder was procured from Torrent Pharmaceutical Ltd, India and was dissolved in MiliQ water with 0.24(N) HCl solution through vigorous vortexing. The dose was calculated 0.2mg/100gm body weight/day.[18] All chemical reagents were of analytical grades purchased from Sigma Chemical Co. (St. Louis, Mo, USA), Merk (Germany) and BDH (England).

Animals

Three months old albino male rats (100-120gm) were obtained from National Institute of Hydrabad (CPCSEA approval No. 154/99/CPCSEA) Animals were housed in large size polyethylene cages under standard hygienic condition. All rat were acclimatized to the laboratory environment for two weeks prior to the starting of the experiment, where they were adapted to the controlled environmental conditions at room temperature of 25 ± 2 °C, relative humidity 60-70 % and at normal photoperiod 12 h/d. The methodology of this work was approved by the Institutional Animal Ethics Committee (IAEC) on Animal. All animals were fasted for 1 hour prior to drug administration.

Experimental Design

For treatments with alprazolam rats were divided into two groups in either sex: normal healthy group (group 1) and drug (Alp) treated group (group 2), each of 6 rats. Alprazolam drug was administered intraperitonially using a single dose of 0 .2mg/100gm body weight/day.

Monitoring of the Signs of Toxicity

All rats of the experiment were closely observed and carefully examined daily throughout the experimental period in order to see whether any apparent behavioural changes and/or signs of toxicity are present or not.

Body Weights and Different Organs Weights

Body weights of all experimental rats were recorded weekly during the period of treatment. Means of the body weights and body weight gains were estimated. At the end of the experiment, both control and experimental groups were sacrificed and dissected. The organs were excised out quickly, weighed and the absolute and the relative weights were calculated according to Matousek (1969) I.W. = organ weight (g)/100×body weight (g).

Collection of Sample and Biochemical Study

After 60 days blood samples were collected at the end of the experiment via cardiac puncture from each anaesthetized rat (Ketamin hydrochloride with Xylazin) after fasting 8-12 h, using disposable syringes. Samples were centrifuged at 3500 rpm for 15 min and then the clear serum was collected in sterilized disposable plastic tubes and stored in a freezer set at -20ºC for subsequent measurement of serum biochemical parameters.

Serum ALT and AST were measured by colorimetric method by using commercially available standard

diagnostic Kit (Spain diagnostic limited, India). The above parameters were also measured from tissue extract of liver, heart and kidney. At the end of experiments, rats were sacrificed Under controlled anesthesia and the liver, kidney and heart samples were collected, minced and homogenized in either ice cold distilled water using a glass homogenizer. The homogenates were centrifuged for 15 min at 10000g. at 4 degree C and the supernatants and was used for different biochemical analysis.

Determination of NO by Griess Method

The total nitrite was determined in the tissue extracts according to the standard biochemical protocol of Greiss method as a measure of NO.

Statistical Analysis

Statistical analysis was done using the ANOVA and test for comparison of data in the control group with the experimental groups. The results were expressed as mean ± S.E.M (standard error of means). P-value less than 0.05 were considered significant and are written in the parentheses.

RESULTS

Effect of Alprazolam on Signs of Toxicity

The results revealed prominent signs of toxicity in rats administered with 0.2 mg/100g alprazolam (ALP), where most treated animals became progressively less active and showed general weakness. We also noticed that they had lost their appetite during and after the experiment of treatment with the drug and noticed marked loss in their body sizes. Control rats showed no mortality during the experimental period, however, there was few mortality observed in ALP-treated rats.

Effect of Alprazolam on Body Weight Change

At the end of the experiment, rats treated with ALP showed marked significant decreases in the body weight change, comparing to the control (Table 1).

Effect of Alprazolam on Relative Different Organ Weight

In this study statistically significant difference in organ weight of the liver, kidneys and heart were found by the intoxication of alprazolam compared to untreated animals. The liver, kidneys and heart were considerably lighter in the mice from the group exposed to Alprazolam (ALP) compared to the rats from the control group (Table 2).

Effect of Alprazolam on Serum and Tissue Protein and Enzyme Estimation

The serum level of total protein and levels of ALT and AST in serum and tissue extracts of liver, heart and kidney in experimental animals showed on (Table 3 and 4). However, the levels of ALT and AST in serum were all significantly higher in alprazolam treated

Table 1: Effect of Alprazolum on Body Weight of rat.

Group	Initial Body Weight (g)	Final Body weight (g)	Net Gain (g)	Average Food Intake (g)
Control	122.56	152.89	30.33±4.32	13.68±1.72
Treated	122.52	136.41	13.89±2.14	15.21±1.49***

All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance. *p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control

Table 2: Effect of Alprazolum on Wet Weight of Different Organ of rat.

Group	Heart (g)	Liver (g)	Kidney (g)
Control	2.86±0.22	6.45±0.66	4.22±0.06
Treated	2.74±0.34	7.56±0.67***	4.18±0.05***

All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance. *p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control

Table 3: ALT (IU/L) in serum and tissue extract of rat.

Group	Serum (IU/L)	Heart (IU/L)	Liver (IU/L)	Kidney (IU/L
Control	51.84±2.76	145.33±9.72	190.17±8.82	48.17±1.15
Treated	62±6.68	426.33±22.86***	265.17±15.54**	169.167±3.2***

All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance. *p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control.

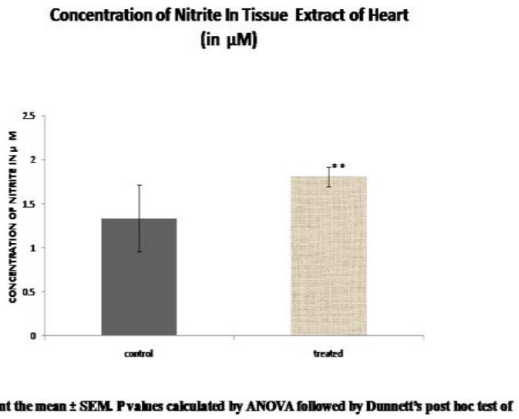
Table 4: AST (IU/L) in serum and tissue extract of rat.				
Group	**Serum (IU/L)**	**Heart (IU/L)**	**Liver (IU/L)**	**Kidney (IU/L**
Control	211.84±11.86	271±17.2	182.3±4.86	154.16±9.22
Treated	246.34±14.20	352.17±20.22**	229.83±8.66**	290.5±16.46

All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance. *p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control.

animals when compared to control values. The increases of the above two enzyme concentrations were also noted in the liver, kidneys and heart in comparison with control group.

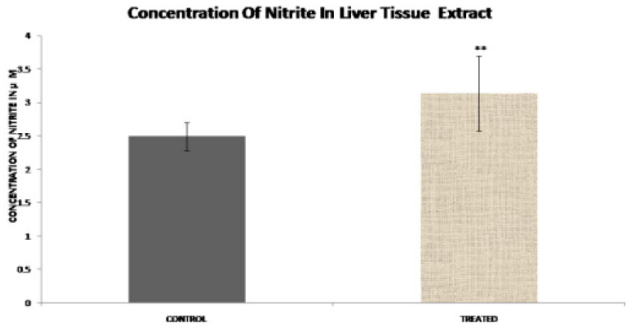
Effect of Alprazolam on Tissue NO Concentration

The effects of alprazolam on tissue NO levels (in terms of total nitrite concentrations) were given in Figure 1-3. Alp. treatment caused a significant increase in total nitrite concentration at a dose of 0.2 mg/100g alprazolum compared with control group in the tissue extracts of heart, liver and kidney (Figure 1-3).



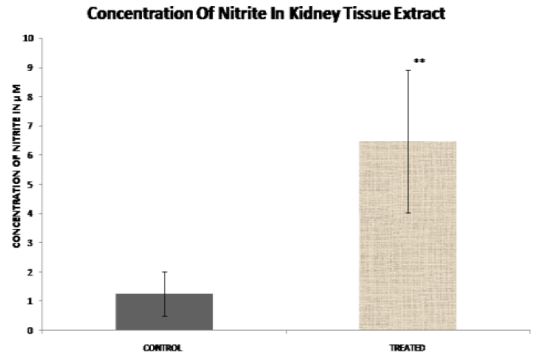
All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance
*p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control.

Figure 1: Estimation of total nitrite in rat heart tissue extract after treatment of Alprazolam as compared with control animals.



All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance
*p<0..05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control.

Figure 2: Estimation of total nitrite in rat liver tissue extract after treatment of Alprazolam as compared with control animals.



All values represent the mean ± SEM. P values calculated by ANOVA followed by Dunnett's post hoc test of significance
*p<0.05 as compared with Control, **p<0.01 as compared with control, ***P<0.001 as compared with control.

Figure 3: Estimation of total nitrite in rat kidney tissue extract after treatment of Alprazolam as compared with control animals.

DISCUSSION

Alprazolam is highly prescribed sedative drug used in the treatment of anxiety disorders. It is readily absorbed in intestinal tract. After absorption, the drug is transported through blood to different tissues. In central nervous system the drug binds with the receptor, which is a GABA receptor.[20] As a result chloride channel at the membrane of the neuron opens. This causes the hyperpolarisation of the membrane. Thus, depolarization of the membrane or stimulation of the neuron becomes difficult. In this process NO acts as a mediator. But in other tissues also, there are PBR. The drug binds with that receptor also. So, the objective of the study was to see whether the binding of drug alprazolam with PBR increases the level of NO in other tissues and blood also.[21] From the result of the study it has been seen that NO level increased in the tissue extracts of the vital organs like heart, liver and kidney of rat after prolonged administration of Alprazolam. NO is a very important chemical messenger and cellular signaling molecule. Increased level of NO in the tissues of vital organs can modulate the normal physiological process and homeostasis of the body in many ways.[22] So, it's a very important finding from the clinical point of view. The NO mediated macrophage activation may also be influenced by the BDZ group of drugs. Further studies can be done to trace the mechanism of generation of NO and it's effects on important physiological processes.

Prolonged use of Alprazolam shows different contra-indications and affects different vital organs and systems of the body. To know how the drug affects heart, liver and kidney the levels of two clinically important enzymes like ALT and AST were measured in serum and tissue extracts of rat. These two enzymes are very good indicator of cardiac and hepatic health. Results of the experiments shows that levels of both the enzymes increased in serum and tissue extracts, particularly in the tissue extract of heart. These are the important and also novel findings of the study.

So, from the study it can be concluded that before frequent and prolonged administration of Alprazolam detailed and further study is needed regarding its effect on different vital organs and cellular and physiological processes.

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CONFLICT OF INTEREST

Authors have none to declare

ABBREVIATIONS

Alp: Alprazolam; **ALT:** Alanine Aminotransferase; **AST:** Aspartate Aminotransferase; **BDZ:** Benzodiazepine; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **GABA:** Gamma-Aminobutyric Acid; **IAEC:** Institutional Animal Ethics Committee; **IL-2:** Interleukin-2; **NO:** Nitric Oxide; **PBR:** Peripheral benzodiazepine receptor; **ROS:** Reactive Oxygen Species; **SGOT:** Serum Glutamate Oxaloacetate Transaminase; **SGPT:** Serum Glutamate Pyruvate Transaminase; **SRBC:** Sheep Red Blood Cells.

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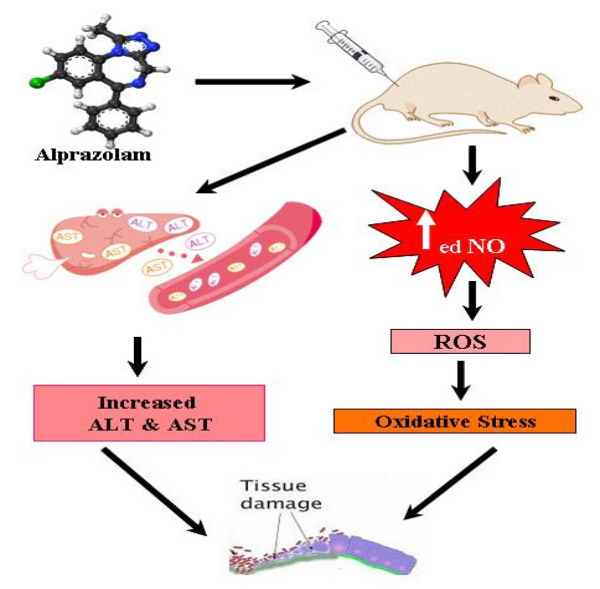
PICTORIAL ABSTRACT



Summary

- Adult male rats were divided into two groups (n=6). One group was treated with measured dose of Alprazolam for two months along with a placebo group (n=6). After the treatment was over blood samples were collected. The rats were sacrificed then and the tissues of heart, liver and kidney were also taken. The levels of diagnostically marker enzymes ALT/SGPT, AST/SGOT, which indi¬cate tissue damage and the signaling molecule NO were estimated in serum and tissue extracts of heart, liver and kidney of rat. It was seen that the levels of all these three parameters were significantly increased in all the treated rats in comparison to control in blood and tissue extracts. The study suggested that prolonged admin¬istration of Alprazolam on rat caused serious damage to the tissues of vital organs. NO is a very important chemical messenger and cellular signaling molecule. It can be envisaged that there is a relation between increase of NO and the increase of levels of clinically important enzymes and so with the damage of the tissues of the vital organ studied. Further detailed study on this relation can reveal the role of NO in molecular mechanism of tissue damage of the vital organ studied.

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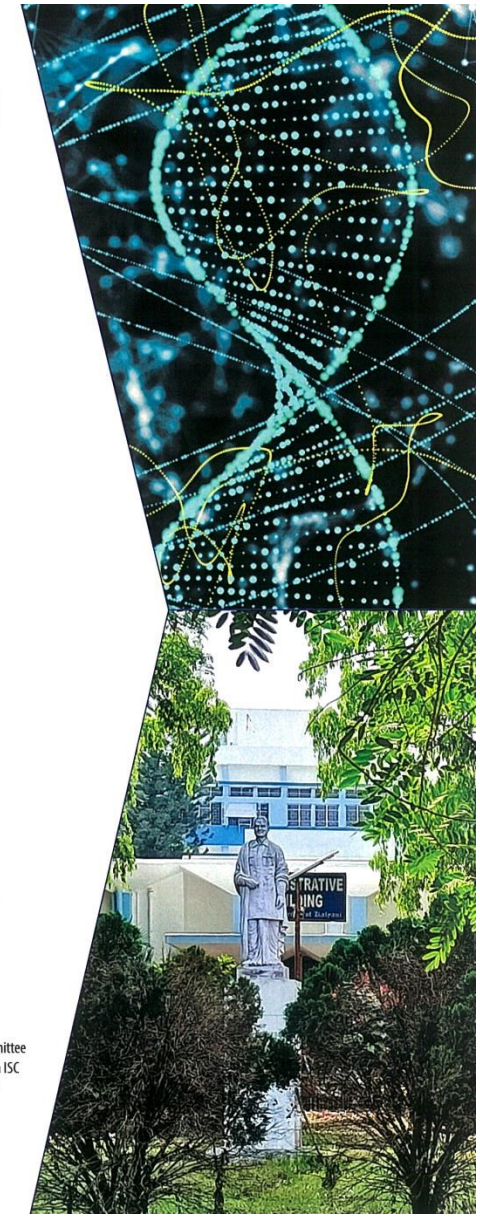

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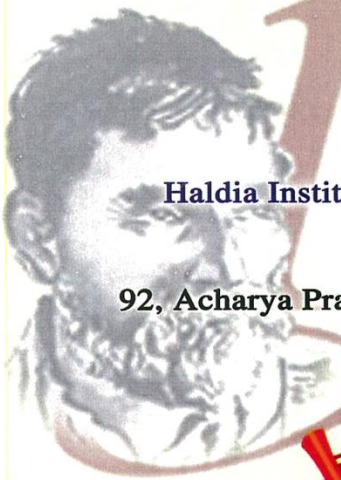

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