The medicinal implication of a Himalayan herb *Gymnadenia orchidis* Lindl against type 2 diabetes mellitus

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled**"The medicinal implication of a Himalayan herb *Gymnadenia orchidisLindl* against type 2 diabetes mellitus"** submitted by **Mrs. Tania Parvin** is an original research work conducted by her during the period of her tenure in our Department as a full-time research scholar under my guidance and supervision for the degree of**Doctor of Philosophy**.

This work or any part thereof has not been submitted elsewhere for any other degree, diploma or fellowship or other similar titles.

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DECLARATION

I do hereby declare that the work entitled in this thesis **"The medicinal implication of a Himalayan herb *Gymnadenia orchidis Lindl* against type 2 diabetes mellitus"** which is being submitted for the degree of **Doctor of Philosophy (Science)** has been carried out by me under the supervision of **Prof. (Dr.) Brajadulal Chattopadhyay** of the Biophysics Laboratory, Department of Physics, Jadavpur University, Kolkata, India. The thesis is neither in it's entirely nor any part thereof has been presented anywhere earlier for any degree or award whatsoever.

Tania Parvin

Date: 28/6/22 **(Tania Parvin)**

DEDICATION

I dedicate this thesis to my parents, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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I bow in reverence to Almighty, whose blessings enabled me to have successfully completed this thesis.

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CONTENTS

LIST OF ABBREVIATIONS

DM	:	Diabetes mellitus
LDL	:	Low density lipoprotein
HDL	:	High density lipoprotein
STZ	:	Streptozotocin
ROS	:	Reactive oxygen species
SOD	:	Superoxide dismutase
CAT	:	Catalase
GPx	:	Glutathione peroxidase
GSH	:	Reduced glutathione
WHO	:	World Health Organization
ADA	:	American Diabetes Association
FPG	:	Fasting plasma glucose
NGSP	:	National glycohemoglobin standardization program
DCCT	:	Diabetes control and complications trial
FPG	:	Fasting plasma glucose
OGTT	:	Oral glucose tolerance test
T1DM	:	Type 1 diabetes
T2DM	:	Type-2 diabetes
GDM	:	Gestational diabetes mellitus
MODY	:	Maturity onset diabetes of the young
AMP	:	Adenosine monophosphate
FTIR	:	Fourier transform infrared spectroscopy
HbA1c	:	Glycosylated hemoglobion
MS	:	Mass spectroscopy
FTNIR	:	Fourier transform near infrared spectroscopy
NMR	:	Nuclear magnetic resonance
IDDM	:	Insulin dependent diabetes mellitus
AST/SGOT	:	Aspartate transaminase/serum glutamic oxaloacetic transaminase
ALT/SGPT	:	Alanine transferase/ serum glutamate pyruvate transaminase

ALP	:	Alkaline phosphatase
GFR	:	Glomerular filtration rate
TG	:	Triglyceride
NADPH	:	Nicotinamide adenine dinucleotide phosphate
PUFA	:	Polyunsaturated fatty acid
TBA	:	Thiobarbituric acid
CAT	:	Catalase
ACP	:	Acid phosphatase
ALP	:	Alkaline phosphatase
SGOT	:	Serum glutamic oxaloacetic transaminase
SGPT	:	Serum glutamic pyruvate transaminase
EDTA	:	Ethylene diamine tetra acetic acid
ELISA	:	Enzyme linked immuno sorbent assay
THb	:	Total hemoglobin
OD	:	Optical density
DAM	:	Diacetylmonoxime
GK	:	Glycerol Kinase
NBT	:	Nitrobluetetrazolium
GSSG	:	Glutathione disulfide

LIST OF FIGURES

LIST OF TABLES

SYNOPSIS

Title of the work "The medicinal implication of a Himalayan herb *Gymnadenia orchidis Lindl* against type 2 diabetes mellitus"

Diabetes Mellitus (DM) is a syndrome characterized by abnormal insulin secretion, action or both with disarrangement in carbohydrate, lipid and protein metabolism and is diagnosed by the presence of hyperglycemia. Diabetes is a major worldwide health problem predisposing to markedly increased atherosclerosis, coronary heart disease and mortality related to development of nephropathy, neuropathy and retinopathy. Nowadays prolonged uses of synthetic drugs are costly as well as causing many adverse side-effects. To achieve complications free treatment without any side effects, our study has been designed to explore the medicinal efficacy of root extracts of *Gymnadenia orchidis* Lindl on type 2 diabetes mellitus. Type 2 diabetes was induced to Wistar albino rats and mice by subcutaneous injection of Streptozotcin (60 mg/ kg /body weight). Acute toxicity study was conducted which confirmed that root Salep was safe even when administered up to 1000 mg/ kg body weight without showing any kind of abnormal physiological features and mortality. Effective dose of root Salep of *Gymnadenia orchidis* Lindl, which showed the optimum anti-hyperglycaemic activity was determined as 200 mg/kg body weight. Terpenoid was extracted from the ethanolic root extracts by fractional distillation method and confirmed by TLC. Characterization of terpenoid was conducted by UV- VIS spectroscopy, FT-IR and Mass spectroscopy confirming the chance of presence of active compound Eugenol in the extracted terpenoid of root Salep. Root Salep (containing terpenoid) and extracted crude terpenoid both revealed the anti-hyperglycaemic effect in the STZ-induced diabetic animals by showing improvement in the glycemic profiles like fasting blood glucose, plasma insulin, total haemoglobin and glycosylated haemoglobin (HbA1c) etc. Improvement in the renal function, kidney function, liver function and lipid profile were also observed in the diabetic animals when treated with root Salep of *Gymnadenia orchidis* Lindl. Improvement in the anti-oxidant defence mechanism, liver glycogen content and carbohydrate metabolising enzyme G6PD were also observed in the root Salep treated diabetic animals. Histopathological analysis of pancreas, liver and kidney were carried out after sacrificing the experimental animals. Histopathological analysis confirmed the cyto-protective nature of *Gymnadenia orchidis* Lindl root Salep regenerating the structure and function of pancreas, liver and kidney. Terpenoid plays the key role for improving the condition in diabetic

induced rats. The root Salep of *Gymnadenia orchidis* Lindl or its terpenoids may be used as potentially herbal therapeutic agent for long term and effective solution against type-2 diabetes mellitus.

OBJECTIVE OF THE PROPOSED RESEARCH WORK

1) To study the hypoglycemic activity of *Gymnadenia orchidis* Lindl root Salep against type 2 diabetes and its toxicological role by oral supplementation in STZ induced diabetic animals (Wistar rats and mice).

2) To identify the phytochemical compounds and to isolate, identify and characterize the anti-diabetic active lead compound(s) from the roots of *Gymnadenia orchidis* Lindl thus establishing that *Gymnadenia orchidis* Lindl as a potent therapeutic agent against type 2 diabetes mellitus

3) To study the anti-diabetic activity of the isolated active lead compound(s) of *Gymnadenia orchidis* Lindl on STZ-induced Wistar rats and mice by assessing the biochemical parameters.

4) To assess the effect of isolated active compound(s) on restoration of histopathological alterations in major organs (liver, kidney and pancreas etc.) of STZ-induced Wistar rats and mice and confirmation of cyto-protective activity of nature of the active compound(s) in diabetic conditions.

5) To assess the action of the active lead compound of *Gymnadenia orchidis* Lindl in regulation of blood glucose level.

CHAPTER 1

General introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia resulting from the defects in pancreatic insulin secretion, insulin action or insulin receptor on peripheral tissues [1]. Due to deregulations in carbohydrates, proteins and fats metabolism, elevated blood glucose level from pancreatic β-cell damage [2], diabetes mellitus causes long term microvascular and macrovascular complications like retinopathy, nephropathy, neuropathy and cardio vascular diseases thus causing maximum death worldwide [3]. Deficiency in insulin action may result from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action [4]. Disparity between generation of oxygen free radicals and endogenous antioxidant defence system in diabetes results in increased oxidative stress thereby manifests in severe diabetic complications [5]. Due to sedentary lifestyle and food habits, diabetes is taking an ever-increasing pandemic form in developing countries thus causing surge in incidence of diabetes mellitus [6].

The global prevalence of diabetes for all range of age-groups in 2000 was estimated to be 2.8% and expected to reach 4.4% in 2030 [7]. In India number of diabetics patients was 40.6 million in 2006 and will increase to 79.4 million by 2030[8]. Diabetic patients suffering from kidney diseases have exceptionally high rates of cardiovascular morbidity and mortality [9]. Although diet, physical activity, synthetic oral hypoglycemic drugs and insulin therapy helps in management of diabetes but conventional synthetic drugs for diabetes available in the market causes various side effects like weight gain, gastrointestinal disturbances, hypoglycemia, hypersensitivity,rise in hepatic enzymes, hematological diseases, liver and heart failure, nausea and diarrhoea etc.Nowadays it has become a potential challenge near

medical fraternity to control diabetes without causing any side effects [10]. Further, administration of these synthetic drugs has completely failed to serve the reduction of glycemic control in diabetic patients [11]. Clinical studies also reported that herbals medicines along with diet, alternative and complementary therapies recovers the homeostasis of glucose metabolism in diabetic patients [12]. Herbal extracts enriched with phytochemicals like flavonoids, alkaloids, phenolic compounds, terpenoids, glycosides and coumarins helps in the regulation of carbohydrates metabolism efficiently thereby combating the deleterious effects of diabetes with fewer side effects [13]. Medicinal plants have been extensively used by the traditional medicine practitioners for the control, management and/ or treatment of diabetes and nowadays recognised as an alternative treatment for managing diabetes [14,15]. Various investigations also established that phytochemicals extracted from medicinal plants also helps in in prevention and treatment of various diseases like diabetes, cancer, heart disease, high blood pressure, etc.It is also reported that when diabetic patients are supplemented with polyphenol-rich herbal extracts like green tea extract, pomegranate extract, and ascorbic acid etc, LDL reduces and HDL increases thereby favouring the prevention of cardiovascular and other complications in diabetic patient thus favoring herbal extracts as an reliable source for new drug development [16]. *Gymnadenia orchidis* Lindl is one kind of antidiabetic perennial herbs belongs to the *Orchidaceae* family. *Orchidaceae* family is highly developed and considered as the largest family of plant kingdom. This plant is locally known as Himalayan Fragrant Orchid / Panchamlay (N). This whole plant is used by the local Bhutia people as a folk medicine against diabetes and the tubers of this plant is mainly eaten by the local people as it has some medicinal usage like astringent, demulcent and highly nutritious. The tuber is eaten with honey as an aphrodisiac and tonic. It is also useful in gastric, liver and urinary disorders [17]. Powdered forms of roots, pseudobulbs are also used in treating minor cuts and wounds.

Gymnadenia orchidis Lindl is enriched with various kind of anti oxidative phytochemical constituents like terpenoids, polyphenols, steroids, cardiac glycosides, tannins, vitamin C, carbohydrates and proteins [18].

Till date so far no scientific report has been published using extracts of *Gymnadenia orchidis* Lindl for its antidiabetic effect against streptozotocin induced type 2 diabetes. Although long and traditional usage of *Gymnadenia orchidis* Lindl is known but no systemic studies has been conducted on hypoglycemic, hepatoprotective, hypolipidemic, antioxidant, nephroprotective activity, cytoprotective activity of these drug. Hence our main objective was to elucidate the antihyperglycemic, antilipidemic and antioxidant efficacy of terpenoid rich extracts *Gymnadenia orchidis* Lindl in restoration of pancreatic islets function in diabetic condition and on biochemical and enzymatic parameters of STZ induced diabetic rats and mice models. Our study has also focused on the histopathological alterations of the pancreas, liver, kidney of terpenoid treated STZ induced diabetic animals and established the cytoprotective nature of terpenoid in diabetic conditions. Therefore our study has confirmed that root extracts enriched with terpenoid is safe and effective against type 2 diabetes. So our future work will emphasize on formulation of safe and complication free drug using Eugenol (terpenoid) by further clinical investigations.

CHAPTER 2
Review of Literature

Diabetes mellitus

Diabetes mellitus is a group of metabolic disorders caused by deficiencies in pancreatic insulin secretion, decreased insulin sensitivity to peripheral tissues or both leading to deregulations in carbohydrates, proteins and fats metabolism resulting in hyperglycaemia [19]. Diabetes is often known as disease of three poly's like Polyuria, polydipsia and polyphagia [20]. Diabetes causes the development and progression of various micro vascular (involving small vessels, such as capillaries) and macrovascular (involving large vessels, such as arteries and veins) diseases. Macro vascular complications include coronary artery disease, peripheral arterial disease, and stroke and microvascular complications include diabetic nephropathy, neuropathy, and retinopathy. Diabetic vascular complications are initiated mainly by chronic hyperglycaemia and insulin resistance and these complications involves several mechanisms : (i) amplified formation of Advanced Glycation End-Products (AGEs) activation of the Receptor for Advanced Glycation End-Products (RAGE) AGE-RAGE axis, (ii) oxidative stress, and iii) inflammation [21].

Diabetes leads to diabetic complications which are associated with oxidative stress persuaded by free radical generations [22]. Diabetes cause chronic complications, dysfunction, and loss of multiple organs, especially the eyes, kidneys, nerves, heart, and blood vessels happen which leads to disability and premature death thus resulting in an increase in mortality and morbidity [23]. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes [24]. Excessive production of reactive oxygen species (ROS) causes cell damage leading to diabetic complications and these damages are combat by the antioxidant enzymes like superoxide dismutase (SOD), catalase

(CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) by scavenging the free radicals [25].

Prevalence of diabetes

Diabetes has nowadays in the whole world has turned into pandemic form. According to the World Health Organization major burden of diabetes will occur in the developing countries and there will be a 42% increase (from 51 million to 72 million) in the developed countries and 170% increase (from 84 to 228 million) in the developing countries [26]. Survey conducted by World Health Organization and American Diabetes Association in several countries reported that in 2010, 285 million people were diabetic and this will increase to 439 million by the year 2030 [27]. Number of diabetic patients are increasing at an alarming rate in India, 40.6 million people were affected by diabetes in 2006 and 79.4 million people will be diabetic in 2030 [28].

This alarming increment in the incidence of type-2 diabetes is mainly due to unprecedented rates of urbanization, which results in environmental and lifestyle changes. In India more than 62 million people have been diagnosed with diabetes in the year 2014 and gaining status of potential epidemic [29]. From the last three decades diabetes has been increasing faster in low and middle income countries in comparison to higher income countries and many associated risk factors like obesity, overweight etc. are also increasing. Uncontrolled or untreated diabetes may lead to kidney failure, blindness, lower limb amputation and other long-term consequences which affects extensively on the quality of life [30]. WHO also has projected that diabetes will be responsible for the seventh leading cause of death in worldwide [31].

Pre- diabetes

According to American diabetes Association (ADA) pre-diabetes are those individual who comprises borderline glycaemia measured by any of three measures-fasting plasma glucose (FPG) 100-125 mg/dL (5.6-6.9 mmol/L), 2-h plasma glucose 140-199 mg/dL (7.8-11.0 mmol/L), or HbA_{1c} 5.7-6.4% (39-46 mmol/mol) [32]. Here the person is always at risk of developing diabetes. Those individuals are also at risk of cardiovascular disease.

Requirement for diagnosis of diabetes mellitus

According to American diabetic association condition for diagnosis of diabetic are:

1. HbA1c ≥ 6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP **(**National Glycohemoglobin Standardization Program**)** certified and standardized to the DCCT **(**Diabetes Control and Complications Trial**)** assay.*

Or

2. FPG (Fasting plasma glucose**)**>126 mg/dl (7.0 mmol/L). Fasting is defined as no calorie intake for at least 8 hr.

Or

3. 2 hr plasma glucose ≥ 200 mg/dl (11.1 mmol / L) during OGTT (Oral glucose tolerance test).The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75-gm anhydrous glucose dissolved in water.

(In the absence of unequivocal hyperglycaemia, criteria 1-3 should be confirmed by repeat testing)

Or

4. In a patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥ 11.1mmol/L (≥200 mg/dL).

	HbA1c (percent)	Fasting Plasma Glucose Level (mg/dl)	Oral Glucose Tolerance Test(mg/dl)
Diabetes	6.5 or above	126 or above	200 or above
Prediabetes	5.7 to 6.4	100 to 125	140 to 199
Normal	About 5	99 or below	139 or below

(Adapted from American Diabetes Association. Standards of medical care in diabetes-2012.*Diabetes care*.2012; 35 (Supp 1): S12) [33].

Types of Diabetes

On the basis of etiology and clinical presentation diabetes mellitus are classified into four classes. They are as follows:-

I. Type-1 (Insulin dependent diabetes mellitus)

II. Type-2 (Non-insulin dependent diabetes mellitus)

III. Gestational diabetes mellitus

IV. Other specific types

- **Type-1 diabetes (T1DM)**

Type-1 diabetes mellitus is an insulin dependent process where irreversible destruction (T-cell mediated autoimmune attack) of the insulin secreting pancreatic beta islet cells occur thus causing insufficient insulin production in the body [34]. As a result pancreatic beta cells become less responsive to all insulin secretory stimuli, rate of glucose uptake decreases into the muscles and adipose tissue. T1DM patients are more susceptible to ketoacidosis and they need exogenous insulin for survival and to decrease blood glucose level. T1DM commonly found in children and adolescents [35]. It usually occurs after 30

years of age, but the peak incidence of this disease occurs during puberty, around 12-14 years in boys and 10-12 years of age in girls. The symptoms of type-1 diabetes are hyperglycemia, ketoacidosis, increased thirst, hunger, frequent urination, unusual weight loss, extremes fatigue and irritability.

- **Type-2 diabetes (T2DM)**

Type-2 diabetes (non-insulin dependent diabetes or adult onset diabetes) most commonly form of diabetes is mainly characterized by decreased insulin secretion, insulin resistance and excessive hepatic glucose production. Insufficient insulin secretion from pancreatic beta cells in T2DM manifests insulin resistant in body, disturbing blood sugar level thus causing hyperglycemia and glycosuria [36]. In T2DM all the target tissues becomes less responsive to the normal circulating levels of insulin in the blood [37]. In diabetic condition, insulin resistance leads to elevated free fatty acids in plasma, decreasing glucose transport into muscle cells thus increasing hepatic glucose production, lipolysis and proteolysis. Person having type-2 diabetes do not need insulin treatment initially and often throughout lifetime to survive.Treatments for type-2 diabetes includes diet, exercise, medication and insulin therapy. About 90% people are affected with T2DM which totally occur among adults but nowadays it is also increasing among children (due to child obesity) [35]. The increasing proportions of the aging population, consumption of calorie rich diet, obesity and sedentary lifestyle have led to a tremendous increase in the number of diabetics worldwide. Cardiopathy, neuropathy, retinopathy etc. are the outcomes of type-2 diabetes. The symptoms of type-2 diabetes are thirst, frequent urination, extreme hunger, abrupt weight gain, irritability and fatigue, blurred vision and noticeable signs of diabetes like decelerated healing, recurrent gum and bladder infections, skin and yeast infections. In some cases, there may be no symptoms. Other symptoms of T2DM are vaginal infections in women, sexual dysfunction in Men, Numbness/Tingling in hands and feet and Itchy or Flaky Skin.

- **Gestational diabetes mellitus (GDM)**

Pregnancy induced diabetes or gestational diabetes is characterized by carbohydrate intolerance of variable intensity with onset or first recognition during pregnancy due to inadequate insulin secretion and relative response like type-2 diabetes [38]. Gestational diabetes may improve or disappear after delivery and it occurs in about 2%-5% of all pregnancies.

- **Other specific types of diabetes**

For specific condition, a few rare kinds of diabetes can be found. These types of diabetes account for only 1% to 5% of all cases of diabetes [39].

1. Diseases and defects of the exocrine pancreas (e. g. Chronic pancreatitis, Cystic fibrosis etc)
2. Drug induced (e. g. glucocorticoids, alpha-interferon's, Thyroid hormone, Statins) [40].
3. Genetic defects of the β-cell function (e.g. MODY form Maturity onset diabetes of the young)
4. Genetic defects of insulin action-defects in insulin processing/insulin action, in proinsulin conversion, Insulin gene mutations, insulin receptor mutations.
5. Infections - Cytomegalovirus infection, Coxsackie virus B. Certain viruses have been associated with β-cell destruction.

Conventional synthetic anti-diabetic drugs

All the conventional anti-diabetic drugs treat diabetes by reducing the glucose level in blood. Except insulin, Exenatide, and Pramlintide all other drugs are administered orally to patients as oral hypoglycemic drugs. According to the nature of diabetes, age and condition

of the patients and other factors various drugs are chosen for the treatment. In type-1 diabetic patients (insulin deficient) insulin is administered either by injection, inhalation or by pump method. In type-2 diabetes, body becomes insulin resistant so anti-diabetic agents are administered orally so they can increase the pancreatic insulin secretion, increases the sensitivity of the target organ to insulin and decreases the glucose absorption rate from the gastrointestinal tract. Various synthetic oral drugs are available for diabetes treatment which exerts hypoglycaemic effects through various mechanisms and they are sulfonylureas (e.g., Tolbutamide), biguanides (e.g., Metformin), α-glucosidase inhibitors (as Acarbose and Miglitol), thiazolidinediones(e.g., Pioglitazone and Rosiglitazone), and non-sulfonylureas secretagogues [41]. Oral sulfonylureas (e.g., glimepiride and glyburide) reduces the blood sugar level by increasing insulin release from islets of Langerhans cells through binding with cell's sulfonylurea receptor which confers closing of adenosine triphosphate-dependent potassium channels. Therefore depolarization of cell membrane causes calcium influx, secretory granules secretes stored insulin within the cells which are mediated by insulin [42-43].

Besides these available drugs, insulin therapy is also implemented for diabetes treatment. Administration of insulin is done by three primary methods and they are injection (subcutaneously), inhalers or by pumps method. In some cases insulin is also administered intravenously in critical condition.

Metformin is a kind of biguanides/insulin sensitizers e.g. which reduces blood glucose level by decreasing hepatic glucose production, reducing intestinal absorption of glucose and thereby enhances insulin sensitivity by peripheral glucose uptake and utilization [44]. Glucose tolerance level is improved by Metformin in type-2 diabetic patients by reducing basal and post prandial plasma glucose level. Metformin exerts glucose lowering effect by inhibiting hepatic mitochondrial respiratory chain, activating AMPK, improving insulin

sensitivity (via effects on fat metabolism), lowering cAMP, reducing expression of gluconeogenic enzymes and opposing glucagon action. The AMPK-independent effects of Metformin on liver cell are mediated by inhibition of fructose-1, 6-bisphosphate by AMP [45]. Therefore in current clinical use, Metformin is most popular prescribed insulin sensitizing agent as it facilitates disposal of glucose in skeletal muscle by insulin stimulated process and regulation of blood glucose level by insulin mediated suppression of hepatic glucose production [46].

Besides those drugs, diabetes can be managed by making certain changes in lifestyle like proper knowledge about diabetes, regular checkup, monitoring diabetes, following proper treatment for long term diabetic problems and exercises. Exercise helps in reducing stress, keeps blood sugar level at control, helps in losing weight, reduces the cholesterol level and blood pressure, etc., helps in increasing tissue sensitivity to insulin and helps in utilization of glucose from blood to targeted cells. Convention synthetic oral hypoglycemic drugs along with insulin therapy have been used to control diabetes but they have encountered complications and unwanted side effects like gastro intestinal discomfort, nausea, diarrhea, weight gain, severe hypoglycemia, hypersensitivity, liver and heart failure [47]. Other drawback of synthetic drugs are reduced therapeutic potency due to inappropriate or futile dosage regimen, low potency and altered side effects due to drug metabolism and lack of target specificity, solubility and permeability [48]. Thus managing diabetes without any adverse side effects has become a challenge to the medical fraternity [49]. Also present diabetic treatments are expensive and synthetic drugs cannot meet the demands of increasing number of patients in near future. Diabetes is creating economic burden in accordance to its health system costs, decreasing quality of life, increasing mortality rate and loss of loved ones thus creating a void in the family. Therefore opportunities are coming more for exploration of newer anti-diabetic drugs from naturally occurring resources [50]. Nowadays opportunities

are coming to explore certain active compounds from plants which can be used as an effective and safe treatment against diabetes.

Medicinal plants as an alternative medicine for diabetes treatment

Medicinal plants are source of active therapeutic constituents as it provides remedies for different ailments and diseases. Queries for discovering lead active hypoglycaemic compound from plant always play the crucial role in the imminent drug development program [51-53]. Plant derived herbal preparations are easily available, inexpensive with less side effects and gaining importance as alternative therapeutic options in developing and underdeveloped countries where modern treatment methods are expensive [54]. Many medicinal plants are enriched with bioactive chemicals free from detrimental side effects and acquire potent pharmacological activity [55-61], providing direct or indirect source for formulating currently available drugs [62,56-58]. According to WHO recommendation, researches on medicinal plant derived anti-hyperglycaemic drugs are gaining popularity and ethno botanical data has reported that about 800 plant species may possess anti-diabetic properties [64] and some plants are useful in diabetic treatment due to confirmation as anti-diabetic, anti-inflammatory, anti-oxidant, anti-hyperlipidemic remedies [64-65]. Anti-hyperglycaemic properties of these plants are due to their ability to preserve pancreatic tissue function by increasing insulin production, inhibiting glucose absorption in the intestine, or facilitating metabolites in insulin-dependent processes [64]. Medicinal plants enriched with antioxidants, nutrients, phytochemicals like flavonoids, phenolic compounds, terpenoids, alkaloids, glycosides, carotenoids, coumarinsetc [66] can regulate carbohydrates metabolism [67] efficiently thereby combating the deleterious effects of diabetes with fewer side effects.

As phytochemicals are naturally found in bark, leaves, stem, root, flower, fruits, seeds, etc. [68], so qualitative and quantitative phytochemical analysis of medicinal plants are

done to extract the desired drug or active component [69]. Vaghasiya *et al.,* (2011) done the qualitative phytochemical analysis for various phyto constituents like alkaloids, tannins, cardiac glycosides, steroids and saponins of 53 types of medicinal plants from Western region of India [70]. Yadav *et al.* (2011) also done the phytochemical estimation of seven medicinal plants selected from North-eastern region of India and determine the total phenolic and flavonoid contents of the selected medicinal plants [71]. Clinical studies have also confirmed that plant extracts efficiently maintained glucose homeostasis, along with following proper diet, an alternative and complementary therapy in diabetic patient [72-73]. Today consciousness regarding natural products is increasing as it is potentially safer options for medicines with negligible side effects in comparison to semi-synthetic conventional drugs. Comprehensive studies for the development of potent, efficient natural bioactive leading compound is required which has specific molecular targets for diabetes and thus helping in diabetes treatment. Therefore herbal medicines as an alternative treatment for diabetes have opened a new opportunity for the scientific researchers throughout the world. Some important medicinal plants like *Aeglemarmelos* (L.) Correa (Wood apple), *Coriandrum sativum*L. (Coriander), *Zingiberofficinale* Roscoe (Ginger), *Syzygium cumini* (L.) Skeels (Black plum), *Murraya koenigii* (L.) Spreng (Curry tree), *Gymnema sylvestre* (Retz.) R.Br. ex Sm., *Phyllanthus emblica* L. (Indian gooseberry), *Cinnamomum Verum* J. Presl (Cinnamon tree), *Momordica charantia* L. (Bitter melon), *Ocimum tenuiflorum* L. (Holy basil) etc have shown antidiabetic properties for herbal formulation of drugs [73].

Plant profile

- ### Plant description: *Gymnadenia orchidis* Lindl

Gymnadenia orchidis Lindl is one kind of anti-diabetic perennial herbs belongs to the *Orchidaceae* family which is highly developed and considered as the largest family of plant kingdom [17,74-77]. This is a terrestrial plant and mainly found in Asia at an altitude of 3000 to 4400 m. It is commonly known as Himalayan Fragrant Orchid / Panchamlay (N) by the local people.

- ### Taxonomical status

The taxonomic classification of *Gymnadenia orchidis* Lindl is as follows:

Kingdom : Plantae

Division : Tracheophyta

Class : Liliopsida

Order : Asparagales

Family : Orchidaceae

Genus : Gymnadenia

Species : *Gymnadenia orchidis* Lindl

- ### Geographical distribution

This plant is mainly found in the subtropical, temperate and alpine regions of Asia including India (North East India, Sikkim, West Bengal); Bhutan, China and Nepal at an altitude of 3000 to 4400 m. This species mainly spread in the Himalayas from Jammu and Kashmir to Sikkim [78-82]. In Darjeeling Himalaya region it is mainly found in the Sandakphu, Phalut forest.

- **Ethno botanical uses**

The tubers of plant are highly nutritious and used as astringent, demulcent. It is used in treatment of gastric, liver and urinary disorders. The tuber is eaten with honey as an aphrodisiac and tonic [82].

Morphological characteristic of *Gymnadenia orchidis* Lindl

Gymnadenia orchidis Lindl is a terrestrial plant that grows upto 60 cm in height. This is a perennial herb and its tuberous root is divided into 2 to 3 lobes. Tubers are ovoid-ellipsoid in shape and stout. Stems are stout, leafy and sheathed. Number of sheaths may vary from 1 to 2, unequal, tubular-conicular in shape, 4-9 cm long, sub acute. Number of leaves may vary from 3 to 8. Shape of the lamina is elliptic oblong to linear-oblong, 4-1 7. 5 x 1.5-4.5 cm, acute to subacute, scattered, slightly narrows to long sheath. Inflorescence of this orchid is many flowered, raceme is lax or dense, peduncle is short in size, and length of rachis is 3-14 cm long. The floral bracts are lanceolate, 0.7-2.7 cm long, acute, and longer than the stalked ovary, often papillose. Flowers are pinkish in color, 3-10 mm across in size, often papillose, sepals are subequal and 3-nerved; dorsal sepal is elliptic-oblong or lanceolate in shape, and size is 2-5 x 1-3 mm, obtuse or acute angled and concealed with petals. Sometimes lateral sepal joined to the column to form a saccate, conical or spur-like mentum. Lateral sepalis ovate to ovate-lanceolate in shape, 2.5-6 x 1.5-3 mm, obtuse or subacute angled. Shape of petals is rhombic orbicular to broadly ovate, 1.5-4 x 1-3.5 mm, acute angled; lip is variable and 3-lobed at apex, shape of lip is broadly oblong, size is 2.5-5 x2-4 mm; mid-lob is prominent or obsolete in nature and acute to rounded, generally papillose throughout the surface, spurred; spur is narrow, slender and size is 0.6-1.7 cm, equal in shape or larger than ovary. This spur generally found curved bent forward with variable angle or remain twisted; column ca. is 2 mm long; shape of pollinia is ovoid; caudicle is slender [83].

Phyto-constituents of *Gymnadenia orchidis* Lindl and its usage as medicine

Gymnadenia orchidis Lindl is enriched with phytochemical constituents like alkaloids, terpenoids, steroids, cardiac glycosides, tannin, polyphenols, vitamin C, carbohydrate (s), and protein [84]. Flavonoids, alkaloids, saponin and free amino acids were not detected in the root Salep. Qualitative analysis of the root revealed the presence of these phytochemical constituents. The plant roots are traditionally used against gastric, gonadic and urine disorders [82] and also used in the effective treatment of wounds [85]. The plant roots are grinded and mixed with water forms a thick 'Salep' which is traditionally used by the local people to get some relief against diabetes. But there is no scientific study on this orchid that establishes the role of this plant on diabetes. Toxicological studies on rats have already proven that root Salep is safe and can be used in edible form [84]. The optimum dose of root Salep (200mg/ kg body weight)plays a major role in normalising the levels of blood glucose, increases the body weight in STZ induced diabetic rats and improve the liver, kidney, pancreas function and lipid profile [84]. Our study has confirmed that active component i.e., terpenoid (Eugenol) might play the major role controlling the blood glucose level and normalising other biochemical functions. Eugenol (terpenoid) was extracted from ethanolic fraction of powdered root of *Gymnadenia orchidis* Lindl root by fractionation method in a separating funnel using pet ether and distilled water as a solvent.Extracted terpenoid was characterized by UV-Vis, FTIR and Mass spectroscopy methods.

Chemical structure of Eugenol (alcoholic terpenoid)

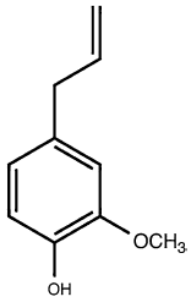


Figure 1:- Structure of Eugenol

Chemical formula:	$C_{10}H_{12}O_2$
Class:	Phenylpropanoid
IUPAC name:	4-Allyl-2-methoxyphenol
Molecular mass:	164.2 g mol^{-1}
Solubility in water:	Partially soluble
Solubility in organic solvents:	Highly soluble
Boiling point:	254 °C (489 °F; 527 K)
Acidity (Pka):	10.19 at 25 °C
Melting point:	−7.5 °C (18.5 °F; 265.6 K)
Color:	Clear to pale yellow
Body metabolism:	Absorption *via* small intesti
Excretion and elimination:	through urination and as expired CO_2 [90]

Eugenol is a kind of phenolic compound belonging to phenyl propanoids class generally extracted from essential oils of clove tree cinnamon tree, bay leaf, nutmeg, tulsi and many other plants [86]. Eugenol is pale yellow coloured, oily liquid aromatic in nature [87]. Significant amount of Eugenol is found in extracts of medicinal herbs and due to its

pharmacological properties it is gaining importance among the researchers. Eugenol has the property of strong analgesics, antibacterial, antifungal, anti-inflammatory, anti-carcinogenic effects, hypolipidemic and anti-diabetic activity, anti-hypertensive and neuroprotective activities [87]. World Health Organization (WHO) has already declared Eugenol as GRAS (generally recognized as safe) and non-mutagenic [86]. Eugenol can be extracted by steam distillation, Solvent extraction [88], Hydro distillation [89-90], Microwave-assisted extraction of Eugenol [91], Supercritical carbon dioxide extraction [92], Ultra-sound assisted extraction of Eugenol [93-94]. Extracted Eugenol can be confirmed and identified by administering modern technique like spectroscopic techniques like Fourier transform infrared spectroscopy (FTIR), Mass spectroscopy (MS), Fourier transform near infrared spectroscopy (FTNIR), and nuclear magnetic resonance (NMR) [95].

Role of terpenoids as well as Eugenol as antidiabetic agent

Terpenoids (plant secondary metabolites) are derived from repeated units of isoprene with vast structural diversity [96] and classified into monoterpenes, hemiterpenes, diterpenes, sesquiterpenes, sesterpenes, tetraterpenes, triterpenes, and polyisoprenes and have the ability to prevent various diseases and possess many significant properties [97]. Terpenes exhibits antioxidant properties, insulin mimetic activities, promotes insulin secretion from existing β-cells, decreases the intestinal glucose absorption, decreases α-glucosidae activity and also increases the insulin signalling pathway [98]. Seikh *et al.*(2015) showed that Trans-anethole (terpenoid) isolated from petroleum ether fraction of the *Foeniculumvulgare* extracts improves blood glucose level, lowering levels of lipid profile, HbA1c etc in STZ induced diabetic rats [99]. Motaal *et al.* (2011) showed that terpenoids isolated from chloroform fraction of *Cleome droserifolia* enhanced the basal glucose uptake from cultured C2C12 skeletal muscle cells and 3T3L1 adipocytes as insulin like property is seen in the peripheral tissues [100]. Eugenol also shows *in vitro* anti-diabetic activity by inhibiting pancreatic α-

amylase, lipase as well as ACE activity and reduces amylase in serum, pancreas and intestine [101]. Srinivasan *et al.* (2014) reported that Eugenol helps in reducing fasting blood glucose level in diabetic rats by enhancing insulin secretion from existing β-cells and improves glucose utilisation by the target tissues, also improves plasma level and amount of immuno-reactive insulin secreting pancreatic cells gets increased which is confirmed by histopathologial investigations[102]. Eugenol improves the hexokinase activity in diabetic animal by preventing STZ- induced hepatic tissues damage [102-103], helps in elevating haemoglobin (Hb) levels by reducing the formation of glycosylated hemoglobion (HbA1c) [102-104].

Eugenol can reduce the activity of glucose-6-phosphatase and fructose-1,6-bisphosphatase (gluconeogenicenzymes) which are responsible for increased hepatic glucose production(gluconeogenesis) in liver and kidney of diabetic rats thus normalizing the glucose metabolism by amplifying utilization of glucose and reducing hepatic glucose production through insulin secretion [102,107].

Mechanisms of STZ action

STZ is an antibiotic produced by the bacterium *Streptomyces achromogens* and is used to induce both insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) in experimental animals [105]. The glucose molecule (in deoxy form) of streptozotocin linked to an extremely reactive methyl-nitroso-urea moiety exerts cytotoxic effect by directing the chemicals to the pancreatic β cells. STZ enters pancreatic β cell through GLUT2 receptors and generates reactive oxygen species like hydroxyl radical and exerts cytotoxic effects to β-cell. STZ causes pancreatic β-cell's DNA damage by inducing activation of poly ADP–ribosylation (poly ADP-ribose polymerases/PARPs) which causes depletion of cellular NAD and ATP and leading toβ-cell dysfunction and possible

necrosis [106]. Due to existence of GLUT2 in liver and kidney, high doses of STZ could also impair the functions of liver and kidney [107].

After inducing diabetes in experimental animal's we have conducted studies to investigate the efficacy of the desired plant extracts of *Gymnadenia orchidis* Lindl on STZ induced diabetic animals by investigating the following parameters like glycemic control, glycosylated hemoglobin, hemoglobin concentration, liver function enzymes like AST, ALT, acid phosphatase, alkaline phosphatase, protein estimation, kidney function parameters, lipid profile, antioxidant enzymes, G-6-PDH level (carbohydrate metabolism enzyme), insulin level, glycogen content in coordination with histological investigations of liver, kidney and pancreas.

Plant derived herbal medicine if taken above certain concentration then it is toxic to the body. So it is necessary to rule out the effective concentration of the desired drug to get optimum results without side effects. Finding proper concentration of root Salep of *Gymnadenia orchidis* Lindl is a major role in diabetes treatment. Investigation of effective dosage of the desired drug for standardizing the optimum activity of the drug is mandatory. So to find the optimum dose acute toxicity studies of root Salep of *Gymnadenia orchidis* Lindl was carried out in Wistar rats.

Acute toxicity studies

Medicinal plants are rich source of valuable phyto-constituents which paved the way for novel drug development. Nevertheless, accurate proof is available regarding the possible toxicity of the medicinal plant products used by the consumers [108]. In the path of drug discovery and development, concerns of several groups like pharmaceutical industry, health personals and patients taking natural drugs should be taken into consideration [109]. Due to unwanted side effects of synthetic drugs, patients are giving importance to natural derived

herbal medicine as these medicines are efficient, easily accessible with low toxicity and have been used in traditional medicine from long time. However many studies have already showed that medicinal plant products may also reveal adverse side effects on the people [110-111]. Therefore emphasis is also given that every plant cannot be used for medicinal purpose as safety of such plant is not guaranteed. Short term or long term use of any medicinal plants for therapeutic purposes is now raising concern regarding their toxic side effects. Lead compounds isolated from medicinal plants should be properly investigated and their acute and sub chronic toxicity should be evaluated before formulating them as drug for safer human consumption [112]. Assessment and authentication of toxicological effects of medicinal plant derived lead compounds is necessary before using them for animal or human consumptions [113].

Blood glucose monitoring

Blood glucose monitoring is necessary in Streptozotocin induced diabetic rats to assess whether diabetes has been induced or not. This monitoring helps in both hypoglycemic and hyperglycemic state by providing rapid information about glucose level and helps in adjusting glucose to a near-normal range [114]. The normal range of blood glucose is from 70 to 100 mg/dL (2·5 to 7·3 mmol/L). Blood sugar level beyond 50 mg/dL is hypoglycaemic and blood sugar levels higher than 120mg/dl is hyperglycaemic. Various techniques have been developed for measuring blood glucose like:-1) Invasive methods, 2) Minimally Invasive methods, 3) Non-invasive methods [115]. Glucometer is one of the most conventional invasive methods, widely used in hospitals, outpatient clinics, etc and in home self-monitoring. In experimental diabetes blood sample is collected by pricking clean tail vein and test strip is hold near the blood drop and glucose level is expressed in glucometer. Working principle of this glucometer depends on the enzymatic reaction of glucose with enzyme glucose oxidase/ glucose dehydrogenase/ hexokinase. Blood glucose which is sucked

through the capillary tube reacts with glucose oxidase present in the test strip and forms gluconic acid which is detected in the glucometer. The monitoring of blood sugar in diabetic patient is done at two stages: - pre-prandial plasma glucose (before meal) and postprandial glucose (after 2 hours of meal). The level of pre-prandial plasma glucose is 80-130mg/dl (4.4-7.2 mmol/L) and level of post-prandial plasma glucose is less than 180mg/dl (< 10.0 mmol/L). P. Akpojotor *et al*. (2021) used Accu-Chek Active blood glucose meter and Accu-Chek Active test strips (Roche Diabetes Care GmbH SandhoferStrasse 116 68305 Mannheim, German) to measure the blood glucose in STZ-induced diabetic male wistar rats after 72 hrs of streptozotocin administration by collecting blood from the tail vein [116].Roy *et al*(2021) has used Glucometer (Wockhardt Ltd. Mumbai) and Pulsatom blood gluco-strip to measure the blood glucose in STZ induced diabetic animals [117].

Glycosylated haemoglobin

Glycosylated haemoglobin (HbA1c, A1C, or commonly known as HbA_{1c}, $HgbA_{1c}$, or Hb1c, etc.) is haemoglobin molecule where glucose molecule (excess in blood) condensed non-ezymatically with the N-terminal valine residue of the hemoglobin beta chain (glycation process) [118]. This conversion occurs throughout the life span of red blood cells (about 120 days). Measurement of HbA_{1c} is done for the average last three months blood sugar level to diagnose diabetes. Higher concentration of HbA_{1c} indicates low glycemic control in diabetic patient and its association with various diseases [119]. The normal range of HbA_{1c} level for non-diabetic's people is between 4% and 5.6%. An HbA1c level between 5.7% and 6.4% indicates a higher chance of getting diabetes. If the levels of HbA_{1c} is 6.5% or higher then it indicates diabetes. Due to glycosylated haemoglobin in diabetes, highly reactive free radicals formation alters the blood cell membrane properties causing blood cell aggregation, increases blood viscosity as a result blood flow is impaired. It also causes inflammation resulting in atherosclerotic plaque (atheroma) formation [120].Measurement of HbA_{1c} in laboratory is

done by several techniques like high-performance liquid chromatography, enzymatic assay, capillary electrophoresis or boronate affinity chromatography. People with shortened red blood cell life spans show low level of HbA_{1c} due to glucose-6-phosphate dehydrogenase deficiency (causes anemia), sickle–cell disease or any other condition causing premature cell death. Hasan *et al.* (2016) have shown that Saudi date seed extract given to STZ induced experimental rat's causes reductions in blood sugar level and glycosylated percentage [121]. Malini *et al.* (2011) evaluated the antidiabetic effect of ellagic acid in streptozotocin induced diabetic rats and showed that ellagic acid helps in reducing the glycosylated haemoglobin of treated diabetic animal compared to diabetic control [122]. Nabietal *et al.* (2013) showed that aqueous extract of *Piper longum* root given to STZ induced rats reduced HbA_{1c} level compared to untreated group [123].

Estimation of haemoglobin

Haemoglobin (Hb) is the iron-containing metalloprotein found in RBC, helps in transporting oxygen from the lungs to the rest of the body (like muscles), and releases oxygen which is utilized in aerobic respiration providing energy for functioning of organism (metabolism). It is important to monitor the amount of hemoglobin in RBC as too little hemoglobin causes weakness, shortness of breath. Anaemia occurs due to blood loss or decrease in red blood cell production in the bone marrow or impaired production of red blood cells. Development of anaemia is associated with diabetes-related kidney disease (nephropathy) and nerve damage (neuropathy). So estimation of haemoglobin is necessary in diabetes. Many researchers have done the estimation of haemoglobin in STZ induced diabetic animals treated with plant extracts and they have shown plants extracts are good in increasing the total haemoglobin. Sabitha *et al.* (2011) showed that peel and seed powder *of Abelmoschus esculentus* L. When administered to Streptozotocin (STZ)-induced diabetic rats for 28 days, haemoglobin percentage has increased nearer to normal level in comparison to

diabetic control [124]. Gandhi *et al*. (2012) showed that extract of *Merremiae marginata* Burm. F. administered to STZ-induced diabetic rats for 28 days; haemoglobin level was elevated [125].

Liver functioning enzyme serum glutamic oxaloacetic transaminase (SGOT/AST)

Aspartate transaminase or aspartate amino transferase (AST/AAT) also known as (serum) glutamic oxaloacetic transaminase (GOT/GOT) is an enzyme which is mainly found in liver, heart, skeletal muscle, kidneys, brain, and red blood cells. It catalyses the transamination of L-Aspartate and α-ketoglutarate (α-KG) to form Oxaloacetate and L-Glutamate. It is an important liver functioning enzyme and so tests are done to assess the health of liver condition. When there is any kind of inflammation or damages in liver, SGOT comes into the bloodstream from the liver thus increasing AST level. The normal levels of AST are:- Men**: -** 10 to 40 units/L Women: 9 to 32 units/L. Higher AST levels indicate viral hepatitis, liver injury from medicines or toxins, or shock liver (damage caused due to lack of oxygen / blood supply to liver). Due to loss of insulin effect in diabetes, liver is unable to reduce blood glucose level as a result glucose oxidation, glycogenesis, lipogenesis decreases. Insulin resistance in diabetes causes abnormal storage of triglycerides and enhances lipolysis, resulting fatty liver due to accumulation of non-esterified fatty acids along with elevation in transaminase activities. Juárez-Rojop *et al.*(2014) evaluated that extract of *Carica papaya* leaf when administered to STZ induced diabetic rats, it reduced the AST level of treated group compared to diabetic control [126]. Abolfathi *et al.*(2012) showed that green tea extract administered to STZ induced diabetic rats reduced the AST level in treated diabetic animals compared to diabetic non-control [127]. Karan *et al.* (2013) reported that increased levels of SGPT in the plasma due to STZ-induced hepatic damage and leakage of this enzyme into the cytosol was cured and reduced by extracts of *Streblus asper* [128].

Liver functioning enzyme serumglutamic pyruvate transaminase (SGPT/ ALT)

Serum glutamic-pyruvate transaminase (GPT)/Alanine-transaminase (ALT)/alanine amino transaminase (ALAT) is a transaminase liver functioning enzyme, present in plasma and in various body tissues. It catalyses the transamination of L-Alanine and α-ketoglutarate (α-KG) to form Pyruvate and L-Glutamate.The ratio between serums (AST/ALT ratio) are clinically measured as biomarkers for liver health. ALT (liver) helps in the breakdown and absorption of protein in the body. If liver gets damaged or inflamed ALT leaks into the blood stream. Elevated levels of ALT (SGPT) indicate severe health problems like viral hepatitis, diabetes, congestive heart failure, liver damage etc. Due to insulin resistance (IR) in type-2 diabetes liver is unable to suppress gluconeogenesis whereas glycogenesis process is retarded. Due to IR in adipose tissue and obesity, lipolysis process increases and free fatty acid flux increases in adipocytes leading to enhanced lipid synthesis and storage in hepatocytes causing fatty liver disease [129]. Jayaraman *et al.* (2018) evaluated that hesperetin, a citrus flavonoid when given to STZ induced rats, it reduced the ALT level in treated diabetic animal compared to diabetic control [130]. Kurup *et al.* (2017) also showed that *Averrhoa bilimbi* fruits reduce the ALT level in STZ induced diabetic rats almost to nearer to normal level compared to diabetic control [131].

Acid phosphatase (ACP)

Acid phosphatase is phosphomonoesterase enzyme (lysosomal enzyme) found in cellular components of bone, spleen, kidney, liver, intestine, blood. It functions by fusing with endosomes in optimum acidic ph converting orthophosphoric monoester and water into alcohol and phosphoric acid. Lysosomal enzymes catalyze the hydrolytic cleavage of glycosaminoglycans, glycoproteins, glycolipids thus playing an important role in

glycoconjugate metabolism. Excessive production of free radicals in diabetes due to elevated advance glycation end products (AGEs) induces oxidative stress and promotes destabilisation of lysosomes resulting in leakage of hydrolases and cellular damages. Altered lysosomal enzymatic function due to oxidative stress enhances the intracellular and extracellular glycoprotein content. Accumulated glycoproteins due to elevation in intracellular protein content leads to formation of diabetic vascular lessions. So higher acid phosphatase levels in diabetes indicate cellular damages. Chougala *et al.* (2012) showed that curcumin and quercetin reduced the acid phosphatase (ACP) levels along with other lysosomal enzymes in STZ induced diabetic animals in compared to diabetic control [132]. Balamurugan *et al.* (2011) also showed that γ-sitosterol isolated from Lippianodiflora L. reduced the acid phosphatase level in STZ induced diabetic rats in compared to diabetic control [133].

Alkaline phosphatase *(ALP)*

Alkaline phosphatase (ALP/ALKP/ALPase, AlkPhos) is a hydrolytic enzyme acts optimally at ph-10 and removes phosphate groups from different types of molecules. It exists predominantly in the liver biliary ducts cells and also in bone, small intestine, kidneys and placenta. It acts as a protein phosphatase on deformation of protein phosphatase activity. ALP also possesses phosphate hydrolysis activity and trans phosphorylase activity. ALP helps in intestinal fats digestion and calcification of bones. Due to diabetes, insulin resistance enhances lipolysis in adipocytes (FFA level increases) promoting uptake of very-low density lipoproteins and synthesis of triglycerides in liver. Accumulated fat due to elevated FFA (free fatty acid) leads to fatty liver resulting in non-alcoholic fatty liver disease (NAFLD) which finally deteriorates to cirrhosis, hepatocellular carcinomas [134]. This condition is toxic to the hepatocytes and causes increment in transaminases and diminished synthetic capacity of liver. Oxidative stress (ROS formation), tissue injury followed by non-enzymatic protein glycosylation, glucose autoxidation and impaired glutathione metabolism resulted in liver

inflammation therefore ALP activity gets altered. So increased ALP levels indicates the liver damage. Ahmad *et al.* (2014) showed that crude methanolic extract; chloroform fraction of *Artemisia indica* significantly reduces the ALP level (hepatic) in STZ induced diabetic rats in comparison to non-treated diabetic control [135]. Hosseini*et al.* (2017) also estimated the ALP level and showed that extracts of *Rheum turkestanicum* Janischew root reduces the ALP level in serum in STZ induced diabetic rats compared to diabetic control [136].

Total protein by Lowry method

Utilization of glucose is impaired due to lack of insulin secretion or its action, which results in the alterations of enzymatic activities in diabetic patients. Insulin enhances the uptake of amino acids by various tissues and facilitated their transportation across the cell membrane thus stimulates protein synthesis by increasing aminoacyl t-RNA formation, ribosomes number and their translational efficiency in muscles. Insulin also suppresses proteolysis in skeletal & cardiac muscles and liver. In diabetes due to insulin deficient condition protein synthesis decreases and also protein catabolism gets enhanced in muscles and tissues which ultimately lead to decrease total protein concentration. Minor imbalance between protein synthesis and degradation causes a profound effect over cell viability and metabolism, thus changes in protein metabolism are held responsible for many chronic diabetic complications and it severely affects the repair of tissue after injury or infection. Previously scientific studies have revealed that in STZ induced diabetic rat, total concentration of protein decreases due to protein catabolism and this ultimately leads to influx of amino acids into liver as a substrate for gluconeogeneis [137]. It is also reported that unstable glucagon-mediated regulation of cyclic AMP due to insulin deficiency enhances proteolysis [138]. Katiyar Deepti *et al.* (2019) have reported that when STZ-induced diabetic rats were supplemented with Formulation A-3 (125mg/kg), total protein content was increased due to inhibition of proteolysis caused by insulin deficiency [139]. Irudayaraj *et al.*

(2012) estimates the protein by Lowry method and showed that extracts of *Toddalia asiatica (L.)* Lam. leaves increases the total protein of STZ induced diabetic rats in comparison non-treated diabetic control [140]. Christudas *et al.* (2012) showed that plumbagin extracted from *Plumbago zeylanica* L. root increases the protein content in STZ-induced diabetic rats in comparison to the diabetic control (non-treated) [141].

Kidney function marker urea

In diabetes due to insulin deficiency glucose molecules are unable to reach extra hepatic tissues so it stimulates gluconeogenesis process for supply of glucose. Along with this process enhanced proteolysis (breakdowns of muscles and other tissue proteins into amino acids) also increases the level of free amino acids in the plasma and these free amino acids are deaminated in the liver thus forming urea. This proteolysis is due to derangement of glucagon mediated regulation of cyclic AMP in insulin deficient condition causes more protein loss in body. So urea level in the blood increases. Amino acids are oxidised by transaminase in liver where alpha-amino nitrogen of amino acids are removed forming ammonia. Being toxic to the body, ammonia reacts with carbon dioxide and is converted into urea and water. Urea excretes through urine from our body and small amount of urea is also excreted through sweat. Urea level indicates the degeneration of kidney. Adaramoye *et al.* (2012) have shown that methanolic extract of African mistletoe (*Viscum album*) reduces the urea level in STZ-induced diabetic rats in comparison to diabetic control [142]. Sugumar *et al.* (2016) also showed that hydroethanolic extract of *Senna alata* also decreases the urea level in TZ induced diabetic rats in comaparison to non-treated diabetic control [143].

Kidney function marker Creatinine

Creatinine is a waste product (or byproduct) which is produced from non-enzymatic catabolism of phosphocreatine (creatine) in the muscle cells. Creatinine gets filtered through

kidney. Higher levels of creatinine in the blood reflect that kidney function is hampered. In diabetes kidney gets enlarged and the glomerular filtration rate (GFR) becomes disturbed. In diabetic patients higher levels of creatinine indicates renal dysfunction. The amount of creatine per unit skeletal muscle is constant, the breakdown of creatine is also constant so plasma creatinine level is also stable and it reflects the mass of skeletal muscle. Skeletal muscle being the major target site of insulin, lower volume of skeletal muscle provides fewer target sites for insulin leading to insulin resistance which causes type-2 diabetes. Somani *et al.* (2012) estimated creatinine and showed that extracts of *Asparagus racemosus* Willd (Liliaceae) reduces the creatinine level in STZ induced diabetic rats in comparison to non-treated diabetic control [144]. Premanath *et al*. (2015) also estimated creatinine in an experiment where ethanol extracts of leaves of *Andrographis paniculata* Nees reduced the creatinine level in STZ induced diabetic rats in comparison to non-treated diabetic control [145].

Cholesterol

Cholesterol is a type of lipid or sterol, synthesized in higher amounts in the hepatic cells (liver) and intestines. Cholesterol is transported in the blood in the form of lipoproteins. Cholesterol acts as precursors for synthesizing steroid hormones, vitamin D (Calcitriol) and bile salt glycocholate [146]. Cholesterol is categorised into two groups; LDL (low density lipoprotein or bad cholesterol develops in the blood vessels) and HDL (high density lipoprotein or good cholesterol which helps in removing excess LDL from the blood). By cholesterol test the level of LDL and HDL are measured. Insulin stimulates the absorption of cholesterol from the gut but in insulin deficient condition (type-2 diabetes) cholesterol synthesis increases and absorption of cholesterol decreases leading to rise in the LDL level in the blood. This increased cholesterol level is associated with cardiovascular disease as cholesterol gets deposited inside the blood vessels and arteries. Type-2 diabetic patient have

different cholesterol metabolism than non-diabetic patients as synthesis of cholesterol increases in diabetic condition. Marella *et al.* (2015) showed that Mcy protein extracted from the fruits of *M. cymbalaria* reduced the cholesterol level in STZ induced diabetic rats in comparison with diabetic control [147] and estimated the cholesterol by the by CHOD/ POD method. Dholi *et al.* (2014) also showed that gymnemic acid extracted from leaves of *Gymnemic sylvestre* reduced the cholesterol in STZ induced diabetic rats in comparison with diabetic control [148].

Triglycerides

A triglyceride (TG) also known as triacylglycerol (TAG) / triacylglyceride is a type of blood fat/lipid and forms the major components of body fat in vertebrates, of human skin oils and vegetable fat. It is a flexible source of energy (ester), mainly synthesized in the endoplasmic reticulum of cells by the attachment of three fatty acids to glycerol molecule and also produced from acetyl-CoA, remains stored as a fat (lipogenesis). Both these processes take place in liver and adipose tissue. Insulin enhances lipogenesis by stimulating fatty acid synthesis through increasing ATP citrate lipase activity which provides NADPH for fatty acid synthesis. Insulin facilitates incorporation of fatty acids into triacylglycerol (TAG) and inhibits hormone sensitive lipase by reducing cAMP levels in adipocytes. But in diabetic insulin deficient condition due to increase lipolysis, mobilization of free fatty acids increases from adipose tissue into circulation and reaches liver for catalyzation into acetyl CoA and then into TAG. Increased levels of TAG gets converted into LDL and expressed in the plasma. Higher levels of TAG causes arteriosclerosis and also responsible for heart diseases, stroke etc. Mabel Parimala *et al.* (2014) revealed that extracts of *Nymphaea nouchali* Burm. F. seeds in STZ induced diabetic rats reduced the triglyceride level compared to diabetic control [149]. Hossein Ashraf *et al.* (2014) also reported that aqueous fruit extracts of

Berberis integerrima Bge STZ induced diabetic rats reduced the triglyceride level compared to diabetic control [150].

HDL cholesterol

High-density lipoprotein (HDL) cholesterol also known as "good" cholesterol is one of the classes of lipoproteins that remove other form of cholesterol (LDL cholesterol) from the blood. HDL also help in transporting fat from cells, from the artery walls, reduces accumulation of macrophage thus HDL plays a vital role in prevention of atherosclerosis development. HDL delivers LDL to liver for its processing and excretion into bile by converting into bile salts which is then absorbed in the intestines. HDL mainly delivers cholesterol to liver or other organs like adrenals, ovary and testes. When the levels of HDL cholesterol is greater than 60 mg/dl that level is considered as good for health. HDL cholesterol reduces the risk of heart attack, stroke etc. Impaired insulin action due to type-2 diabetes causes lipid abnormalities which reduce the level of HDL cholesterol from blood thus increasing the risk of cardiovascular diseases [151]. So in epidemiological studies low levels of HDL cholesterol are associated with the development of type-2 diabetes [152-153]. Low levels of HDL cholesterol and high levels of triglycerides manifests in the development of diabetic dyslipidemia [153-156]. In different experimental and human settings it has been observed that HDL cholesterol enhances pancreatic β-cell insulin secretion and modulates glucose uptake in skeletal muscles [157-160]. Komeili *et al.* (2016) showed that hydro alcoholic extracts of *Peganum harmala* seeds increases the HDL level in comparison to STZ induced diabetic control [161]. Omonkhua *et al.* (2013) also revealed that extracts of *Carica papaya* leaves increases the level of HDL in treated diabetic rats in comparison to STZ induced diabetic control [162].

Antioxidant enzyme lipid peroxidation

Lipids are generally targets of reactive oxygen species (ROS). Diabetes mellitus is associated with elevated lipid content of the adipose tissues during obese condition thus causing enlargement of adipocytes, activating phospholipase A2 which in turn initiates the process of lipid peroxidation i.e. oxidative damage of lipids (mainly membrane lipids) by ROS [163] and in response to the extent of membrane lipid peroxidation and according to specific cellular metabolic circumstances and repair capacities,cells may promote cell survival or induce cell death according to their defence mechanism [164]. Lipid peroxidation is mediated by both enzymatic and non-enzymatic methods and mainly causes damages to glycolipids, phospholipids (PLs), and cholesterol (Ch) as they undergo peroxidative modifications.In lipid peroxidation, free radical/ reactive oxygen species (ROS) through chain reaction system attacks carbon-carbon double bond(s) of lipid molecule especially polyunsaturated fatty acids (PUFAs) by abstracting hydrogen from a carbon, or addition of an oxygen radical forming lipid peroxyl radicals and hydroperoxides. Hydroperoxides are toxic to cells by exerting direct and through degradation to highly toxic hydroxyl radicals. Hydroperoxides forms stable aldehydes, such as malondialdehyde (MDA) by reacting with transition metals like iron or copper and damages the cell membranes [165]. Other products of lipid peroxidation are acrolein, propanal, hexanal, 4-hydroxynonenal (HNE), 4-oxononenal (ONE), and isolevuglandins (IsoLGs) [166]. These MDA is reported as a biomarker for free radical mediated lipid damage (lipid peroxidation) and oxidative stress [167]. Elevated levels of MDA in diabetes may be responsible for diabetic complications due to peroxidative injury and enhanced lipid peroxidation also deteriorates defence mechanisms of enzymatic and non-enzymatic antioxidants [168]. Along with formation of MDA, prostaglandins like endoperoxides are also produced from polyunsaturated fatty acid (PUFA) with two or more double bonds [169]. In diabetic patients elevated levels of MDA has been detected in plasma,

serum and many other tissues [170-171]. Baynes (1991) and Ramesh *et al.* (2012) have documented that in diabetes, many secondary chronic complications like atherosclerosis and neural disorders are induced by lipid peroxidation [172-173]. Yang *et al.* (2009) evaluated elevated serum lipid peroxidation in terms of MDA in hyperglycemic mice thus suggesting that enhanced lipid peroxidation aggravates myocardial infarction through NADPH oxidase activation [174]. MDA level is detected by TBARS assay using thiobarbituric acid (TBA) as a reagent where MDA react with TBA (thiobarbituric acid) forming a pink chromogen (TBARS), which is measured at 532–535 nm. Thiobarbituric acid-reactive substances (TBARS) are also a byproduct of lipid peroxidation. In diabetic condition, elevated levels of TBARS are observed in erythrocytes, serum which is responsible for diminished erythrocyte antioxidant enzyme activities [175-176].Clinical study conducted by Bandeira and his coworkers (2012) also reported that elevated lipid peroxidation levels is associated with high glucose levels as observed by the fasting glucose and HbA1c levels [171]. As ageing is associated with lipid peroxidation due to ROS so diabetes patients are more prone to ageing [177]. Kyznetsova *et al.* (2015) showed that aqueous extracts of *Phaseolus vulgaris* pod in STZ induced diabetic rats reduces the lipid peroxidation level in treated diabetic rats in comparison with diabetic control[178]. Gomathi *et al.* (2013) also showed that when STZ induced diabetic rats were treated with ethanolic extracts of *Evolvulus alsinoides (L.)*, lipid peroxidation level were reduced in treated diabetic rats in comparison with diabetic control [179].

Antioxidant enzyme superoxide dismutase

Superoxide dismutase (SOD) is an antioxidant enzyme which catalyzes breakdown/dismutation of superoxide anion (O2−) into hydrogen peroxide and molecular oxygen [180-181]. SOD is critical for preventing the cell against toxic products of aerobic respiration and plays defensive role against cellular and histological damages generated by

ROS. SOD promotes the formation of hydrogen peroxide from superoxide radicals and in the presence of other enzymes it is converted into oxygen and water [182]. SODs are mainly of three types in mammalian tissues: Cu-Zn-SOD, Mn-SOD and extracellular SOD, EC-SOD and each are product of specific gene [183, 184]. EC-SOD or SOD 3 (EC 1.15.1.1) exists in the extracellular matrix of pancreas, skeletal muscle, and blood vessels; Cu-Zn-SOD or SOD 1 (EC 1.15.1.1) is localized in cytosol, Mn-SOD or SOD 2 (EC 1.15.1.1) in mitochondria [185-186]. Oxidative peroxynitrite radical are formed when superoxide reacts rapidly with nitric oxide (NO), reducing NO bioactivity [187]. SOD acts as a major defender against superoxide in the kidneysduring the progression of murine diabetic nephropathy and down regulation of renal SOD (SOD 1 and SOD 3) may play a major role in the pathogenesis of diabetic nephropathy [188]. Over expression of SOD or antioxidants supplements like SOD mimetics helps in overcoming oxidative stress, reduction in ROS level and amplified antioxidant enzymes have proven to restrict diabetes mellitus [189]. R. A. Kowluru have reported that higher SOD level helps in reducing oxidative stress, reduces cytochrome C release from mitochondria and apoptosis in neurons; prevents diabetes-induced glomerular injury in mice thus implying the major role of SOD in the apoptosis regulation [190]. Many studies have also reported that SOD level gets reduced in diabetic tissue and blood [191-193]. Kim (2013) reported recently that relatively small amount of extracellular protein is expressed in the diabetic skin tissues indicating extracellular SOD is responsible for altered metabolic state in diabetic skin, ultimately elevating ROS production [194]. Lucchesi *et al.* (2013) conducted study and reported that enhanced oxidative balance reduces the activity of SOD and other antioxidant enzymes in diabetic rats [195]. Premanath *et al.* (2015) found that when STZ induced diabetic animals treated with extracts of *A. paniculata,* SOD level increases in treated group in comparison to diabetic control animals [196]. Omodanisi *et al.*

(2017) also showed that the level of SOD increases in STZ induced diabetic rats when treated with methanolic leaf extracts of *Moringa oleifera* in comparison to the diabetic control [197].

Antioxidant enzyme Catalase (CAT)

Catalase is an important antioxidative enzyme found in all living organisms which plays a protective role against oxidative stress-generated complications such as diabetes and cardiovascular diseases [198]. Catalase mainly found in the liver and in the major hydrogen peroxide production sites in cell like peroxisomes, mitochondria, cytosol and chloroplast (higher plants). Catalase catalyses the reduction of two hydrogen peroxides into one molecule of oxygen and two molecules of water in a two-step reaction [199-200] and neutralises it. Hydrogen peroxide, natural product of energy metabolism is a highly reactive small molecule and high concentration of it is hazardous for protein, DNA, RNA and lipids [201]. But low levels of hydrogen peroxide in muscle cells facilitate insulin signalling. Catabolism of hydrogen peroxide prevents the cells from oxidative damage by preventing pancreatic β cells from hydrogen peroxide injury [202-203]. Catalase also exhibits peroxidase activity and catalyses the oxidation of various hydrogen donors in the presence of relatively lower concentrations of hydrogen peroxide. It removes cellular superoxide and peroxides thus preventing formation of more reactive species by carrying reaction between superoxide and peroxides with metal catalysts. Patients having deficiency of Catalase are more prone to the risk of diabetes. Deficiency of Catalase in the β-cell enhances oxidative stress as β-cell are enriched with mitochondria (source of ROS) and ultimately causes β-cell failure [204] as pancreatic β-cells are protected from damage of hydrogen peroxide by Catalase [203]. Concentration of hydrogen peroxide is reduced by Catalase, but Catalase allows functioning of hydrogen peroxide at low, physiological concentrations. Patients with schizophrenia and atherosclerosis suffer from low Catalase activities and prolonged oxidative stress develops late onset disorders like type-2 diabetes [205-206]. Pouraboli *et al.* (2016) have shown that

methanol extract of *D. Polychaetum* increases the CAT level in STZ induced diabetic animals in comparison to diabetic control animals [207]. Marzouke*et al.* (2013) also showed that dry seed powder of Fenugreek (F), *Trigonella foenum graecum,* and Termis (T), *Lupinus albus,* increased the CAT level in in STZ induced diabetic animals in comparison to diabetic control animals (non-treated) [208].

Antioxidant enzyme glutathione peroxidase (GPx)

Glutathione peroxidase (GPx) is a cytosolic enzyme having peroxidase activity which protects organism from oxidative damages by reducing lipid hydroperoxides to their corresponding alcohols and reduces free hydrogen peroxide to water[209]. GPx found in the cytoplasm, endothelial cell, lungs, mitochondria and nucleus. It catalyses hydrogen peroxide to water in presence of reduced glutathione (GSH) as a hydrogen donor. Reduced glutathione (GSH) in presence of GPx is converted into oxidised glutathione (GSSG) by forming disulfide bridge with another glutathione and converts hydrogen peroxide into water. Glutathione disulfide (GSSG) is converted back to glutathione (GSH) by glutathione reductase, using the cofactor NADPH generated by glucose-6-phosphate dehydrogenase. It acts as an important indicator of oxidative stress level. Selenocysteine forms the active residue of GPx catalytic domain. In diabetes due to oxidative damages hydrogen peroxides are formed, so levels of GPx decreases to combat the damages and their activity increases. RBC suffers from haemoglobin auto oxidation due to high rate of endogenous H_2O_2 production which gets elevated in cells containing unstable hemoglobins. Glutathione peroxidase along with Catalase protects erythrocytes from peroxides which are produced intracellularly or exogeneously [210]. For maintaining the H_2O_2concentration under normal physiological conditions and after oxidative stress, GPx activity plays the primary protective role [211]. Due to oxidative stress, GPx activity reduces in obese and diabetic patients in comparison to in normal patients [212-214]. Also obese Type-2 diabetic patients show

diminished GPx activity than non-obese Type-2 diabetic patients revealing higher oxidative stress in obese patients [215-217]. Jayachandran *et al.* (2018) showed that guava leaf extract (*P. Guajava*) increased the GPx activity of STZ induced diabetic rats in comparison to non-treated diabetic control [218]. Seedevi *et al.* (2020) showed that crude polysaccharide and rhamnose enriched polysaccharide extracted from *G. lithophila* increases the activity of GPx in STZ induced diabetic rats in comparison to diabetic control (non-treated) [219].

Antioxidant enzyme reduced glutathione (GSH)

Glutathione (γ-glutamyl-cysteinylglycine) is an important intracellular antioxidant enzyme occurring in most mammalian cells at 1-10 mM concentrations (highest concentration in liver). It is the most plentiful non-protein thiol that combats the deleterious effect of oxidative stress by scavenging the free radicals in the body [220-222].It is a ubiquitous tripeptide (γ-Glu-Cys-Gly) of L-glutamine, L-cysteine, glycine formed in two major steps in presence of ATP [222]. In first step, enzyme glutamyl-cysteine ligase (GCL)/gamma-glutamyl-cysteinyl-synthetase (GCS) forms gamma-glutamyl cysteine by gamma peptide linkage between the carboxyl group of the glutamate side chain and cysteine. In second step carboxyl group of the cysteine residue of gamma-glutamyl cysteine is linked with glycine by normal peptide linkage to form GSH in presence of GSH synthetase (GS) enzyme [223].The cysteinyl portion of GSH containing sulfhydryl (SH) group is responsible for strong electron donation. After losing electrons, GSH is oxidised and two molecules of it are dimerized by disulfide bridge forming glutathione disulfide (GSSG) in presence of enzyme glutathione peroxidase (GPx).Glutathione disulfide is reduced back to glutathione (GSH) by glutathione reductase, using the cofactor NADPH generated by glucose-6-phosphate dehydrogenase. This ratio of GSH to GSSG controls redox dependent cell signalling. When level of oxidative stress increases then conversion of GSSG to GSH is disturbed and exocytosis of GSSG occurs or reaction between GSSGwith protein sulphydryl

groups happens to prevent a major shift in the redox equilibrium [222] thereby reducing cellular GSH. The homeostatic balance of GSH molecule is tightly controlled both intracellular and extracellular and dynamic balance is necessary to be maintained between GSH synthesis, it's recycling from GSSG/oxidized glutathione, and its utilization.GSH acts as a cofactor for variety peroxidise enzymes to detoxify peroxides, generated from ROS attack on biological molecules; acts as a trans hydrogenase to reduce the oxidised centres on DNA, protein and other biological molecules etc., maintains SH groups of proteins in a reduced state, participate in amino acid transport, detoxify free radicals, lipid peroxides, heavy metals thus preventing cellular components and tissue damage [224].Within the cells, the ratio of reduced glutathione to oxidised glutathione is considered as a measure of cellular toxicity. Several studies have revealed that type-2 diabetic patients have reduced erythrocyte glutathione concentration and impaired glutathione metabolism [225-230].The competition between aldose reductase and glutathione reductase for NADPH, a cofactor and enhanced oxidative stress is responsible for the reduction in the reduced GSH level [228].Reduced GSH level is responsible for the oxidative damage of DNA in type-2 diabetes [229]. Unregulated type-2 diabetes cause deficient GSH synthesis thus limiting availability of precursor. In severe hyperglycaemia, dietary supplementation with GSH precursor amino acids re-establishGSH synthesis and reduces the oxidative damage [230].

Carbohydrate metabolism enzyme glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydogenase (EC1.1.1.49; D-Glucose-6-phosphate: $NADP^+$ oxido-reductase) is one of the most important antioxidant enzyme require for cell survival. Itis the principal producer of NADPH which is the key cellular reductant and fuel for glutathione recycling within the cells thus providing antioxidant defence mainly by promoting antioxidation of peroxides [231]. G6PD plays the role of key rate-limiting enzyme in the pentose-phosphate pathway producing ribose-5-phosphate and NADPH [232]. Besides

playing the central role in cell metabolism, G6PD also have a role in the pathogenesis of diseases like diabetes, aldosterone-induced endothelial dysfunction, and cancer. G6PD provides NADPH, a hydrogen carrier necessary for essential cellular systems like nitric oxide synthesis, p450 system, and others [233]. Rather producing ribose, G6PD activity is more important for NADPH production for defence against oxidative stress. Activity of G6PD decreases in high glucose level and hyperglycaemia in kidney cortex of experimental STZ induced diabetic animal and this reduction is due to enhance adenylate cyclase activity which in turn increases cellular cAMP levels, cAMP activates protein kinase A, an inhibitor of G6PD causing phosphorylation of G6PD [234]. Decreased activity of G6PD is also correlated with reduced NADPH and reduced glutathione ultimately responsible for kidney cell damage. Zhang *et al*. (2010) also reported that high glucose level is responsible for decrease expression and activity of G6PD in human pancreatic islets cells [235]. Zhang *et al.* also reported in his experiment that protein kinase A in culture of bovine endothelial aortic cells is activated at high glucose concentration which finally causes phosphorylation of G6PD and reduction of G6PD activity [235]. Xu *et al.* (2005) has reported that chronic hyperglycaemia of experimental diabetes animals has shown reduction of G6PDH in kidney cortex which finally responsible for increased oxidative stress [236]. In hyperglycaemic condition, glucose is more utilised in polyol pathway consuming NADPH which is necessary for GSH regeneration by GR enzyme, therefore hyperglycaemia is indirectly responsible for GSH depletion. Therefore more consumption of NADPH in polyol pathway reduce NADPH, as glucose is reduced to sorbitol by NADPH in the first step of polyol pathway and depletion of NADPH balances GSH level in reduced state [236]. Also G6PD plays a vital role in the survival of RBC, as RBC lacks citric acid cycle and only has the pentose phosphate pathway (PPP) for NADPH production. In PPP, NADPH is produced when D-glucose-6-phospate is converted into D-glucono-1,5-lactone-6-phosphate(6-phosphogluconolactone) this NADPH is

crucial for reduced glutathione generation which protects RBC from oxidative damages. So G6PD deficiency reduces NADPH level thus reducing activity of reduced glutathione (GSH) against oxygen free radical damage.

Glycogen content of liver

Glycogen is the storage form of glucose (polysaccharide structure/branched biopolymer) acting as a major source energy storage in animals, fungi, and bacteria. It is mainly stored in liver and skeletal. Glycogen consists of linear chains of glucose residues where approximately 8-12 glucose units are linked with each other by α(1→4) glycosidic bonds. In one molecule of glycogen around 2,000-60,000 residues are present. Branches are joined to the chains and branching starts from the existing chain by α(1→6) glycosidic bonds between the first glucose of the new branch and a glucose on the stem chain. Excess glucose from blood enters into liver through portal vein and converted into glycogen catalysed by enzyme glycogen synthase. This synthesis is en endergonic process stimulated by insulin. This insulin mediated stimulation is carried out by protein kinase B which inactivates glycogen synthase kinase-3,an inhibitory regulator of glycogen synthase via phosphorylation [31]. Cyclic AMP level is lowered by insulin thus glycogen synthase activity is stimulated and glycogen phosphorylase activity inhibited thereby facilitating glycogen storage [237]. So in diabetic hyperinsulinemia (type-2 diabetic condition) glycogen content of liver and muscle decreases due to reduced insulin signalling [238]. However diabetes causes the accumulation of glycogen in other organs like heart and pancreatic β-cells [239], adipose tissue, kidney. This accumulation may also results from defects in the enzymes responsible for glycogen synthesis and breakdown [240-242]. It is reported that accumulated glycogen in kidney may prevent advanced glycation of proteins thus inhibiting formation advanced glycation end (AGE) products. Vinayagam (2018)

showed that extracts of *Psidium guajava* (PG) Linn increases the liver glycogen content in treated STZ induced diabetic rats by regulating insulin secretion [243].

Insulin

Insulin is the most efficient anabolic peptide hormone produced by beta cells of the pancreatic islets of the body. Human insulin (single protein monomer) having molecular formula $C_{257}H_{383}N_{65}O_{77}S_6$, molecular mass of 5808 Da and consists of 51 amino acids. It is made of two peptide chains (dimer), an A-chain (21 amino acids) and a B-chain (30 amino acids) which are joined with each other by two disulphide bonds. Insulin plays the key role by regulating carbohydrates, fats and protein metabolism thereby promoting glucose absorption from blood into liver, fat and skeletal muscle cells. The absorbed glucose in these tissues either converted into glycogen via glycogenesis or fats (triglycerides) via lipogenesis or both in liver. Insulin also facilitates the storage and synthesis of carbohydrates, protein and lipids and inhibits breakdown and release into the circulation [244]. The activity of insulin is mostly seen in muscle and liver cells, mediates fatty acid uptake in adipose cells. In human body insulin responsive target cells having metabolic functions are macrophages, endothelial cells, and insulin-producing pancreatic β-cells and insulin receptors possessing cells are present. Insulin helps in enhanced energy storage or utilization of energy by regulating the transport of glucose through facilitative glucose transporter GLUT4 into cell. Glucose uptake is increased by insulin through increasing the GLUT4 proteins concentration at the plasma membranes. As insulin stimulates the glycogen synthesis in skeletal muscle by mediating glucose transport and utilization and inhibiting protein catabolism. Insulin also facilitates glucose transport in adipose tissue and lipogenesis by inhibiting lipolysis. Also insulin retards glucose synthesis and fatty acid oxidation in liver thus stimulating glycogen production and lipogenesis. By inhibiting lipolysis of fat and protein catabolism in muscle, insulin helps in reducing supply of substrate for gluconeogenesis in hepatocytes (liver) [243-244]. But in

insulin deficient condition all these processes get altered leading to development of various complications like fatty liver, obesity, polycystic ovary syndrome etc. along with diabetes. Metabolic stress, hyperglycemia, hyperlipidemia, ketonemia are some of the outcomes of insulin deficiency and or insulin resistance in diabetic condition. Also high blood pressure, high cholesterol develops due to insulin resistance. Nowadays synthetic insulin is used as insulin therapy to counter the effects of diabetes.

Chapter 3
Materials & Methods

Chemicals

The chemicals used in the study were purchased from the following sources: Streptozotocin (STZ) was purchased from Sigma Chemical Company Inc. USA. All other analytical grade chemicals were purchased from standard chemical companies. Blood glucose level was measured by using digital Breeze2 glucometer supplied by Bayer Healthcare. Glycosylated Haemoglobin (Hb1Ac) kit was provided by Biosystems, Barcelona, Spain (Bisse and Abraham, 1985) and Coral Clinical Systems, India (Ion Exchange Resin method). SGOT (AST) and SGPT (ALT) test kits were supplied by Piramal Healthcare Limited, Mumbai, India and ARKRAY Healthcare Pvt. Ltd., India. Total serum protein was measured using the method given by Lowry etal (Lowry OH, *et al.*. 1951). Total Cholesterol was measured using kit provided by Accurex Biomedical Pvt. Ltd., Mumbai, India and BEACON DIAGNOSTICS PVT.LTD.INDIA. Triglyceride was measured using kit provided by Merckotest, Merck, Goa, India and ARKRAY Healthcare Pvt. Ltd. India. Lipid peroxidation was determined from the Thiobarbuturic Acid test (TBA test) with modification by Kumar and Das (Kumar KV *et al.* 1993). Superoxide Dismutase (SOD) activity was assayed by the method based on the reduction of nitro blue tetrazolium (NBT) to blue pharmazone by superoxides, produced phytochemically in the reaction system (Beauchamp C *et al.* 1971). Reduced Glutathione (GSH) was determined by using the method of Davila *et al.* (Davila *et al.* 1991) and Glutathione Peroxidise (GPx) was estimated by using the method of Levander *et al.* (Levander *et al.* 1983). Acid phosphatase was assayed according to the method of Bessey et.al (1946) illustrated by Bergmeyer&Bent (1963) using þ- nitrophenyl phosphate as substrate. Alkaline Phosphatase, Urea, Creatinine test kits were supplied by ARKRAY

Healthcare Pvt. Ltd., India. Glucose-6-Phosphate dehydrogenase test kit was supplied by Reckon Diagnostic Pvt. Ltd. Insulin test system was supplied by Monobind Inc. USA. Analytical grade Acetonitrile (HPLC grade), water (Milli-Q) and glacial acetic acid (HPLC grade) were purchased from Merck Ltd. (Mumbai, India). Standard oleanolic acid was purchased from Sigma Aldrich (St. Louis, MO, USA).

Plant Collection and authentication

The fibrous roots of the *Gymnadenia Orchidis* Lindl were collected from local market of Darjeeling roots and were immediately transferred into a container with dry ice, brought to the laboratory and stored in a -20 ^{o}C refrigerator until used. The plant was submitted to Botanical survey of India, Sikkim Himalayan Regional Centre, Gangtok, India for phytochemical analysis. The plant was identified and authenticated by Dr. D.K. Agarwal as *Gymnadenia Orchidis* Lindl. of the *Orchidaceae* family and its Accession No.: 0046 dated 26.09.2014. V.No. SHRC-5/02/2012- Tech. – 195.

Preparation of root Salep and terpenoid rich extract of the roots

- ### Root Salep

The fibrous roots were air dried and grinded in a mortar pestle placed in ice bath and different concentrations of Salep as per requirement was freshly prepared by adding sterile deionised water to the powder root before use.

- ### Terpenoid rich extract of the roots

Extraction process was carried out by differential fractionation method where the powder of the roots of *Gymnadenia orchidis* Lindl (40 gm) were macerated with ethanol (80% v/v) and kept at room temperature for 6-8 days in clean, flat bottomed glass container accompanied by constant shaking and stirring. The ethanolic extracts (filtrate) were

subsequently filtered through Whatman filter paper (Bibby RE200, Sterilin Ltd., UK) and ethanol was evaporated over water bath. The concentrated filtrate was suspended with distilled water (10 ml) and partitioned with petroleum ether (50 ml) in a separating funnel. These two solvents were shaken vigorously for 1 mins, and standing time (5-8 mins) were given for separation of two solvents. Expected terpenoid was extracted with the elution of pet ether. Rest of the solution again suspended with distilled water and petroleum ether and the process repeated for 10–14 times. Thus pet ether eluates containing terpenoid was separated and pet ether removed from the extract by distillation process. Thus crude terpenoid was recovered and preserved in sterile glass container at 4° C for further analysis. Confirmation of terpenoid was done by TLC process with two known standards menthol and limoneme. From 40 gm of dried roots approximately 30 mg of crude terpenoid was extracted.

Phytochemical investigation (qualitative analysis) of the aqueous extract of *Gymnadenia orchidis* Lindl root.

- **Qualitative analysis of *Gymnadenia orchidis* Lindl**

The qualitative analysis of aqueous extracts was carried out by following the methods of Trease and Evans (1996) and Harborne (1998) for the identification of different phytochemicals in the root salep (aqueous extract) of *Gymnadenia orchidis* Lindl.Aqueous extract of the dried roots was made for three different concentrations (1mg/ml, 10mg/ml and 25mg/ml) and analyzed in triplicate for each concentration to get statistical average.The presence of active constituent'slike terpenoids, steroids, cardiac glycosides, tannin, polyphenols, vitamin C, carbohydrate(s) and protein(s) were determined in the root extracts of *Gymnadenia orchidis* Lindl by the standard laboratory methods (Harbone, 1998; Trease and Evans, 1996). Also qualitative analysis was done by HPLC and TLC.

(A) Test for terpenoids

Salkowski test: - 3ml of extract was mixed with 1 ml of chloroform and 1 ml of conc. H_2SO_4 was mixed carefully to form a layer. At the interface reddish brown color was observed for all concentrations of the sample indicating the presence of terpenoids in significant amount. Here Eugenol was taken as positive control [245]

(B) Test for Steroids

Salkowski test: - 10 mg of the extract was dissolved in one ml of chloroform and one ml of conc. H_2SO_4 was added gradually. The solution was allowed to stand. Formation of brown ring by chloroform layer and green color fluorescence by the acid layer indicated the presence of steroids [245].

Libermann Burchard's test: - Small amount of extract was dissolved in 1 ml of chloroform and resulting solution was treated with 1 ml of acetic anhydride. The solution was boiled and allowed to cool. Then 1 ml of conc. H_2SO_4 was added. Formation of bluish green color solution confirms the presence of steroids. Here cholesterol was taken as positive control.

(C) Test for Cardiac glycosides

(i) Kellar kiliani test: - small amount of extract was treated with 2 ml glacial acetic acid and one drop of ferric chloride solution. Then 1 ml of conc. H_2SO_4 was added to the solution. Very faint brown ring appear at the interface indicating the deoxysugar characteristic of cardenolides. Appearance of a violet ring below the brown ring and a greenish ring in the acetic acid layer confirmed the presence of cardiac glycosides.

(ii) Methanolic extract of the sample was mixed with chloroform and then conc. H_2SO_4 was added. A brown ring was observed at the interface indicating the presence of cardiac glycoside.

(D) Test for tannin

Dried sample was boiled with 20 ml water in a test tube and then filtered. Few drops of 0.1% ferric chloride were added to the filtrate. Faint brownish green color or blue black color was observed which indicated the presence of tannins.

(E) Test for polyphenols

10 mg of sample extract was dissolved in 10 ml of triple distilled water (TDW). 0.1 ml of this solution was transferred to a test tube and 0.05 ml 2N of Folin-Ciocalteu reagent was added. 0.15 ml 20% of Na_2CO_3 solution was added and volume makes upto 0.8 ml with TDW followed by vigorous shaking. Finally it was allowed to stand for 2 hrs. The absorbance was taken at 765nm (Spectroscopic determination). Here low absorbance was observed. Total polyphenol content was determined using standard curve obtained from various concentrations of gallic acid. And low amount of polyphenol was present.

(F) Test for proteins

Protein was estimated using Bradford's reagent. The Bradford protein assay is used to measure the concentrations of total proteins in a sample. Here proteins molecules present in the sample binds with Coomassie dye under acidic condition and this results in a color change from brown to blue. Blue color indicates the presence of proteins. Here BSA was taken as standard.

Biuret test: - here sample extract was treated with 1ml of 40% sodium hydroxide solution and two drops of 1% copper sulphate reagent. Violet color was developed indicating the presence of proteins.

(G) Test for vitamins

Sample extract was mixed with dinitrophenyl hydrazine. Then conc. H_2SO_4 was added to dissolve in the solution and yellow color precipitate formed. This yellow color precipitate indicated the presence of vitamin C. Here Ascorbic acid was taken as standard.

(H) Test for carbohydrate

Extract heated with Benedict's reagent (Copper sulphate, Sodium citrate &Sodium carbonate) in water. Brown or red color precipitate developed which indicated the presence of carbohydrate. Here sucrose was taken as standard

(I) Test for saponins

Froth test: - the extract was diluted with 20 ml water and shaken in a graduated test tube for 15 minutes. If the sample would contain saponin, it would have formed 1 cm froth and this froth would have disappeared with addition of few drops of olive oil. But no frothing was seen with addition of few drops of olive oil indicating the absence of saponins.

(J) Tests for flavonoids

(i) About 3 mL of dilute ammonia was added to 2 mL aqueous filtrate of plant root extract and yellow color developed. This was followed by addition of 1 mL concentrated sulphuric acid (H_2SO_4) and yellow color disappears in extract showed the absence of flavonoids.

(ii) **Shinoda test:** 0.5 ml of extract with 5-10 drops of diluted HCL in test tube and small piece of ZnCl or magnesium were added to this test tube and the solution

was boiled for few minutes. No colour developed confirming the absence of flavonoids.

(iii) Alkaline reagent test: 1 ml of extract and few drops of sodium hydroxide were taken in a test tube. An intense yellow color develops in the test tube which remains same on the addition of dilute HCL indicates the absence of flavonoids.

(iv) Ferric chloride test: few drops of ferric chloride were added to the extract solution and no intense deep green color developed thus confirms the absence of flavonoids. Quercetin was taken as positive control and the entire test conducted here showed no results. Thus flavonoid is absent.

(K) Test for alkaloids

(i) Dragendroff's test: (a) 8.0 gm of bismuth nitrate (Bi $(NO_3)_3 5H_2O$) was dissolved in 20 ml of nitric acid (HNO_3). (b) 27.2 gm of potassium iodide was dissolved in 50 ml of distilled water. (a) and (b) were mixed and allowed to stand until KNO_3 crystals formed. The supernatant was decanted off and their volume was adjusted to 100 ml with distilled water (Dragendorff's reagent). 0.5 ml of extract was mixed with 2ml of HCL. 1 ml of Dragendorff's reagent was treated to this acidic medium and no red or orange color precipitate developed thus confirmed the absence of alkaloid in the sample.

(ii) Hager test: saturated solution of picric acid in distilled water was made. Extracts were treated with Hager's reagent. No yellow coloured precipitate developed indicates the absence of alkaloids.

(iii) Mayer's test: (a) 1.36 gm of mercuric chloride was dissolved in 60 ml of distilled water (b) 5.0 gm of potassium iodide was dissolved in 20 ml of distilled water. This two solutions (a) and (b) were mixed together and diluted to 100 ml

with distilled water.1**.**2 ml of the extract was taken in a test tube, 0.2 ml of HCL and 0.1 ml of Mayer's reagent were added. No yellow colour precipitate developed and thus confirms the absence of alkaloid.

(iv) Wagner test: 1.2 gm of iodide and 2.0 gm of potassium iodide were dissolved in 5 ml of sulphuric acid and the solution was diluted to 100 ml (Wagner's reagent). 10 ml of the extract was acidified by adding 1.5% v/v of HCL and a few drops of Wagner's reagent. No yellow or brown color precipitate was formed and thus no alkaloid was present. Here Strychnine was taken as positive control.

(L) Test for amino acids

In 1 ml of extract 2 drops of freshly prepared 0.2% Ninhydrin reagent was added and heated. No purple color developed and it indicated the absence of amino acids.

- **HPLC (C18) for root sample (*Gymnadenia orchidis* Lindl)**

HPLC is a multipurpose, vigorous, and broadly used chromatographic technique for the isolation of natural products and can separate a mixture of compounds. It is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture.HPLC finger printing of the methanolic extract of ingredient of *Gymnadenia orchidis* Lindl was performed for the qualitative estimation of phyto constituents like terpenoids, steroids, cardiac glycosides, tannins, polyphenols, vitamins, proteins, vitamin, and carbohydrate. Here RP HPLC (Waters, Milford, MA, USA) was developed using reverse phase $\mathbf{C_{18}}$ column, 600 controller pumps, a multiple-wavelength ultraviolet-visible (UV-Vis) detector equipped with an in-line degasser AF 2489 and a rheodyne 7725i injector having 20 mL loop volume. Empower 2 software programs were used for quantitative estimation using the external calibration method. Pore size of the membrane filters was 0.45 mm (Millipore) used for filtration of the mobile phase and for the

filtration of the sample Whatman's syringe filters (NYL 0.45 mm) were used. Eyela (Tokyo, Japan) rotary vacuum evaporator and Milli-Q academic water purification system (Bedford, MA, USA) equipped with 0.22 mm Millipak express filter were also used [262]. HPLC process showed the presence of terpenoids, steroids, cardiac glycosides, tannins, polyphenols, vitamin C, carbohydrates and proteins in the root sample [Table1].

- **HPLC (C18) analysis of terpenoids**

Root sample of *Gymnadenia orchidis* Lindl was dried in oven at 50^{o}C for 4 hrs, and then crushed to fine powders. Methanol was added in a ratio of 15 mg/gm dry weight of root. Samples (0.5gm) were extracted in an ultrasonic water bath for 30 mins. Extracts were evaporated approximately to dryness in rotary vacuum evaporator and transferred to 100 ml flask where sample solutions were diluted to the mark with ethanol. Solutions were centrifuged at 10000 rpm for 10 mins. **A)** The flow rate of the mobile phase was kept at 1 ml/min. Temperature maintained at 30^{o}C. Mobile phase A was distilled water containing 0.05% formic acid and mobile phase B was Acetonitrile containing 0.05% formic acid in gradient mode as follows:- 0-1 min hold at 30% B; 1-24 min to 100% B; 24-25 min to 30% B. The effluent was monitored at wavelength 254 nm. Injection volume was 10µl. Reference standards used were triptolide, tripchlorolide, demethylzelastral, wilforlide B and wilforlide A. 5 µl of each sample solution was injected into HPLC. **B)** Mobile phase of solvent A was distilled water with 0.05% formic acid and mobile phase B was actonitrile with 0.05% formic acid in gradient mode as follows:- 0-5 min at 35-40% B; 5-30min at 40-50% B; 30-50min at 50-60%B; 50-65min at 60-95% B; Hold for 1 min at 95%. Flow rate was maintained at 0.8ml/ min. Injection volume was 5 µl. The retention time (RT) values for standard compounds were fixed 10 min. prior to each run, the HPLC-UV/Visible system was allowed to warm, and the baseline was monitored until it was stable before sample analysis.

- **HPLC (C18) analysis of Cardiac glycosides**

Root sample of *Gymnadenia orchidis* Lindl was crushed to fine powders and weighed. Crude extracts were extracted by sonicating 0.5 g of ground sample with 10 mL of 70% (v/v) aqueous methanol in an ultrasonic bath at room temperature for 15 min following solid phase extraction. After sonication, centrifugation was done at 4000 rpm for 3 min and supernatant solution was filtered under vacuum. The remaining residue recovered in the same way. From both extractions filtrates were mixed and concentrated to dryness under reduced pressure at 45^{o}C. The residue was diffused in 2ml of water-acetonitrile (9:1, v/v) and cleaned-up by solid phase extraction. Here reference standards for cardiac glycosides used were Digoxigenin, Deacetyllanatoside-C,Digoxigenin-bis-digitoxoside, Gitoxigenin, Digoxin, Lanatoside-C, Digitoxigenin, α–Acetlydigoxin, β-Acetyldigoxin, Lanatoside B & A, Gitoxin, Digitoxin. The phytochemical analysis was carried out in C_{18} column. The mobile phase comprised of (A) distilled water and (B) Acetonitrile and the flow rate was maintained at 1ml/min. The gradient elution was maintained as 0-35 min linear gradient from A: B (80:20) to A: B (70:30); 35-45 min linear gradient from A: B (70:30) to A: B (60:40) held for 3 mins. The column temperature was maintained at 20^{0} C and the injection volume was 10µl. The effluent was monitored at wavelength 220 nm. The total running time was 48 mins and post running time was given 5 mins. For each sample three injections were performed.

- **HPLC (C18) analysis of glycosides**

Root samples were taken, dried and grounded to fine powders. 1 gm. of fine root powder of *Gymnadenia orchidis* Lindl were kept in 250 ml of Erlenmeyer flask, mixed with 100 ml of ethanol (EtOH) 70% (w/w) and placed in 70^{o}C water bath, shaken for 30 mins for extraction. After cooling, 10 ml of solution was filtered using Whatmanfilter paper and syringe filter, nylon 0.22 µm. 10 µl of the sample was injected for HPLC analysis. Mobile

phase comprised of (A) Acetonitrile and (B) distilled water with 0.05% trifluroacetic acid, the flow rate was maintained at 0.9 ml/min. The gradient elution was maintained as A: B from 20:80 to 45:55 in 40 mins. Refernce standards used were stevioside and rebaudioside A.

- **HPLC (C18) analysis of tannins**

Root of *Gymnadenia orchidis* Lindl was dried and crushed to fine powder and then dissolved in 70% aqueous methanol (1 mg/ ml) in a ratio 1:1 (w/v) ratio. The mixture was homogenized in blender and then filtered through a Whatman no. 1 filter paper under vaccum and then it is filtered through Whatman's syringe filters (NYL 0.45 mm). The final filtrate was stored at -20^0 C prior to HPLC analysis. The HPLC analysis was carried out at 30^0 C in the isocratic elution mode using water, acetonitrile and acetic acid mixture in the ratio of 89:10:1 as mobile phase. The flow rate was 0.8ml/ min and eluent was detected at 280 nm and 230 nm. The injection volume was 10µl. Standards used for tannins were gallic acid, ellagic acid, catechin, epicatechin, quercetin.

- **HPLC (C18) analysis of Polyphenols**

2 gm of root powder of *Gymnadenia orchidis* Lindl was mixed with methanol (8 ml) and 2 ml of water in a tube. The tube was capped and shaken in water bath at 200 rpm for 30 min at 60^{o}C. After removing the tube from water bath, it is vortexed and centrifuged at 2,000 rpm for 2 min.

The samples were filtered through a 0.45 mm Millipore syringe filter (Whatman, Inc., Clifton, NJ). Mobile phase comprised of (A) 2% Acetic acid in distilled water and (B) methanol, the flow rate was maintained at 1 ml/min. The samples were eluted at the following gradient:-95% A and 5% B as initial conditions 0-2 min; 75% A and 25% B for 2-10 mins; 60% A and 30 %B for 10-20 min; 50% A and 50% B for 20-30 min; 0% A and 100% B for 30-35 min. And this was continued at 0% A until completion of the run. The

eluent was detected at 278 nm and 340 nm and the identification of compounds was achieved by comparing their retention time values with those of standards. Injection volume for HPLC was 20µl**.** The column temperature was maintained at 25^{o}C. Standards used were Gallic acid (GA), (+)-catechin hydrate (CH), vanillic acid (VA), caffeic acid (CA), (-)-epicatechin (EC), p-coumaric acid (PCA), rutin hydrate (RH), ellagic acid (EA). GA, CH, VA, CA, and EC detection was done at 280 nm and PCA, RH, and EA was detected at 320 nm.

- **HPLC (C18) analysis of Flavonoids**

Roots of *Gymnadenia orchidis* Lindl was dried at 50^{o}C. 1.5 gm pulverised root sample was taken in a round bottom flask and mixed with 60 ml methanol. It was then heated under reflux for 3 hrs; methanol solution was added, mixed properly and then filtered. The filtrate was evaporated to dryness in a rotary vacuum evaporator. Filtrate collected and was dissolved in 100 ml distilled water. The sample was defatted with 60 ml of pet ether for 3 times. The extracted solution was evaporated and dissolves in 5 ml of ethanol. Then it was kept in room temperature for 20 min and then filtered through 0.45µm membrane. Standards for flavonoids used were eriodictyol, hesperetin, myricetin, quercetin, epigallocatechin, catechin, epicatechin. Mobile phase comprised of solvent (A) 0.3% glacial acetic acid in water and solvent (B) Acetonitrile and the flow rate was maintained at 1 ml/min. Temperature of column was maintained at 25^{o}C.The drift tube temperature for ELSD was 105^{o}C, and the nebulizing gas flow rate was 2.7 L min^{-1}.The samples were eluted at the following gradient:- 0-12 min at 18%B; 12- 35 min at 18-30% B; 35-43min at 30% B; 43-60 min at 30 -55% B; 60-70min at 55-70% B; 70-80min at 100% B. Injection volume of the sample was 15µl. Eluent was detected at 340 nm.

Thin layer chromatography

Thin layer chromatography is an easy and highly useful technique in research laboratories to separate and identify unknown compounds. Thin layer chromatography is for the separation of a mixture into individuals components using a stationary and mobile phase (Sadasivam and Manikam, 1992).

- **Principle**

The principle behind the separation of unknown compound depends upon adsorption process.

A chromatographic plate firstly is coated with a thin layer of adsorbent and one or more compound is then spotted on the TLC plate. Due to capillary action mobile phase solvent flows towards the adsorbent. The movement of the component depends upon the affinity of the component towards the stationary phase. Components which shows high affinity towards the stationary phase travels through the plate slowly and components showing weaker affinity towards the stationary phase travels faster. Thus thin layer chromatographic plate helps in the separation of the components based on the affinity towards the stationary phase.

- **Procedure**

The optimized conditions were used for the identification of active constituents present in the plant extract. Crude terpenoid was extracted and collected from fraction separation method and then subjected to thin layer chromatography (TLC) as per conventional one dimensional ascending method using silica gel (Belenki *et al.*; 1990). Three respective spots of crude terpenoid, menthol and limonene (known standards) were spotted using glass capillaries to TLC (silica coated) plate by taking 1 µl volume of each sample at a

distance of 1 cm from the base. In the solvent chamber pet-ether and ethyl acetate (99%: 1%) is used as solvent system (mobile phase). The solvent system was given a standing time of 20 min for pre-saturation. After pre-saturation the spotted TLC plates were kept in-side the solvent chamber and the solvent was allowed to rise for 20 min to the maximum height of the TLC plate, then the plate was removed from solvent chamber, dried and the spots were detected by placing the TLC plate in a chamber containing iodine vapour (Figure 2).

- **Rf values**

The behaviour of an individual compound in TLC was characterized by a quantity known as R_f and expressed as a decimal fraction. The R_f value was calculated by dividing the distance the compound travelled from the original position by the distance the solvent travelled from the original position (the solvent front). (Table 2)

Characterization of the crude sample

For the Characterization of the extracted crude sample we have performed UV-Vis, FTIR, and Mass Spectroscopic Analysis.

UV – Vis Spectroscopy

Ultraviolet –(UV-Vis) spectroscopy is used to obtain the absorption spectra of a compound in solute as a solid. What is actually being observed spectroscopically is the absorbance of light energy or electromagnetic radiation, which excites electrons from the ground state to the first singlet excited state of the compound or material. Ultra-violet radiation energy which gets absorbed is equivalent to the energy difference between the ground state and excited state. Thus through UV spectroscopy, an unknown compound can be identified by comparing the spectrum of unknown compound with the reference compound's spectrum. When both spectrums are identical then unknown substance is identified. Also

through UV-Vis spectroscopy, different types of bonds like sigma-bonds, pi-bonds and lone pair of electrons, chromophores and aromatic rings whether present in a compound is also determined.The UV-region of energy for the electromagnetic spectrum covers 1.5-6.2 eV which relates to a wave length range of 800-200 nm. The beer's law is the principle behind absorbance spectroscopy.

- **Principle**

It uses light in the visible and adjacent (near-UV and near-infrared ranges). The absorption or reflectance in the visible range directly affects the perceived colour of the chemicals involved in this region of the electromagnetic spectrum the molecules undergo transitions.

- **Instrument details**

-Instrument made by JASCO

-Model –V-700

- **Procedure**

UV-Visible investigation was preformed for the identification of phytochemicals present in the root extracts of *Gymnadenia orchidis* Lindl. UV-Vis Spectroscopy was performed using JASCO V-700 Spectrophotometer. Ethanol was used as a solvent and as a reference for this experiment. The crude extract was diluted into two different concentrations. At first 50 µl of crude sample was diluted to 2000µl ethanolic solution i.e. 2.5% (v/v) thereafter 100µl crude sample was diluted to 2000µl ethanolic solution and UV spectra was recorded as A and B respectively in Figure 3 in the range between 200 to 600 nm. The peak values of the UV-VIS were recorded. For the spectrum confirmation, every analysis was performed in triplicate (Trease and Evans, 1983).

Analysis of the functional groups by Fourier Transform Infrared Spectroscopy (FTIR)

Fourier-transform infrared spectroscopy (FTIR)is a procedure which is utilised to acquire an infrared spectrum of absorption or emission of a solid, liquid or gas. FTIR spectrometer can gathers high spectral resolution data over a wide range, generally between 5000 and 400 cm^{-1} for mid-IR region wavelength, and between 10,000 and 4000 cm^{-1} for near-IR region wavelength. This ability of collection of data over wide range makes FTIR spectrometer more advantageous than dispersive spectrometer, which can only evaluate intensity over a narrow range of wavelengths at a time.

- **Principle**

The infrared spectrum originates from the vibrational motion of the molecule. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for characterization of organic, inorganic and biological compounds. The band intensities are proportional to the concentration of the compound and hence quantitative estimations are possible. The interference pattern obtained from a two beam interferometer as the path difference between the two beams is altered, when fourier transformed, gives rise to the spectrum. The transformation of the interferogram into spectrum is carried out mathematically with a dedicated on-line computer.

- **Procedure**

FTIR spectroscopy was performed using Bruker Tensor II FTIR spectrophotometer in the scan range of 400- 4000 cm^{-1}. Spectra were recorded in ATR mode. One drop of crude extract (ethanolic extract) was taken for the experiment and placed in the crystal head of the instrument. The FTIR spectrum of the sample was analyzed on the basis of peak values in the region of infrared radiation.

Mass Spectroscopy

Mass spectrometryis a powerful analytical technique in chemistry, biochemistry, pharmacy, medicine and many related fields of science. Mass spectrometry is useful in quantification of known materials, identification of unknown compounds within a sample, elucidating the structure and chemical properties of different molecules specially unknown substances, environmental and forensic analytes, quality control of drugs, foods and polymers.

- **Principle**

In this process sample is converted into gaseous ions with or without fragmentation, and their characterization are done according to their mass to charge ratios (m/z) and relative abundances. Through this technique molecular weight of the sample components, molecular mass of a compound are precisely determined and also helpful in identification of isotopes indirectly. In this technique ions are generated from either inorganic or organic compounds, separated by their mass-to-charge ratio (m/z) and qualitative as well as quantitative detection are done according to their respective m/z and abundance. The ionization of the analytes may be done thermally, by electric fields or by impacting energetic electrons, ions or photons. Here status of ions may be single ionized atoms, clusters, molecules or their fragments or associates. Separation of ions is executed by static or dynamicelectric or magnetic fields.

For analyzing biomolecules mass spectrometry has become a vital field in scientific world. Other techniques like electrophoresis, chromatography or ultracentrifugation although provides similar information but they only provides information about characterization rather than molecular weight. Exact molecular weight can only be calculated by determining the chemical structure of a particular compound. One type of mass spectroscopy is Electrospray ionization mass spectrometers (ESI MS) which is also known as “soft” ionization technique

as it is widely utilized in biological mass spectrometry. It helps in the ionization of large bio molecule like proteins without breaking them, thus helps in intact analysis.

In Electrospray ionization mass spectrometers (ESI MS) the targeted molecules which will be analysed is allowed to pass through mass spectrometers in a fine spray, subsequently separated through chromatographic process either through traditional liquid chromatography, HPLC, or nano-LC. After emerging from ESI source in the form of spray, molecules get ionised by the nozzle's electrically charged tip. Like the travelling of mist and its evaporation, electrostatic repulsion causes repulsion between like-charged ions which ultimately drives the molecules apart. Then the molecules are analysed by various analysers including quadrupoles, ion traps, time-of-flight, and Fourier transform-based instruments.

- **Procedure**

ESI mass spectra were recorded from a Water HRMS model XEVO-G2QTOF#YCA351 spectrometer. For Experimental procedure the crude extract was diluted with Acetonitrile and spectrum was measured at room temperature.

Acute toxicity study

Acute studies were carried out on adult female albino rats of Wistar strain having standard body weights (130-150g)and age (4-5 months) (having fasting blood sugar levels < 110mg/dl) were procured from the animal housing facility of Jadavpur University. Animals were housed in propylene cages in an air conditioned room under standard conditions of temperature and humidity (25±2^{o}C with 12 h-light and 12 h-dark cycle). Animals were fed with normal protein diet (18% casein, 70% carbohydrate, 7% fat, 4% salt mixture and 1%vitamin mixture) and water ad libitum(Hawk PB *et al.*, 1954). All the animals were acclimatized to normal laboratory conditions for 7 days before commencement of the experiment.Animals were maintained according to the guidelines of Institutional Animal

Ethics Committee of Jadavpur University, Kolkata, India (constituted as per the "Gazette of India "notification part II Sec.3 (ii) 17 of the Ministry of Environment & Forestry, Government of India, dated 8th 1998 for the "Prevention to cruelty to animal 1968"). Also the study of the anti hyperglycemic effect of the extracted crude terpenoid was conducted on female albino mice (each 30-40gm). The experimental protocols were performed in accordance and approval by the Institutional Animal Ethical committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), under the Ministry of animal welfare division, Government of India, New Delhi, India. Thirty female Wistar albino rats were selected for the acute toxicity study. They were arbitrarily divided into one normal control group and five treated groups, containing six animals in each group and were given standard normal pellet diet provided with water ad libitum. They were permitted to acclimatize for seven days to the laboratory condition prior to the experiment. The treated groups were orally supplemented with varying doses of root Salep of *Gymnadenia orchidis* Lindl (100 mg, 200mg, 300mg, 500mg and 1 g respectively of dry root powder/ kg body weight/day) for 10 days. Normal control group were treated with water. Body weight of animals were weighed and recorded. Animals were observed every day to observe any changes in autonomic or behavioral responses. After oral administration of the root Salep, the animals were observed individually for a period of 24 hours for the signs of toxicity or mortality. Gross activity, posture and tone, eye ball movement, reaction and reflexes of the animals, behavioral pattern (salivation, fur, lethargy, and sleep), changes in physical appearance, injury, pain, and signs of illness were noted in every day.

Experimental design of animals for the toxicity study

Dust root powder was prepared from the tuberous root by grinding the dry roots in a mortar placed in ice bath and different concentrations of Salep as per requirement was freshly

prepared with sterile deionized water to the powder root before use. Each group contains six rats.

Groups	Experimental design
Group I	Control (not supplemented with root salep)
Group II	Root Salep 100 mg of dry root powder /kg body weight/day
Group III	Root Salep 200 mg of dry root powder /kg body weight/day
Group IV	Root Salep 300 mg of dry root powder /kg body weight/day
Group V	Root Salep 500 mg of dry root powder / kg body weight/day
Group VI	Root Salep 1000 mg of dry root powder / kg body weight/day

- **Assessment of body weight in toxicity study**

During the acclimatization period, body weight of each rat was measured and recorded by using a sensitive balance during the adaptation period, once before starting the administration of drug, once weekly during drug administration period and once on the day of sacrifice. Measurement of body weights of rats was recorded to evaluate regular increment in body weight [246].

- **Clinical and mortality observation in toxicity study**

In toxicity study even though the rats appeared healthy and normal, it is mandatory to monitor the rats daily for 10 days to find out the toxicity. During the drug administration period of 10 days, all the animals were carefully evaluated daily for mortality patterns and clinical signs once before drug administration, immediately after drug administration and up to 4 hour after drug administration [247,248]. Mortality during the experimentation period of 10 days was also recorded and the percentage of mortality in each group was noted. Animals were observed every day to observe any changes in autonomic or behavioral responses. After oral administration of the root Salep, the animals were observed individually for a period of 24 hours for the signs of toxicity or mortality. Gross activity, posture and tone, eye ball

movement, reaction and reflexes of the animals, behavioral pattern (salivation, fur, lethargy, and sleep), changes in physical appearance, injury, pain, and signs of illness were noted in every day.

- **Collection of serum samples**

At the end of the investigation study period (11^{th}day) the rats were sacrificed with mild anesthesia. Blood samples were collected from the heart using appropriate needle from the ventricle slowly so that collapsing of heart could be avoided. Blood samples were collected in two different tubes and stored in normal BD Vacutainer and EDTA (5.4 mM/ 3ml blood) for further analysis of both biochemical analyses, respectively. Biochemical analyses were done by

- **Assessment of serum emzyme marker analysis and kidney function**

Blood sugar levels of all the animals (control and treated with graded dose of root Salep) were noted on initial and final day of experiment. Biochemical parameters like liver function enzymes such as acid phosphatase (ACP), alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) and kidney function test (urea and creatinine) were determined from serum by using the supplied kits for toxicological analysis of the Salep treated animals.

Experimental design of animals for antidiabetic study using root Salep on three groups namely control, diabetic control and diabetic group treated with root Salep.

Female albino rats (40 in number and having fasting blood sugar levels < 100 mg/dl) were procured similarly from animal housing facility of Jadavpur University. Animals were maintained according to the same guidelines of Institutional Animal Ethics Committee of Jadavpur University, Kolkata, India. Animals were acclimatized to standard laboratory

conditions and were given normal protein diet with water ad libitum (Hawk PB *et al.*, 1954).Out of 40 animals, 10 were taken for control (Group A) and rest of the diabetic induced animals were then clustered into two experimental groups (Group B and Group C) having 10 rats in each group whose fasting blood sugar levels were > 200 mg/dl. The experiment was thus conducted on those 3 groups of rats for 32 days.

- **Induction of experimental diabetes**

The rats were adapted to the diet and acclimatized in the standard laboratory conditions for 2 weeks before the experiment began. After 16 hrs of fasting, diabetes was induced by intraperitonal injection of Streptozotocin (STZ) at 60 mg/ kg body weight dissolved in 0.1 M citrate buffer at pH 4.5 [249]. The induced diabetes was confirmed by measuring the fasting blood glucose level from the tail vein by blood glucometer after 3 days. Animals that demonstrate blood glucose level more than 150mg/dl were selected as diabetic for experiment. Normal rats having blood glucose level less than 150 mg/dl were injected with citrate buffer**.**

Groups	Experimental setup
Group A	Control**,** normal healthy rats
Group B	STZ induced diabetic rats not supplemented with root salep.
Group C	STZ induced diabetic rats supplemented with root salep (200mg powder root /kg body weight.

- **Measurement of blood glucose**

The blood glucose level in 12 hour fasting condition of each rat was measured by making a small incision at the tip by using digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. This measurement was repeated in every 3 days till the completion of

the experiment. Body weight and general health conditions were also monitored every day in the fasting state.

- **Collection of blood from the rats**

After the treatment period of 32 days, all the rats were sacrificed after mild anesthesia and the blood samples were collected from the heart and stored in normal BD Vacutainer and EDTA (5.4 mM/ 3ml blood) containing BD Vacutainer for further analysis. Serum was isolated from the blood stored in normal BD Vacutainer by centrifuging at 3000 rpm for 20 mins to separate serum and were used for biochemical analysis.

- **Biochemical parameters, serum enzyme, antioxidant enzyme analysis**

Glycosylated haemoglobin (Hb1Ac) was measured using the kit and procedure provided by Biosystems, Barcelona, Spain [250]. Total serum protein was measured using the method given by Lowry *et al.* [251]. Total Cholesterol was measured using the kit provided by Accurex Biomedical Pvt. Ltd., Mumbai, India. Triglyceride was measured using kit provided by Merckotest®, Merck, Goa, India. Urea, alkaline phosphatase (ALP), SGOT and SGPT were measured using individual kits provided by Piramal Healthcare Limited, Mumbai, India. Creatinine was determined from Kit using the modified Jaffi's method provided by Merckotest®, Merck, Goa, India. Acid phosphatase (ACP) was measured by using the kit provided by Accurex Biomedical Pvt. Ltd., Thane, India. Lipid peroxidation was determined from the Thiobarbuturic Acid test (TBA test) with modification by Kumar and Das (1993) [252]. Super oxide dismutase (SOD) activity was assayed by the method based on the reduction of nitrobluetetrazolium (NBT) to blue pharmazone by superoxides, produced phytochemically in the reaction system (Beauchamp and Fridovich, 1971) [253]. Reduced glutathione (GSH) was determined by using the method of Davila *et al.* (1991) and

glutathione peroxidase (GPx) was estimated by using the method of Levander *et al.* (1983) [254,255].

Experimental design for antidiabetic study using extracted terpenoids (as a trial dose) from *Gymnadenia orchidis* Lindl roots

25 Female albino rats (having fasting blood sugar levels < 100 mg/dl) were taken from animal housing facility of Jadavpur University and were acclimatized to standard laboratory conditions and were given normal protein diet with water ad libitum (Hawk PB *et al.*, 1954).Animals were maintained according to the guidelines of Institutional Animal Ethics Committee of Jadavpur University, Kolkata, India. Out of 25 animals, 5 were taken for control (Normal control group) and rest of the STZ-diabetic induced animals were then clustered into four experimental groups named as (Group DC, Group DT, Group DWT and Group DM) having 5 rats in each group whose fasting blood sugar levels were > 200 mg/dl. The experiment was thus conducted for 5 days. Different group of animals were treated with drugs twice a day with 8 hour interval for nine doses of 5 days schedule as described by Chakrabarti *et al*. [255].

Animal Groups	Treatment
Normal Control (NC)	Normal controlhealthy rats with no treatment.
Diabetic control(DC)	STZ induced Diabetic rats with no treatment
Diabetic+terpenoid (DT)	Supplemented with extracted terpenoid from the root (4mg/kg bodyweight as a trial dose).
Diabetic-terpenoid (DWT)	After terpenoid extraction, the root powder was washed repeatedly by deionized distilled water, air dried and supplemented (200mg/kg body weight) to the STZ induced diabetic animals orally.
Diabetic+metformin(DM)	Metformin (100 mg/ kg body weight) was supplemented to STZ induced diabetic animals orally.

- **Measurement of blood glucose**

The blood glucose level in 12 hr fasting condition of each rat was measured by making a small incision at the tip of the tail by using digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. This measurement was done on 1st day, 3rd day and on the 5th (last) day of the experiment. Body weight and general health conditions were also monitored every day in the fasting state.

- **Collection of liver from the rats for liver glycogen estimation**

After the treatment period of 5 days, all the rats were sacrificed after mild anesthesia and the liver samples were dissected out from the rats and washed in ice-cold saline taken on tray for liver glycogen estimation.

- **Preparation of tissue homogenate**

200 mg of the liver was taken and homogenized with 10 ml of 5% trichloroacetic acid in a homogenizer fitted with a Teflon plunger at 600 rpm for 5 min. The homogenate was then filtered and used for glycogen estimation.

Experimental design on diabetic rats by using root Salep with terpenoid, root Salep devoid of terpenoid, metformin (standard drug) on the hepatic enzymes of STZ-induced diabetic rats and histological analysis.

25 Female albino rats (having fasting blood sugar levels < 100 mg/dl) were taken from animal housing facility of Jadavpur University and were taken from animal housing facility, acclimatized to standard laboratory conditions and were maintained at same above mentioned conditions during toxicity study. Animals were given normal protein diet with water ad libitum (Hawk PB *et al.*, 1954). The animals were divided into five groups, each group containing five rats and the experiment was carried out for 5 days. Out of 25 animals, 5 were taken for control (Normal control group) and rest of the STZ-diabetic induced animals

were then clustered into four experimental groups named as (Group DC, Group DT, Group DWT and Group DM) having 5 rats in each group whose fasting blood sugar levels were > 200 mg/dl.Different group of animals were treated with drugs twice a day with 8 hour interval for nine doses of 5 days schedule as described by Chakrabarti *et al*..(2003)[256]. The fasting blood glucose levels and biochemical parameters in serum were analyzed to assess the antidiabetic activity of the root Salep of *Gymnadenia orchidis* Lindl.

Animal Groups	Treatment
Normal Control (NC)	Control**,** normal healthy rats with no treatments.
Diabetic control (DC)	STZ induced diabetic rats with no treatment.
Diabetic+terpenoid (DT)	Supplemented with root Salep containing terpenoid (200mg/kg body weight)
Diabetic-terpenoid (DWT)	After terpenoid extraction from the root, the root powder was washed repeatedly by deionized distilled water, air dried and supplemented (200mg/kg body weight) to the diabetic animals orally.
Diabetic+metformin (DM)	Metformin (100 mg/ kg body weight) was supplemented orally.

- **Measurement of blood glucose**

The blood glucose level in 12 hr fasting condition of each rat was measured by making a small incision at the tip of the tail by using digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. This measurement was done on 1st day, 3rd day and on the 5th (last) day of the experiment. Body weight and general health conditions were also monitored every day in the fasting state

- **Collection of blood from the rats and biochemical analysis.**

After the treatment period of 5 days, all the rats were sacrificed after mild anesthesia and the blood samples were collected from the heart and stored in normal BD Vacutainer and EDTA (5.4 mM/ 3ml blood) containing BD Vacutainer for further analysis. Serum was

isolated from the blood stored in normal BD Vacutainer by centrifuging at 3000 rpm for 20 mins. to separate serum and were used for biochemical analysis (acid phosphatase, alkaline phosphatase, GOT,GPT, kidney function i.e. urea, creatinine and BUN, antioxidant enzyme functions like S.O.D, lipid peroxidation from serum and liver tissue homogenates, lipid profile etc.

- **Histopathological Study.**

On the final day of experiment, rats were sacrificed and organs like liver, kidney, and pancreas were collected for histological studies. Collected tissue were cleaned by washing them with ice-cold normal saline and fixed in Bouin's fluid immediately for at least 24 hour period. Then the tissues were washed with first 70% alcohol then 80% alcohol to clear out the fixing fluid. After fixation and the removal of the fixative by alcohol, the specimens were dehydrated by using graded concentrations of alcohol (50%, 70%, 80% and 95%) and each specimen were kept in each grade of alcohol long enough (12hrs) to dehydrate. Dehydrated specimens were embedded in paraffin at 56-58^0 C. Using rotary microtome the embedded tissues were sectioned serially into 4-5 µm in thickness. These paraffin sectioned tissues were attached to the slide and before staining they were subsequently washed with xylolfor 5 mins to remove paraffin. The slides were then transferred to a mixture of equal parts of xylol and absolute alcohol, then through absolute, 95%, 80%, 70%, 50% alcohols. The slides containing sectioned tissues were then stained with haematoxylin-eosin dye for morphological evaluation under microscope (100X). Differences were observed between the microscopic features of the organ of the treated groups and control groups [113].

Experiment on diabetic mice by using extracted terpenoid (crude)

20 Female albino mice of Wistar strain (having fasting blood sugar levels < 100 mg/dl) were taken from animal housing facility and acclimatized to standard laboratory

conditions and were given normal protein diet with water ad libitum (Hawk PB *et al.*, 1954).Animals were maintained according to the guidelines of Institutional Animal Ethics Committee of Jadavpur University, Kolkata, India. Animals were fed with normal protein diet and water ad libitum (Hawk PB *et al.*, 1954). Out of 20 animals, five animals were taken as control animal and rest of the STZ-diabetic induced animals were then clustered into three experimental groups named as (Group DC, Group DT, and Group DM) having 5 mice in each group whose fasting blood sugar levels were > 200 mg/dl and the experiment was carried out for 5 days.Different group of animals were treated with drugs twice a day with 8 hour interval for nine doses of 5 days schedule as described by Chakrabarti *et al.* (2003)[256]. The biochemical parameters in serum of mice were analyzed to assess the antidiabetic activity of the extracted crude terpenoid of *Gymnadenia orchidis* Lindl.

Animal Groups	Treatment
Normal Control (NC)	Control**,** normal healthy mice with no treatments.
Diabetic control (DC)	STZ-diabetic mice were not treated with crude extracted terpenoid.
Diabetic+terpenoid(DT)	Treated with extracted terpenoid (5 mg/ kg body weight for 5days).
Diabetic+Metformin(DM)	Treated with Metformin 100 mg / kg body wt. for 5 days

- **Collection of blood from the mice and biochemical analysis**

After the treatment period of 5 days, all the mice were sacrificed after mild anesthesia and blood samples were collected from the heart and stored in normal BD Vacutainer and EDTA (5.4 mM/ 3ml blood) containing BD Vacutainer for further analysis. Serum was isolated from the blood stored in normal BD Vacutainer by centrifuging at 3000 rpm for 20 mins to separate serum and were used for further biochemical analysis (GOT, GPT, ACP, S.O.D, Cholesterol, Triglycerides, Urea, Creatinine, BUN). Histopathologiacal analysis of pancreas, liver and kidney were done to see the efficacy of the extracted terpenoid from root on STZ-induced diabetic mice.

Experiment on diabetic mice by using extracted terpenoid (crude) in two different doses.

Here two different doses of extracted terpenoid were taken to see the effect of desired drug on STZ-induced diabetic mice. Twenty adult female albino mice (Wistar strain) were taken from animal housing facility and acclimatized to standard laboratory conditions of temperature and humidity (25±2ºC with 12 h-light and 12 h-dark cycle) for 14 days before commencement of the experiment. Animals were fed with normal protein diet and water ad libitum (Hawk PB *et al.*, 1954). Out of 20 animals, five animals were taken as control animal (NC) and rest of the STZ-diabetic induced animals were clustered into three experimental groups named as (Group DC, Group DT-T2, and Group DT-T4) having 5 mice in each group whose fasting blood sugar levels were > 200 mg/dl and the experiment was carried out for 5 days. Different group of animals were treated with drugs twice a day with 8 hour interval for nine doses of 5 days schedule as described by Chakrabarti *et al.* (2003) [256]. The biochemical parameters in serum were analyzed to assess the antidiabetic activity of the extracted terpenoid of *Gymnadenia orchidis* Lindl.

Groups	Experimental setup
Normal Control (NC)	Control**,** normal healthy mice
Diabetic control (DC)	STZ-diabetic mice were not treated with crude extracted terpenoid.
Diabetic+terpenoid(DT-T2)	Treated with terpenoid extracted (2 mg/ kg body weight for 5days).
Diabetic + terpenoid (DT-T4)	Treated with terpenoid extracted (4 mg/ kg body weight for 5days).

- ### Collection of blood from the mice

After the treatment period of 5 days, all the mice were sacrificed after mild anesthesia and the blood samples were collected from the heart and stored in normal BD Vacutainer and EDTA (5.4 mM/ 3ml blood) containing BD Vacutainer for further analysis. Serum was

isolated from the blood stored in normal BD Vacutainer by centrifuging at 3000 rpm for 20 mins to separate serum.

- **Analysis of key enzymes of carbohydrate metabolism (G6PDH)**

Quantitative estimation of Glucose-6-phosphate dehydrogenase (G6PDH) was done from erythrocytes by reagent kit supplied by Enzopak (RECKON DIAGNOSTICS P. LTD) according to the UV-kinetic method.

- **Glycatedhemoglobin (HbA1c) and haemoglobin concentration**

Glycosylated Haemoglobin (Hb1Ac) was measured by using ion exchange resin method (glycosylated haemoglobin kit) provided by Coral Clinical Systems; India. Haemoglobin concentration was measured using collected blood, Hydrochloric acid and Sahli's hemoglobinometer.

- **Determination of serum insulin**

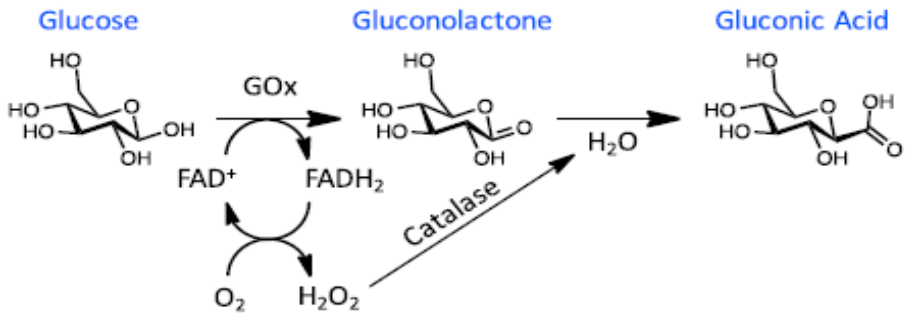
Serum insulin level was assayed by Enzyme linked Immuno sorbent Assay (ELISA) kit using human insulin as a standard and supplied by MonobindInc.USA.

Biochemical estimations in serum samples

Blood glucose determination

Blood glucose level was measured by digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. The blood glucose level in 12 hr fasting condition of each animal was measured by making a small incision at the tip of the tail by using digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. This measurement was done on every alternate day of the experiment. Blood sample is collected from the pricked tail vein and test strip was hold near blood drop. Through capillary test strip blood is sucked and blood containing glucose

reacts with an enzyme electrode containing glucose oxidase /glucose dehydrogenase/hexokinase in glucometer. Thus hydrogen peroxide is generated or an intermediary which reacts with a dye resulting in a colour change proportional to the concentration of glucose in solution and detected in detector. Enzyme glucose oxidase (GOx) catalyses the oxidation of glucose (blood) to form gluconolactone in presence of oxygen. Here hydrogen and water are produced as by-products. The produced gluconolactone reacts further with water producing carboxylic acid product, gluconic acid [275]. GOx utilises flavin adenine dinucleotide (FAD^+) as a cofactor to carry out this oxidation process where FAD^+ act as an electron acceptor which gets reduced to $FADH_2$ during the redox reaction [276]. FAD^+ cofactor is regenerated when $FADH_2$ consequently reacts with oxygen forming hydrogen peroxide. This reaction takes place at the anode and number of transferred electrons is proportional to the amount of H_2O_2 generated and hence the concentration of glucose [276].



Scheme 1: Conversion of glucose to gluconic acid using glucose oxidase [257].

Estimation of glycosylated haemoglobin (HbA1C) (Trivelli *et al.*, 1971)

Glycosylated haemoglobin level was analysed by using ion exchange resin method (glycosylated haemoglobin kit).

- **Principle**

Glycosylated haemoglobin (GHb) / HbA1cis a form of haemoglobin which is chemically linked to sugar molecules by non-enzymatic process i.ecovalent bonding between glucose to the amino-terminal valine of the haemoglobin beta chain. This is an irreversible and slow process which indicates the presence of excessive amount of glucose in blood and acts mainly as a marker of diabetes in patients. This change is stable throughout the RBC life.

$$\text{Hemoglobin (whole blood preparation)} + \text{Cation exchange resin} \xrightarrow{\text{mix for 5 min}} \text{Fast fractions (HbA1a , HbA1b, HbA1c)}$$

GHb reflects the mean glucose level over an extended period of time (average three month). HbA1c measurement acts as a diagnostic test for diabetes mellitus and helps in the evaluation of glycemic control in diabetic people. Glycosylated haemoglobin are defined as "fast fraction" hemoglobins HbA1 (HbA1a, A1b, A1c) which elutes first during column chromatography. Non-glycosylated fraction of haemoglobin (HbAo) forms the bulk of haemoglobin. Glycosylated haemoglobin is estimated by employing a weak binding cation-exchange resin for the rapid separation of glycohemoglobin (Fast Fraction) from non-glycosylated hemoglobin. GHb in blood was estimated by ion-exchange resin method (Goldstein *et al.,* 1994). The whole blood was mixed with lysing reagent containing detergent and borate ions. Hemolysate was further mixed with cation exchange resin. All Hb's are retained by the resin and glycatedhemoglobin was eluted. The % of HbA1c was determined by measuring the ratio of absorbance of the glycohemoglobin (GHb) fraction and total hemoglobin (THb) fraction at 415 nm and results were compared with a standard GHb preparation carried throughout the test.

- **Materials and Reagents**

-Ion exchange resin (L1)

-Lysing reagent (L2)

-Resin seperators

-Sample: Venous blood collected with EDTA was used for the analysis of GHb.

-The control was reconstituted with 1 ml of distilled water and allowed to stand for 10 mins with occasional stirring.

- **Procedure**

A. Hemolysate preparation

0.5ml of lysing reagent (R2) was dispensed into tubes labelled as control (C) and test (T). 0.1ml of the reconstituted control was added and well mixed blood sample into the appropriate labelled tube and mixed until complete lysis was evident, allowed to stand for 5 minutes.

B. Glycosylated Hemoglobin (GHb) separation

The cap was removed from the ion exchange resin tubes and labelled as control and test. 0.1 ml of the hemolysate from step A was added into the appropriate labelled ion exchange resin tubes. Inserted the resin separator (R3) into each tubes, the rubber sleeve was approximately 1 cm above the liquid level of the resin suspension. The tubes were mixed using a vortex mixer continuously for 5 minutes and allowed the resin to settle and then push the resin separator into the tubes until the resin was firmly packed. Then poured the supernatant into a cuvette and read the absorbance at 415 nm against distilled water.

C. Total Hemoglobin (THb) fraction

5ml distilled water was added into the labelled tubes and added 0.02ml of hemolysate from step A into the appropriate labelled tubes. Mixed well and read the absorbance at 415 nm against distilled water.

- **Calculation**

$$\text{Ratio of control } (R_c) = \frac{\text{Abs. Control GHb}}{\text{Abs. Control THb}}$$

$$\text{Ratio of Test } (R_T) = \frac{\text{Abs. Test GHb}}{\text{Abs. Test THb}}$$

$$\text{GHb in \%} = \frac{\text{Ratio of Test } (R_T)}{\text{Ratio of control } (R_c)} \times 10$$

Estimation of haemoglobin

Haemoglobin is the iron-containing oxygen-transport metalloprotein found in the red blood cell of vertebrates. Haemoglobin transports oxygen from the lungs or gills to the rest of the body, such as to the muscles, where it releases the oxygen for cell use. The haemoglobin molecule is an assembly of four globular protein subunits. Each subunit is composed of a protein chain tightly associated with a non-protein heme group. A heme group consists of an iron (Fe) ion (charged atom) held in a heterocyclic ring, known as a porphyrin. The iron ion, which is the site of oxygen binding, coordinates with the four nitrogens in the centre of the ring, which all lie in one plane. The iron is also bound strongly to the globular protein. The iron ion may either be in the Fe^{2+} or Fe^{3+} state, but ferrihemoglobin (Fe^{3+}) cannot bind oxygen. In binding, oxygen temporarily oxidizes Fe to (Fe^{3+}), so iron must exist in the +2 oxidation state in order to bind oxygen (Couture *et al*.., 1999). Haemoglobin is measured in grams per decilitre of blood. Normal haemoglobin levels of blood are as follows:

Men: 13.5 to 16.5 g/dl

Women: 12.1 to 15.1 g/dl

Children: 11 to 16 g/ dl

Pregnant women: 11 to 12 g/dl

Rat : 14 to 16.1 g/dl

- **Material Required**

1) Hydrochloric Acid (HCl) -0.1 N

2) Sahli's Haemoglobinometer

- **Method of Estimation**

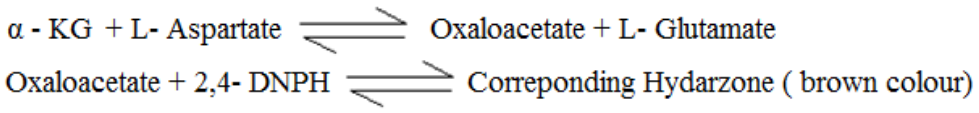
Blood haemoglobin percentage was measured by Sahli's Haemoglobinometer (Chatterjee, 1994). The instrument consists of two tubes, one of which contains 20 cu.mm of blood haemolysed with (N/10) HCL and saturated with CO gas. The colour of this tube was used as standard. In the other tube a little (N/10) HCl was taken and 20 cu.mm of animal blood from each group was collected by a special tube and added. Haemoglobin was converted into acid haematin by HCL. The colour developed was matched against the standard. If the colour of the unknown was stronger, it was diluted with (N/ 10) HCL until the tinge was same in both. The graduation up to which the blood had been diluted gives the percentage of haemoglobin.

Estimation of serum glutamic oxaloacetic transaminase (SGOT/AST)

Diagnostic Reagent Kit supplied by ARKRAY Healthcare Pvt. Ltd, Surat, India for the in vitro determination of Aspartate aminotransferase (AST) in human serum/ plasma. This procedure was according to the 2,4-DNPH (Reitman and Frankel, 1957) Method.

- **Assay principle**

The transamination of L-Aspartate and α-ketoglutarate (α-KG) to form Oxaloacetate and L-Glutamate was catalyzed by Aspartate aminotransferase(AST).The produced Oxaloacetate coupled with 2,4-Dinitrophenyl hydrazine (2, 4-DNPH) and it forms a corresponding hydrazone, a brown coloured complex in alkaline medium and this can be measured colorimetrically.



- **Reagent composition**

-Buffered Aspartate- α-KG Substrates, pH 7.4

-2, 4- DNPH Colour Reagent.

-Sodium Hydroxide, 4 N.

-Working Pyruvate Standard, 6 mM (114 IU/L).

- **Working Reagent Preparation**

Reagent 1, 2 and 4 were ready to use.

Solution I: 1 ml of Reagent 3 was diluted to 10 ml with purified water.

- **Procedure**

Four tubes were taken, Blank (B), Standard (S), Test (T) and Control (C). 0.25 ml of Reagent 1 was added to each tube respectively. 0.05 ml of Serum was added to Test (T) tube and 0.05 ml of Standard was added to the Standard (S) tube. All the above tubes were mixed well and kept for incubation at 37ºC for 60 minutes. Then 0.25 ml of Reagent 2 was added to

each test tube respectively. 0.05 ml of Deionised Water was added to Blank (B)tube. Then 0.05 ml of Serum was added to Control(C) tube. After mixing well, all the tubes were allowed to stand at room temperature (+15ºC to +30ºC) for 20 minutes. 2.5 ml of Solution I was added to each tube respectively. All the tubes were mixed well and the O.D was measured against purified water in a colorimeter using green filter or on photometer at 505 nm, within 15 minutes.

- **Calculation**

$$\text{AST (GOT) activity (in IU/L)} = \frac{\text{Abs. of Test - Abs. of Control}}{\text{Abs. of Standard - Abs. of Blank}} \times \text{Conc. of Standard}$$

Estimation of serum glutamic pyruvate transaminase (SGPT/ ALT)

Diagnostic Reagent Kit supplied by ARKRAY Healthcare Pvt. Ltd, Surat, India for the in vitro determination of Alanine aminotransferase (ALT) in human serum/ plasma. This procedure was according to the 2, 4-DNPH (Reitman and Frankel, 1957) Method.

- **Assay principle**

The transamination of L-Alanine and α-ketoglutarate (α-KG) to form Pyruvate and L-Glutamate was catalyzed by Alanine aminotransferase (ALT). The produced Pyruvate coupled with 2, 4- Dinitrophenyl hydrazine (2, 4-DNPH) and it forms a corresponding

$$\alpha\text{ - KG + L- Alanine} \rightleftharpoons \text{Pyruvate + L- Glutamate}$$
$$\text{Pyruvate + 2,4-DNPH} \rightleftharpoons \text{Corresponding Hydrazone (brown colour)}$$

hydrazone, a brown coloured complex in alkaline medium and this can be measured colorimetrically.

- **Reagent composition**
  - Buffered Alanine -α-KG Substrates, pH 7.4
  - 2, 4- DNPH Color Reagent.
  - Sodium Hydroxide, 4 N.
  - Working Pyruvate Standard, 8 mM (150 IU/L).
- **Working reagent preparation**
  - Reagent 1, 2 and 4 were ready to use.
  - Solution I: 1 ml of Reagent 3 was diluted to 10 ml with purified water.
- **Procedure**

Four tubes were taken and marked as Blank (B), Standard (S), Test (T) and Control (C). 0.25 ml of Reagent 1 was added to each tube respectively. 0.05 ml of Serum was added to tube marked as Test (T) and 0.05 ml of Standard was added to the tube marked as Standard (S). All the above tubes were mixed well and kept for incubation at 37ºC for 60 minutes. Then 0.25 ml of Reagent 2 was added to each test tube respectively. 0.05 ml of Deionised Water was added to test tube Blank (B). 0.05 ml of Serum was added to tube marked as Control (C). All the tubes were mixed well and allowed to stand at room temperature (+15ºC to +30ºC) for 20 minutes. 2.5 ml of Solution I was added to each tube respectively. All the tubes were mixed well and the O.D was measured against purified water in a colorimeter using green filter or on photometer at 505 nm, within 15 minutes.

- **Calculation**

$$\text{ALT (GPT) Activity (in IU/L)} = \frac{\text{Absorbance Of Test - Absorbanmce of Control}}{\text{Absorbance of Standard - Absorbance of Blank}} \text{ X Conc. of Standard}$$

Estimation of Acid Phosphatase (ACP)

- **Principle**

The phosphatase activity in plasma was assayed according to the method of Bessey *et al.* (1946), illustrated by Bergmeyer and Bernt (1963) using p-nitrophenyl phosphate as the substrate. After 30 minutes incubation, the phosphatases were completely inhibited by sodium hydroxide which forms a yellow anion with the p-nitrophenol liberated by the phosphatases. The phosphatase activity is directly proportional to the amount of p-nitrophenol, liberated per unit time. One phosphatase unit is the amount of enzyme contained in 1000ml plasma, which liberates 1 m mole (139.11mg) p-nitrophenol at 37^{o}C.

- **Reagents**

  - Citric acid
  - Sodium citrate
  - Glycine
  - Magnesium chloride ($MgCl_2$, $6H_20$)
  - Sodium hydroxide
  - p-nitrophenyl phosphate, sodium salt
  - p-nitrophenol
  - Sucros

- **Preparation of solution**

(a) Acid buffer substrate solution (0.05 M citrate buffer; $5.5xl0^{-3}$ M p-nitrophenyl phosphate; pH 4.8) : 0.41 g of citric acid, 1.125 g of sodium citrate and 165 mg of Na-p-nitrophenyl phosphate were dissolved in 100 ml of double distilled water

(b) Alkaline buffer-substrate solution (0.05 M glycine buffer; $5.5xl0^{-3}$ M p-nitrophenyl phosphate; pH 10.5) : 375 mg of glycine, 10 mg of magnesium chloride and 165 mg of Na-p-nitrophenyl phosphate were dissolved in 42 ml of 0.1N NaOH and the volume was made up to 100 ml with double distilled water.

(c) p-nitrophenol standard solution (5×10^{-5}M) 696 mg of p-nitrophenol was dissolved in 0.02 N sodium hydroxide and the volume was made to 1000 ml. 10 ml of this solution was diluted to 1000 ml with 0.02 N NaOH, for preparation of the working standard.

(d) 0.25 M sucrose solution: 21.39 g of sucrose was dissolved in 250 ml of double distilled water.

(e) 0.1 N NaOH solution: 0.4 g ofsodium hydroxide was dissolved in 100 ml double distilled water.

(f) 0.02 N NaOH solution: 0.1N NaOH solution was diluted five times to prepare 0.02 N NaOH.

- **Tissue preparation**

50 to 60 mg of tissues were homogenized separately in 2 ml of 0.25 M sucrose solution and then centrifuged at 3000 rpm for 10 minutes. The clear supernatant were used for the assay of acid phosphatise

Procedure for determination of acid phosphatase

First of all for each sample, two test tubes were taken separately, one was marked "sample" and the other "control". 1 ml of acid buffer - substrate solution was added to each of these tubes and they were preheated at 37°C for 5 mins. After that 0.2 ml of the supernatant was added to each tube marked "sample". The incubation process of all marked test tubes was carried out for 30 mins at 37°C. After incubation 4 ml of 0.01 N NaOH were added to each of the tubes to stop the reaction. 0.2 ml of the supernatant was then added to the tubes marked "control". Mixing of the contents of the tubes was done by inverting them several times. O.D measurements of the solution were carried out at 420 nm against a reagent blank. The final O.D was calculated by subtracting the O.D of the corresponding "control" from the "sample" O.D.

In case of plasma, 1ml of ACP buffer was taken in each of sample and control tubes.0.2 ml of plasma was added to the sample tube, and both tubes were incubated as in the above procedure. 0.2 ml plasma was added to the control tube after incubation, and the remaining procedure remains the same.

- ### Preparation of standard curve

By using 1ml, 2ml, 3ml, 4ml, 5ml, 6ml, and 7ml, of working standard solution of p-nitrophenol a standard curve was prepared. 0.02 N NaOH was added to each of these tubes to make the volume 11.1 ml and optical density was measured against 0.02 N NaOH as blank. With µmol PNP on the abscissa and optical density (OD) on the ordinate a standard curve was plotted.

- **Calculations**

Respective amounts of p-nitrophenol liberated was calculated from the standard curve. The amount (unit) of acid phosphatase present in each sample was calculated as follows:

- **Acid phosphatase**

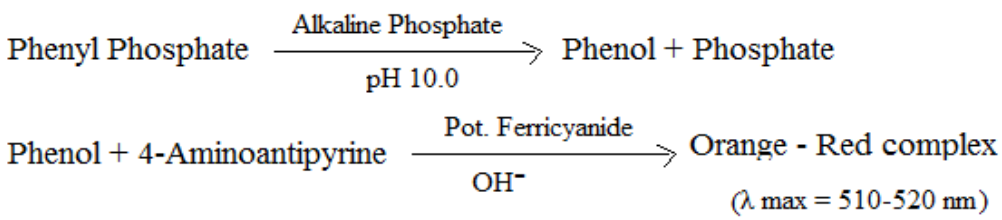
The values were calculated from the standard curve. Unit read off from standard curve were multiplied by 20x (5.2/ll.l) x 1/2 = 4.68 to obtain units of acid phosphatase. It was expressed as p moles/hr/100 ml of serum and mmol/hr/gm of wet tissue.

Estimation of Alkaline Phosphatase (ALP)

Diagnostic Reagent Kit supplied by ARKRAY Healthcare Pvt. Ltd, Surat, India for the in vitro determination of Alkaline Phosphatase in Serum. This procedure was according to the King and King's Method.

- **Principle**

Phenyl Phosphate converts to Inorganic Phosphate and Phenol by alkaline phosphatase from serum at pH 10.0. In alkaline medium this produced Phenol reacts with 4-Aminoantipyrine in presence of the oxidizing agent Potassium Ferricyanide and forms an orange-red coloured complex, which can be measured colorimetrically. The color intensity is proportional to the enzyme activity. The Reaction can be represented as:



- **Preparation of working solution**

One vial of Reagent 1, Buffered substrate was reconstituted with 2.2 ml of purified water. Reagent 2 and 3 were ready to use.

- **Procedure**

A. FOR COLORIMETER

0.5 ml of working buffered substrate solution was added to the vial labeled as Blank (B), Standard (S), Control (C), and Test (T). Then 1.5 ml of purified substrate was added to each above mentioned vial respectively. Then each vial was mixed well and kept for incubation at 37º C for 3 mins. 0.05 ml of serum was added only to the Test (T) vial. 0.05 ml of Reagent 3 was added to the Standard (S) vial. Then it was mixed well and kept for incubation at 37º Cfor 15 mins. 1 ml of Reagent 2 was added to each vial respectively. 0.05 ml of serum was added to Control (C) vial. Each of the vials was mixed well after addition of each reagent and the O.D of Blank (B), Standard (S), Control (C) and Test (T) was measured against purified water using a green filter.

B. For spectrophotometer

Depending on flowcell / cuvette capacity, all the volumes mentioned for colorimetric procedure can be adjusted proportionately and rest of the procedure remains unchanged. The O.D can be measured at 510 nm.

- **Calculations**

Serum Alkaline Phosphatase

$$\text{Activity in K.A Units} = \frac{\text{O.D Test - O.D Control}}{\text{O.D Standard - O.D Blank}} \times 10$$

Estimation of protein (Lowry *et al.*, 1951)

- **Principle**

Protein was estimated by the Folin Ciocalteau reagent (Lowry *et al.*,1951).The blue colour developed by the reduction of the phosphor molybdic phosphotungstic components in the Folin Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the color developed by the biuret reaction of the protein with the alkaline cupric tartrate was measured in the Lowry's method at 660 nm.

- **Reagents**

  - 2% sodium carbonate in 0.1 N NaOH (Reagent A)
  - 0.5% Copper Sulphate in 1% Sodium Potassium Tartarate (Reagent B)
  - Alkaline copper reagent: 50 ml of A was mixed with 1.0 ml of B prior to use
  - **Folin-Ciocalteau reagent:**1 part of reagent was mixed with 2 parts of water.
  - **Stock standard:**50 mg of Bovine Serum Albumin was weighed and made up to 50 ml in a standard flask with saline.
  - **Working standard:** 10 ml of the stock of 50 ml was diluted with distilled water. 1.0 ml of this solution contains 200 μg of protein. A mixture consisting of 100g sodium tungstate ($Na_2WO_4.2H_2O$), 25g sodium molybdate, 700ml water, 50ml of 85% phosphoric acid, and 100ml of concentrated hydrochloric acid was refluxed gently for 10 hours in a 1.5L flask. 150g lithium sulfate, 50ml water and a few drops of bromine water was added. The mixture was boiled for 15minwithout condenser to remove excess bromine. The mixture was cooled, diluted to 1L and filtered. The reagent should have no greenish tint. (The acid concentration of the

reagent was determined by titration with 1N NaOH to a phenolphthalein end-point).

- **Protein solution (Stock standard):**Weigh accurately 50mg of bovine serum albumin (Fraction V) and dissolve in distilled water and make up to 50ml in a standard flask.

▪ Procedure

- **Extraction of protein from sample**

Extraction was carried out with buffers used for the enzyme assay. 500.0 mg of the sample was weighed and grinded well with a pestle and mortar with 5-10 mL of the buffer. Then it was centrifuged and the supernatant was used for protein estimation.

- **Estimation of protein**

1. 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard was pipetted out into a series of test tube.
2. 0.1ml and 0.2ml of the homogenate was pipetted out in two other test tubes.
3. The volume was made up to 1ml in all the test tubes. A tube with one ml of water serves as the blank.
4. 5ml of reagent C was added to each tube including the blank. All the tubes were mixed well and allowed to stand for 10min.
5. Then 0.5mL of reagent D (Folin-Ciocalteau) was added, mixed well and incubated at room temperature in the dark for 30min. Blue color is developed.
6. The reading was taken at 660nm.
7. A standard graph was drawn to calculate the amount of protein in the sample.

- **Calculation**

The amount of protein was expressed in mg/g wet tissue.

Estimation of urea

Diagnostic Reagent Kit supplied by ARKRAY Healthcare Pvt. Ltd, Surat, India for the in vitro determination of urea in human serum. This procedure was according to the DAM Method (Coulambe, G.G. *et al.* 1965 and Donald, R. Wybenga *et al.* 1971).

- **Principle**

In presence of Thiosemicarbazide, urea reacts with hot acidic Diacetylmonoxime and produces a rose-purple coloured complex, which was measured colorimetrically.

- **Reagent composition (supplied in the kit)**
  - Reagent 1: Urea Reagent
  - Reagent 2: Diacetylmonoxime (DAM)
  - Reagent 3: Working Urea Standard, 30 mg%
- **Preparation of Working Solution**

Solution I: 1 mL of Reagent 1 was diluted to 5 mL with purified water. Reagent 2 and Reagent 3 (Standard) were ready to use.

- **Procedure**

A. For Colorimeter: Three tubes were marked as Blank (B), Test (T) and Standard (S). 2.5mL of Solution I was added to each tube respectively. 0.01 mL of Sample was added to Test tube (T). 0.01 mL of Standard (Reagent 3) was added to the Standard tube (S). After mixing each tube well, 0.25 ml of Reagent 2, Diacetylmonoxime (DAM) was added to each test tube respectively. Again the tubes were mixed well and were kept in boiling water

exactly for 10 minutes. Then the tubes were cooled immediately under running water for 5 minutes, and were mixed by inverting them well. The color intensity was measured using a green filter against blank within 10 minutes.

B. For Spectrophotometer: All the volumes mentioned under colorimetric procedure can be adjusted proportionately depending on flowcell/ cuvette capacity. Rest of the procedure remains unchanged. The O.D was mentioned at 525 nm.

- **Calculation**

Serum / Plasma: Urea in mg/100 mL, (A) = $\frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times 30$

Blood Urea Nitrogen in mg/100 mL = (A) X 0.467

Urine: Urea in g/ L, (B) = $\frac{\text{O.D. of Test}}{\text{O.D. of Std}} \times \frac{30 \times 20}{100}$

Urea Nitrogen in g / L = (B) X 0.467

Estimation of creatinine

Diagnostic Reagent Kit supplied by ARKRAY Healthcare Pvt. Ltd, Surat, India for the in vitro determination of Creatinine in Serum, Plasma and Urine. This procedure was according to the Alkaline Picrate8 Method (Bonses, R.W *et al.* 1945 and Toro, G *et al.* 1975)

- **Principle**

Creatinine in a protein free solution reacts with Alkaline Picrate and produces a red coloured complex, which is measured colorimetrically

- **Reagent composition (supplied in the kit)**

  - Reagent 1: Picric Acid
  - Reagent 2: Sodium Hydroxide, 0.75 N

- Reagent 3: Stock Creatinine Standard, 150 mg%

- **Preparation of Working Solution**

Working Standard: 0.1 mL of Reagent 3 (Stock Creatinine Standard) was diluted to 10 mL with purified water and mixed well. All other reagents were ready to use.

- **Procedure**

A. For Colorimeter

Step A. Deproteinization of test sample:

0.5 mL of Serum / Plasma/ Dilute urine was taken in a test tube. 0.5 mL of purified water and 3.0 mL of Reagent 1(Picric A) were added to it and mixed well. Then the tube was kept in boiling water bath exactly for one minute. Tube was cooled down immediately under running tap water and it was centrifuged or filtered. Three tubes were taken and marked as Blank (B), Standard (S) and Test (T).2 mL of Supernatant/ Filtrate (from Step A) was added to tube marked as Test (T). 0.5 mL of Working Standard was added to tube marked as Standard (S).0.5 mL of purified water was added to the tube marked as Blank (B).1.5 mL of Reagent 1 (Picric acid) were added to Blank (B) and Standard (S). 0.5 mL of Reagent 2 (Sodium Hydroxide, 0.75 N) was added to these three test tubes respectively. All the tubes were mixed well and allowed to stand at room temperature (+15º C to +30ºC) exactly for 20 mins and the optical density of Blank (B), Test (T) and Standard (S) against purified water on a colorimeter with a green filter.

B. For Spectrophotometer:

All the volumes mentioned under colorimeteric procedure could be adjusted proportionately depending on flowcell/ cuvette capacity. Rest of the procedure remains unchanged. The O.D could be measured at 520nm.

- **Calculations**

Serum Creatinine in mg/100 mL= $\frac{\text{O.D. Test - O.D. Blank}}{\text{O.D. Std. - O.D. Blank}} \times 3.0$

Urine Creatinine in g/ litre,(A) = $\frac{\text{O.D. Test - O.D. Blank}}{\text{O.D. Std. - O.D. Blank}} \times 0.75$

Urine Creatinine in g/ 24 collection = (A) x 24 hours. Urine volume in litres

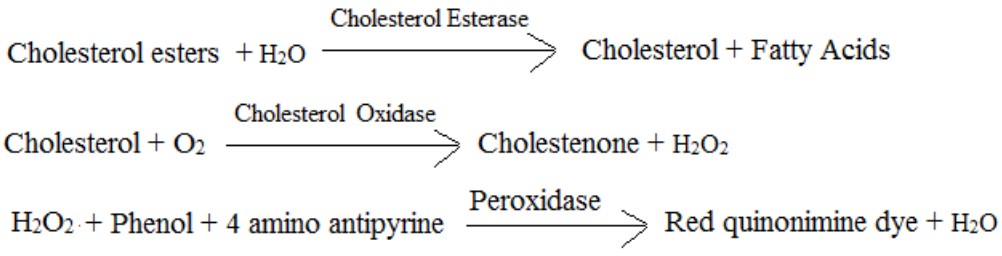
Estimation of Cholesterol (CHOD/POD METHOD)

The reagent kit was supplied by BEACON DIAGNOSTIC PVT.LTD. and intended for the "in vitro" quantitative determination of Cholesterol in serum/plasma.

- **Principle**

Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidised to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly propotional to the amount of cholesterol present in the sample.

- **Reaction:**



- **Reagent composition (supplied in the kit)**

  - Reagent 1: Cholesterol Enzyme Reagent
  - Reagent 2: Cholesterol Standard 200 mg/dl
  - Reagent 3: Cholesterol Precipitating Reagent (Free)

- **Procedure**

Three clean and dry test tubes were taken and marked as Blank (B), Standard (S) and Test (T). 1 mL of Enzyme reagent was added to tube Blank (B), Standard (S) and Test (T). 10 µL of Standard was added to tube marked as Standard (S). 10 µL of Sample was added to the tube marked as Test (T). All the tubes were mixed well and incubated at 37ºC 5mins and the optical density of Standard (Ab.S) and Test (Ab.T) against Reagent Blankat 505 nm.

- **Calculations**

Cholesterol in mg/dL = $\frac{\text{Abs. T}}{\text{Abs.S}} \text{ X } 200$

Estimation of HDL Cholesterol

The reagent kit was supplied by BEACON DIAGNOSTIC PVT.LTD. and intended for the "in vitro" quantitative determination of HDL Cholesterol in serum/plasma.

- **Preparation**

0.5 mL of serum / plasma was taken in to glass tube. 50µL of Cholesterol precipitating reagent was added. The tube was mixed well and left at R.T. for 10 mins. Then it was centrifuged at 3000 r.p.m. for 10 mins. The clear supernatant was taken for HDL cholesterol estimation.

- **Procedure**

Three clean and dry test tubes were taken and marked as Blank (B), Standard (S) and Test (T). 1 mL of Enzyme reagent was added to tube Blank (B), Standard (S) and Test (T). 10 µL of Standard was added to tube marked as Standard (S). 10 µL of Supernatant Sample was added to the tube marked as Test (T). All the tubes were mixed well and incubated at 37ºC

for 5 mins and the optical density of Standard (Ab.S) and Test (Ab.T) against Reagent Blank at 510 nm.

- **Calculation**

$$\text{HDL Cholesterol mg/dL} = \frac{\text{Absorbance of HDL Test}}{\text{Absorbance of Std}} \times 200$$

Estimation of Triglycerides

The reagent kit was supplied by liquid Gold ARKRAY Healthcare Pvt. Ltd. and intended for the "in vitro" quantitative determination of Triglycerides in serum/plasma.

- **Assay principle**

Triglycerides are hydrolysed by Lipoprotein Lipase (LPL) to produce Glycerol and Free Fatty Acid(FFA). In presence of Glycerol Kinase (GK), Adenosine Triphosphate (ATP) phosphorylates Glycerol to produce Glycerol 3-Phosphate and Adenosine Diphosphate (ADP). Glycerol 3-Phosphate is further oxidised by Glycerol 3-Phosphate Oxidase (GPO) to produce Dihydroxyacetone Phosphate (DAP) and H_2O_2. In presence of Peroxidase (POD), Hydrogen Peroxide couples with 4-Aminoantipyrine (4-AAP) and 4-Chlorophenol to produce red Quinoneimine dye. Absorbance of coloured dye is measured at 505nm and is proportional to Triglycerides concentration in the Sample.

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{FFA}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol 3-Phosphate} + \text{ADP}$$

$$\text{Glycerol 3-Phosphate} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$2\,H_2O_2 + \text{4-AAP} + \text{4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4\,H_2O$$

- **Reagent composition (supplied in the kit)**

  - Reagent 1: Triglyceride mono Reagent
  - Reagent 2: Triglyceride Standard

- **Procedure**

Three clean and dry test tubes were taken and marked as Blank (B), Standard (S) and Test (T). 10 µL of Serum/Plasma was added to tube Test (T). 10 µL of Reagent 2(Triglyceride Standard) was added to tube marked as Standard (S). 1000 µL of Reagent 1(Triglyceride mono Reagent) was added to allthree marked tubes. All the tubes were mixed well and incubated at 37ºC for 10mins and the optical density of Standard (Ab.S) and Test (Ab.T) against Reagent Blank at 505 nm.

- **Calculation**

  - **For Triglycerides**

Triglycerides (mg/dl) = $\frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \times 200$

For LDL- Cholesterol using Friedewald's equation:-

$$\text{LDL-Cholesterol} = \text{Total Cholesterol} - \frac{\text{Triglycerides}}{5} - \text{HDL Cholesterol}$$

  - **Conversion Factor**

Triglycerides concentration in mmol/L = Triglycerides concentration in mg/dL x 0.0113

Lipid peroxidation

▪ Principle

The formation of lipid peroxides by lipid peroxidation is an important step which leads to rapid and spontaneous degradation to large numbers of short chain products and specially aldehydes. The extremely simple method for the measurement of lipid peroxidation is Thiobarbituric acid test (TBA test) which was originally called as " Kreiss Test" and developed in 1930s as a test for rancid milk. This method was used by Wilbur et al. in 1949 to demonstrate lipid peroxidation in pure unsaturated fatty acids and tissue lipids. By slightly modification (Kumar and Das, 1993) this method is still used.

▪ Reagent composition

- Reagent 1: Disodium hydrogen phosphate (Na_2HPO_4)
- Reagent 2: Potassium dihydrogen phosphate (KH_2PO_4)
- Trichloroacetic acid (TCA)
- Thiobarbituric acid (TBA)

▪ **Preparation of Solution**

1. Phosphate buffer (0.1 M PB, pH = 7.5):-

- 14.2 gm of anhydrous Na_2HPO_4 was dissolved in double distilled water and volume made up to 1000 ml (solution a).
- 13.6 gm of KH_2PO_4 was dissolved in double distilled water and volume made up to 1000 ml (solution b).
- Solution "a" and "b" were mixed in 7:3 (v/v) ratios to get the required buffer and the pH was checked to 7.5.

2. 20% Trichloroacetic acid solution:-

- 20 gm TCA was dissolved in 100 ml double distilled water.

3. 0.67% Thiobarbituric acid solution:-

- 1.34 gm TBA was dissolved in 200ml hot double distilled water.

▪ Tissue preparation

Tissues were weighed and homogenized in phosphate buffer solution by maintaining a constant tissue concentration. Tissues homogenates were then centrifuged at 3000 x g for 20 mins. The clear supernatants were used for the assay.

▪ Procedure

In each test tube 0.5 ml of TCA, 1 ml TBA and 1 ml plasma were added. The tubes were then heated in a tightly stoppered tube for 10 min. in a boiling water bath. Tubes were cooled and then centrifuged for precipitating the protein. The absorbance of the pink color was then read at 530 nm against blank in a spectrophotometer.

Lipid peroxidation in tissue was determined by taking 2 ml tissue homogenate, 1 ml TCA and 2ml TBA mixture in a test tube and the tubes were heated, cooled and centrifuged. The optical density of the pink color was then measured at 530 nm against blank in a spectrophotometer.

▪ Calculation

The level of malondialdehyde (MDA) formed due to degradation of hydroperoxide is used to assess lipid peroxidation and it is calculated on the basis of molar extinction coefficient of MDA (1.56 x 105 M-1 cm-1) (Sinnhuber *et al..*, 1958). The results were expressed as n mole MDA / ml plasma as n mole MDA / mg of protein.

Superoxide Dismutase (SOD) activity

Beauchamp and Fridovich (1971) derived the SOD assay which was based on the reduction of nitrobluetetrazolium (NBT) to blue pharmazone by superoxides, produced photochemically in the reaction system.

- **Reagent composition**

I. Reagent 1: Phosphate buffer (0.1 mM, pH 7.8)

- Solution A: - In double distilled water,87.09 gm of anhydrous K_2HPO_4 was dissolved and volume made up to 500 ml.
- Solution B: -In double distilled water, 68.05 gm of KH_2PO_4 was dissolved and volume made up to 500 ml. From Solution "A" 90.8 ml was taken and mixed with 9.2 ml of Solution "B" and diluted to 1 litre with distilled water.

II. Reagent 2: Methionine 650 mM

- In distilled water, 2.984 gm of methionine was dissolved and volume was made up to 100 ml.

III. Reagent 3: Riboflavin 900 µM

- In distilled water, 3 3.87 mg of riboflavin was dissolved and volume was made up to 100 ml

IV. Reagent 4: Nitrobluetetrazolium (7.5 mM)

- In distilled water, 613.23 mg of nitrobluetetrazolium was dissolved and volume was made up to 100 ml.

V. Reagent 5: EDTA (200 mM)

- In distilled water 5.845 gm of EDTA was dissolved and volume was made up to 100 ml.

- **Tissue Preparation**

5 ml of 0.1mM phosphate buffer solutions (pH 7.8) was taken. 0.5gm of tissue (liver) were weighed and homogenized in above solutions and thus a constant tissue concentration was maintained. At r. p.m of 7000x g for 20 min tissue homogenates were centrifuged and the clear supernatant thus obtained were used for further assay.

- **Procedure**

This reaction mixture comprises of 1.7 ml phosphate buffer, 150 µl EDTA, 600 µl methionine, 300 µl NBT and 50 µl tissue homogenate. The initiation of reaction took place by adding 200 µl of riboflavin and then keeping all the tubes in front of tube light. Control tube was prepared without adding enzyme source. The optical density was measured against blank at different time intervals at 560 nm. The protein content of tissue was quantified according to the method of Lowry method (1951).

- **Calculation**

SOD activity was calculated in units / min / 100 mg protein.

Catalase activity

Catalase enzyme catalyses the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen (Ref). This enzyme plays an important role in protecting the cell from oxidative damages. According to the method of Cohen, et al. (1970) catalase activity was measured. The decomposition of H_2O_2 in presence of catalase enzyme was measured.

- **Reagent composition**

I. Reagent 1: Phosphate buffer (0.01 M, pH 7)

- Solution A: - In double distilled water, 87.09 gm of anhydrous K_2HPO_4 was dissolved and volume made up to 500 ml.
- Solution B: - In double distilled water, 68.05 gm of KH_2PO_4 was dissolved and volume made up to 500 ml. From Solution "A", 6.15ml of K_2HPO_4 was taken and mixed with 3.85 ml of Solution "B" (KH_2PO_4) and diluted to 1 litre with distilled water to obtain required ph.

II. Reagent 2: H_2O_2 (6mM)

- 0.06 ml 30% H_2O_2 per 100 ml 0.01M phosphate buffer.

III. Reagent 3: H_2SO_4(6N)

- 166 ml concentrated H_2SO_4 dissolved in distilled water and volume was made up to 1 litre.

IV. Reagent 4: $KMnO_4$ (0.01 N)

- 316 mg $KMnO_4$ dissolved in 1000ml double distilled water.

- **Tissue Preparation**

5 ml of 0.1M phosphate buffer solutions (pH 7) was taken. 0.5gm of tissue (liver) from each were weighed and homogenized in above solutions and thus a constant tissue concentration was maintained. At r. p.m of 7000x g for 20 min tissue homogenates were centrifuged and the clear supernatant thus obtained were used for further assay.

- **Procedure**

Three clean and dry test tubes were taken and marked as Test (T), Standard (St) and Blank (B).

In these above three mentioned tubes 0.5 ml of cold sample, buffer and distilled water were added seperately. The initiation of enzyme reaction took place sequentially at fixed intervals by adding and mixing 5ml of cold H_2O_2in each tube. Exactly after 3mins the reactions were stopped sequentially at same fixed intervals by rapidly adding 1 ml 6 (N) H_2SO_4. In each test tube 7 ml of KMnO4 reagent was rapidly added, mixed thoroughly and absorbance was measured within 30 to 60 seconds at 480 nm.

- **Calculation**

Catalase enzyme catalyses the decomposition of H_2O_2 and this can be expressed by the given equation K= Log (S_o/S_3) x 2.3/t, Where K = first order reaction rate constant. T = time interval over which the reaction was carried out viz. 3 mins. S_o = substrate concentration at zero time.

S_3 = substrate concentration at 3 minutes. Catalase activity was expressed in n mol H_2O_2 decomposed / min/ mg protein.

Glutathione Peroxidase (GPx) Activity

Glutathione peroxidase (GPx) comprises the enzyme family having peroxidase activity whose main function is to protect the organism from oxidative damage.Glutathione peroxidase catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols. It also reduces free hydrogen peroxide to water in presence of reduced glutathione (GSH) as a hydrogen donor.This catalyzation involves oxidation of the selenol of a selenocysteine residue by hydrogen peroxide.

- **Reagent composition**

I. Reagent 1: Phosphate buffer (50mM, pH 7)

- Solution A: - In double distilled water, 87.09 gm of anhydrous K_2HPO_4 was dissolved and volume made up to 500 ml.
- Solution B: - In double distilled water, 68.05 gm of KH_2PO_4 was dissolved and volume made up to 500 ml.

From Solution "A", 28.9ml of K_2HPO_4 was taken and mixed with 21.1 ml of Solution "B" (KH_2PO_4) and diluted to 1 litre with distilled water to obtain 50mM phosphate buffer with required ph 7.

II. Reagent 2: Phosphate buffer with EDTA (50mM)

- In 100ml of 50mM phosphate buffer, 11.69mg of EDTA was dissolved.

III. Reagent 3: GSH (200mM)

- 6.146 mg of GSH dissolved in 100µl of distilled water.

IV. Reaction mixture: 11.808 ml of phosphate buffer (50mM) with EDTA was mixed with 20 µl of glutathione reductase. In this solution 64.8 µl GSH (200mM) and 1.2 mg NADPH was added. 108 ml of phosphate buffer (50mM) was mixed with these above solution and the pH was adjusted to 7.

V. H_2O_2 solution (stock):

3994.4µl of distilled water was mixed with 5.6 µl of H_2O_2 (30%) to prepare 0.042% of H_2O_2 from this stock solution.

- **Tissue preparation**

In 5 ml of 50 (mM) phosphate buffer, 0.5 gm of liver tissue was homogenized by maintaining constant tissue concentrations. Centrifugation of tissue homogenates were done at 7000 x g for 20 mins. The clear supernatant was collected and used for the assay.

- **Procedure**

50 µl of tissue homogenates were mixed with 3 ml of reaction mixture. At first the initial absorbance was measured at 340nm. Now 10 µl of H_2O_2 (0.042%) was added. After addition of H_2O_2 absorbance was recorded at 340 nm sequentially at 0", 30" and 60" (seconds). The test tube marked as Blank (B) contained 3 ml reaction mixture in which 50 µl phosphate buffer (50 mM) and 10 µl H_2O_2 (0.042%) were added and the absorbance was measured at 340 nm.Using standard protocol protein concentration was measured.

- **Calculation**

Units /ml enzyme = sample optical density x 2 x total volume of assay x dilution factor 6.22 x volume of sample used.

GPx activity = Units / ml enzyme. Amount of enzyme values were expressed as mole/ min/ mg protein.

Estimation of Reduced Glutathione (GSH) Activity

GSH is the most important antioxidant in most mammalian cells. It is a ubiquitous tripeptide(γ-Glu-Cys-Gly) with a gamma peptide linkage between the carboxyl group of the glutamate side chain and cysteine. The carboxyl group of the cysteine residue is linked by normal peptide to glycine. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals. The disulfide bonds formed within

cytoplasmic protein is reduced to cysteines by Glutathione and Glutathione serves as an electron donor. During this mechanism Glutathione is converted to its oxidised form, glutathione disulfide (GSSG), also called L-(-)-glutathione Glutathione disulfide is converted back to glutathione (GSH) by glutathione reductase, using the cofactor NADPH generated by glucose-6-phosphate dehydrogenase. Within the cells, the ratio of reduced glutathione to oxidised glutathione is considered as a measure of cellular toxicity.

- **Reagent composition**

  - Reagent 1: Phosphate buffer (0.1 M, pH 7.5): Prepared as mentioned before.
  - Reagent 2 : DTNB
    - 594.6 mg of DTNB was dissolved in 100 ml of 0.1 (M) phosphate buffer (pH 7.5)
  - Reagent 3 : NADPH
    - 8.33 mg of NADPH was dissolved in 100µl of 0.1 (M) phosphate buffer (pH 7.5)
  - Reagent 4 : GSH
    - 0.307 mg of GSH was dissolved in 1 ml of distilled water.
  - Reagent 5 : GR (Glutathione reductase)
    - 2.8 µl of GR was dissolved in 67.2 µl of 0.1 (M) phosphate buffer (pH 7.5).
  - Preparation of standard curve: A standard curve was made by using standards containing different amounts of glutathione.

- **Tissue preparation**

5 ml of 0.1(M) phosphate buffer solutions (pH 7.5) was taken. 0.5gm of tissue (liver) was weighed and homogenized in above solutions and thus a constant tissue concentration was maintained. At r.p.m. of 7000x g for 20 min tissue homogenates were centrifuged and the clear supernatant thus obtained were used for further assay.

- **Procedure**

Two clean and dry test tubes were taken and marked as Test (T) and Blank (B).In test tube (T) 612 µl of phosphate (0.1 M, pH 7.5) were added with 333 µl of DTNB, and then 2 µl of NADPH and 3 µl of GR was added. In this mixture 50 µl of tissue homogenate was added. In test tube blank (B) 667 µl of phosphate buffer (0.1 M, pH) was added along with 333 µl of DTNB. Then these to test tubes were kept for incubation at room temperature for 30 min, and the absorbance was measured at 412 nm.

- **Calculation**

Glutathione activity was measured from the recorded optical densities from the standard curve. Values were expressed as µg / mg of protein.

Estimation of insulin

Serum insulin was assayed by the solid phase enzyme linked immunosorbent assay (ELISA) using kit obtained from AccuBind Elisa Microwells Monobind Inc., CA., USA.

- **Principle**

Immunoenzymometric assay (TYPE 3)

The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (Ab), (enzyme conjugated and immobilized), with different

and distinct epitope recognition, in excess, and native antigen (Ag). In this procedure, the immobilization takes place during the assay at the surface of a micro plate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal insulin antibody. Upon mixing monoclonal biotinylated antibody, the enzyme-labelled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies, without competition or stearic hindrance, to form a soluble sandwich complex. The interaction is illustrated by the following equation:

$$\text{Enz}_{\text{Ab}_{(M)}} + \text{Ag}_{\text{Ins.}} + {}^{\text{Btn}}\text{Ab}_{(M)} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} \text{Enz}_{\text{Ab}_{(M)}} - \text{Ag}_{\text{Ins.}} - {}^{\text{Btn}}\text{Ab}_{(M)}$$

${}^{\text{Btn}}\text{Ab}_{(M)}$ = Biotinylated Monoclonal Ab (Excess Quantity)

$\text{Ag}_{\text{Ins.}}$ = Native Antigen (Variable Quantity)

$\text{Enz}_{\text{Ab}_{(M)}}$ = Enzyme labelled Monoclonal Ab (Excess Quantity)

$\text{Enz}_{\text{Ab}_{(M)}} - \text{Ag}_{\text{Ins.}} - {}^{\text{Btn}}\text{Ab}_{(M)}$ = Antigen - Antibodies complex

k_a = Rate constant of Association

k_{-a} = Rate constant of Dissociation

Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavid in and biotinylated antibody. This interaction is illustrated below

$$\text{Enz}_{\text{Ab}_{(M)}} - \text{Ag}_{\text{Ins.}} - {}^{\text{Btn}}\text{Ab}_{(M)} + \underline{\text{Streptavidin}}_{\text{cw.}} \Rightarrow \underline{\text{Immobilize complex}}$$

$\underline{\text{Streptavidin}}_{\text{cw.}}$ = Streptavidin immobilied on well

$\underline{\text{Immobilize complex}}$ = Sandwich complex bound to the solid surface

After equilibrium is attained, the antibody-bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum

references of known antigen values, a dose response curve can be generated from which the antigen concentrations of an unknown can be ascertained.

- ## Materials and Reagents

a) Insulin Calibrators

Six vials of reference insulin antigen at levels of 0(A), 5(B), 25(C), 50(D), 100(E), and 300(F) μIU/ml were reconstituted with 2ml of distilled or deionized water. The reconstituted calibrators were stable for sixty days at 2-8˚C.

b) Insulin enzyme reagent

This contains one vial of enzyme labelled with affinity purified monoclonal mouse x-insulin IgG, biotinylated monoclonal mouse x-insulin IgG in buffer, dye and preservative which were stored at 2-8˚C.

c) Streptavidin coated plate

It is a 96-well microplate coated with streptavidin and packaged in aluminium bag with a drying agent, stored at 2-8˚C.

d) Wash solution concentrate

A vial of surfactant with added preservative in buffered saline and stored at 2-8˚C.

e) Substrate A

One bottle containing tetramethylbenzidine (TMB) in buffer was stored at 2-8˚C until use.

f) Substrate B

One bottle containing hydrogen peroxide (H2O2) in buffer was stored at 2-8˚C.

g) Stop solution

One bottle contains strong acid (1N HCl) and stored at 2-30˚C.

h) Wash buffer

Contents of wash concentrate were diluted to 1000ml with distilled water and stored in a suitable storage container at 2-30˚C upto 60 days.

i) Working substrate solution

The contents of the amber vial labelled solution 'A' was poured into a clear vial labelled solution 'B'.

- ## Procedure

50 µl of calibrators, controls and samples are pipetted out into the assigned wells then 100 µl of the insulin enzyme reagent are added to each well. For 20-30 seconds microplate was mixed gently. The microplate was covered with a plastic wrap and incubated for 120 minutes at room temperature (20-27˚C). The contents of the microplate were discarded by decantation or aspiration, after the completion of the incubation period. The microplate was washed with 350 µl of wash buffer and decanted. For two additional times, this washing was repeated. Into each well 100 µl of working substrate solution was added and this should not be shaked after substrate addition. The contents were kept in room temperature for 15 minutes, after the incubation 50 µl of stop solution was added to each well and mix gently for 15-20 seconds. The absorbance was read within 30 minutes at 450 nm. Insulin concentrations were expressed in µIU/ml.

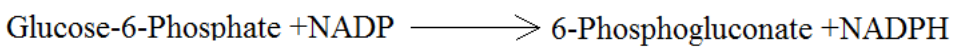
Estimation of Glucose-6-Phosphate Dehydrogenase activity (UV-kinetic method)

Diagnostic Reagent Kit supplied by RECKON DIAGNOSTICS P.LTD, Gowra, Vadodara, India for the quantitative estimation of G6PDH in erythrocytes. This procedure was according to the DAM Method.

- **Principle**

In the red blood cells, Glucoe-6-Phosphate Dehydrogenase (enzyme) is present and it is extracted by lysing the cells using the natural detergent. Glucose-6-Phosphate is oxidized by the extracted enzyme to 6-Phosphogluconate and simultaneously reduces co-enzyme NADP to NADPH giving increase in absorbance at 340 nm.

Enzymatic determination of G6PDH activity is based on the following reaction:-



- **Reagent composition**

  - Reagent 1: Glucose-6-Phosphate , NADP Na_2forms the G6PDH (C0-enzyme-subtrate)
  - Reagent 2 : G6PDH Buffer (pH 7.5 ± 0.1 at 25ºC)
  - Reagent 3 : Lysing Detergent

- **Preparation of Working Solution**

1.1 ml of buffer was mixed with Reagent 1and mixed well. All the reagents were used after 5 mins.

- **Preparation of Red Cell Hemolysate**

0.1 ml of whole blood was washed with 2 ml aliquots of physiological saline (0.9%) for 3 times and then the suspended erythrocytes were washed , packed and centrifuged in pre cooled 0.5 ml of lysing reagent. It was then mixed well and kept in the refrigerator (2-4ºC) for at least 15 mins and maximum for 2 hrs. The lysate was centrifuged at 3000 r.p.m. for 5 mins prior to use.

- **Test Procedure**

Clean and dry test tubes were taken. 1.0 ml of working reagent was pipette out in test tube. 0.025 ml of hemolysate was added to this tube. In this way all the sample test tubes were prepared and mixed well. The absorbance of test sample was recorded after the initial delay of 180 seconds at the interval of one minute for the next 3 mins at 340 nm. The mean change in absorbance per minute is determined and the test results were calculated.

- **Calculation**

(i) G6PDH Activity (U/ 10^{12} RBC)

$$= \triangle A/Min \times \frac{224 \times 10^{12}}{6.22 \times N \times 10^{6} \times 1000}$$

$$= \triangle A/Min \times \frac{36013}{N}$$

Where 224= Total assay volume to sample volume.

10^{12} = Factor for expressing activity in 10^{12} cells.

6.22 = Milimolar absorptivity of NADPH at 340 nm.

N x 10^{6} = Number of erythrocytes / cmm.

1000 = Conversion of cell count from count per cmm to count per ml.

(ii) G6PDH Activity (U/ g Hb)

$$= \triangle A/Min \times \frac{224 \times 100}{6.22 \times Hb\ (g/dl)}$$

$$= \triangle A/Min \times \frac{3601}{Hb\ (g/dl)}$$

Where 100= Factor to convert to 100 ml

224 = Total assay volume to sample volume.

6.22 = Milimolar absorptivity of NADPH at 340 nm.

Hb (g/dl) = Hemoglobin concentration

CHAPTER 4

Results

Gymnadenia orchidis Lindl and its root

Figure 1: Image of *Gymnadenia orchidis* Lindl and its tuberous roots.

Phytochemical screening of the plant root aqueous extract by HPLC method (qualitative analysis).

Qualitative estimation of phytoconstituents was determined by HPLC technique from root extracts of *Gymnadenia orchidis* Lindl.

Table 1: Phytochemical screening of the roots of *Gymnadenia orchidis* Lindl.

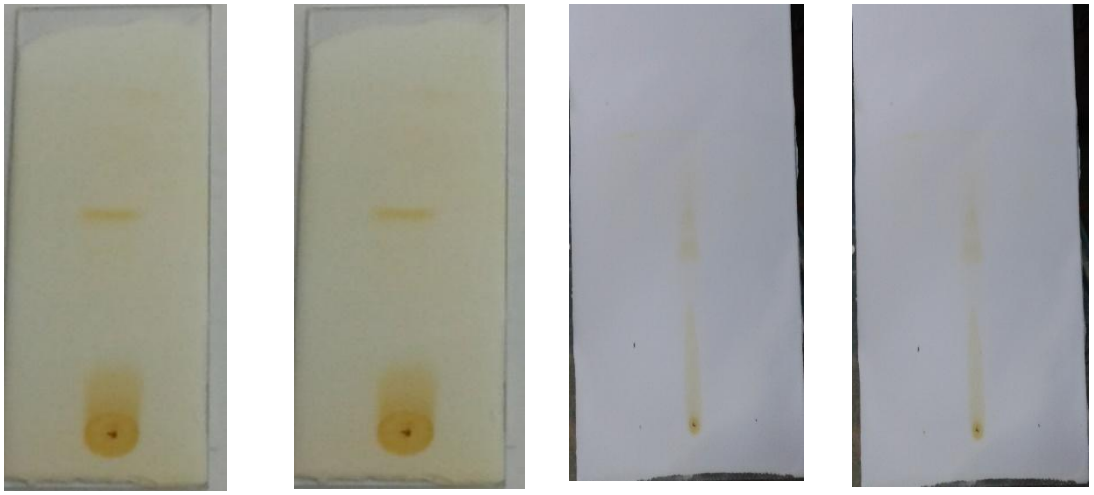
Phytochemicals	Present (+) / Absent (-)
Flavanoids	-
Alkaloids	-
Terpenoids	++
Steroids	+
Cardiac Glycosides	+
Tannins	+
Saponins	-
Polyphenols	+
Vitamin C	+
Carbohydrates	++
Proteins	++
Free amino acids	_

++ Adequately present

The presence of active constituent's like terpenoids, steroids, cardiac glycosides, tanins, polyphenols, vitamin C, carbohydrate(s) and proteins were determined in the root extracts of *Gymnadenia orchidis* Lindl as shown in Table 1.

Separation and characterization of active phytoconstituents of root extracts of *Gymnadenia orchidis* Lindl with chromatographic techniques (TLC).

TLC was carried out in solvent system containing pet-ether and ethyl acetate (99%: 1%) as a solvent system (mobile phase). TLC of *Gymnadenia orchidis* Lindl root extracts showed R_f values of 1.88571, 1.85714, 1.9117 and 1.9705 of slide 1, slide 2, slide 3 and slide 4 respectively.



Slide 1 **Slide 2** **Slide 3** **Slide 4**

Figure 2: TLC image of crude terpenoid using two standards limoneme and menthol.

Table 2: R_f value calculation from TLC slides containing crude terpenoid extracts of *Gymnadenia orchidis* Lindl.

S. No	Solvent system	Extraction process	Distance travelled by the solvent	Distance travelled by the solute	Ratio	Retention factor (R_f)
1.	Pet-ether & ethyl acetate	Differential fractionation method	6.6	3.4	6.6/3.4	1.88571
2.	Pet-ether & ethyl acetate	Differential fractionation method	6.5	3.5	6.5/3.5	1.85714
3.	Pet-ether & ethyl acetate	Differential fractionation method	6.5	3.4	6.5/3.4	1.9117
4.	Pet-ether & ethyl acetate	Maceration and differential fractionation method	6.7	3.4	6.7/3.4	1.9705

Spectroscopy analysis of the crude sample

The UV–VIS spectroscopic investigation of ethanolic extracts of roots of *Gymnadenia orchidis Lindl* is shown in the Figure 3. 2.5% (v/v) and 5% (v/v) of crude root extracts was prepared in ethanol, both of these samples of *Gymnadenia orchidis Lindl* showed intense peak of λ_{max} at 280 nm for the concentrations. From literature it was found that eugenol (terpenoid) also has a characteristic UV-Vis λ_{max} at 280.9 nm, shown in the inset of the Figure 3. Therefore the result of UV-VIS spectroscopic investigation confirms the presence of eugenol (terpenoids) in the ethanolic crude extracts of *Gymnadenia orchidis Lindl.*

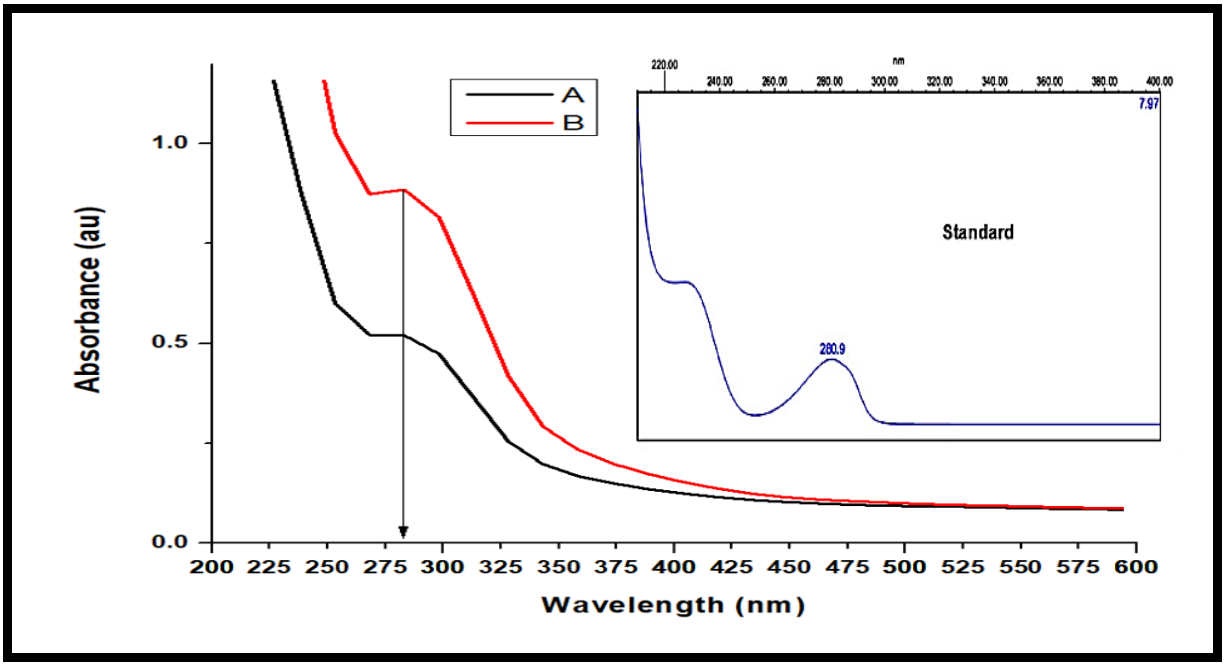


Figure 3: UV-Vis spectra of ethanolic root extracts (crude terpenoid) of Gymnadenia orchidis Lindl. A: crude terpenoid and B: diluted (x 2) crude terpenoids.

FT-IR analysis of the crude extracts

Fourier Transform Infrared Spectroscopy (FT-IR) technique is useful in identification of functional group of the active components based on the peak value in the region of infrared radiation. Through FT-IR analysis of crude extracts of roots, probable functional groups were determined by seeing the data on the peak values and these are shown in table 2. The FT-IR spectrum of root extracts of *Gymnadenia orchidis* Lindl is shown in Figure 4.

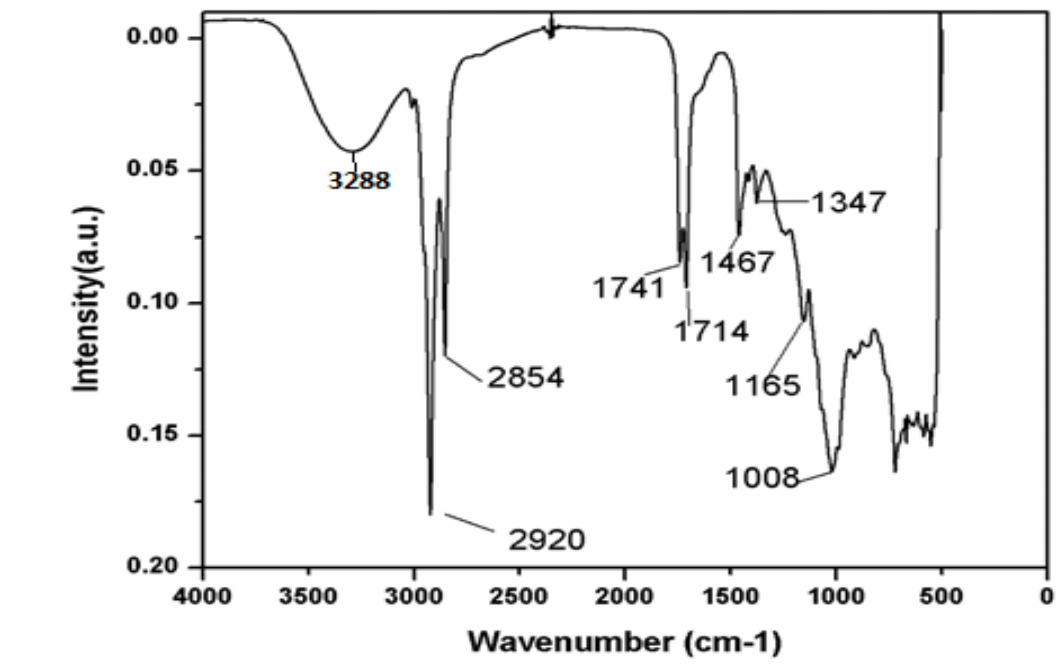


Figure 4: FT-IR spectra of ethanolic root extracts (crude terpenoid) of *Gymnadenia orchidis* Lindl.

The peak values in the infrared radiation region help in the identification of the functional groups of the active components. From the FTIR spectrum of root extracts, a characteristic peak was observed at 3423 cm^{-1} is owing to the stretching frequency of hydroxyl (–OH) groups. The peaks at 2920 and 2854 cm^{-1} are due to asymmetric and symmetric stretching vibrations of C–H groups. The peaks at 1741 and 1714 cm^{-1} arrises may be due to the presence of carbonyl(C=O) stretching vibrations for ester groups. The peaks at 1467, 1347, 1165 and 1008 cm^{-1} are assigned to C=C-C aromatic ring stretch, –OH bending, C–O–C stretching and O-C-C stretch respectively.

Table 3: FT-IR analysis of root extracts of *Gymnadenia orchidis* Lindl.

Frequency, cm^{-1}	Observed Peak value	Bond	Functional group
3500-3200	3423	O-H stretch, H-Bonded	Alcohols, Phenols
3000-2800	2920; 2854	C-H stretch	Alkanes
1760-1665	1741; 1714	C=O stretch	Carboxylic acids/esters
1500-1400	1467	C=C-C aromatic ring stretch	Aromatics compound
1410-1310	1347	O-H bend, Alcoholic group	Phenol/ tertiary alcohol
1210-1100	1165	C-O-C asymmetric stretch, ether group	Cyclic ethers
1100-1000	1008	O-C-C stretch of esters	Esters

Mass analysis of the crude extracts

The most abundant ions are due to the molecular ions of eugenol with m/z value 203.1277. In particular, the ions with m/z 203 are related to Thymol methyl ether, Carvacrol methyl ether, 2-Phenylethyl acetate, 3,4-Dimethoxystyrene, Chavibetol (m-Eugenol), (E)-Anethol epoxide, Methyl perillate and ions with m/z 213 are related to 8,9-Dehydrothymol acetate, 5-Acetoxylinalool, (E)-b-Damascenone, Butylphthalide/(3-n-butylphthalide or NBP) respectively. In addition the glycosides derivatives are also present.

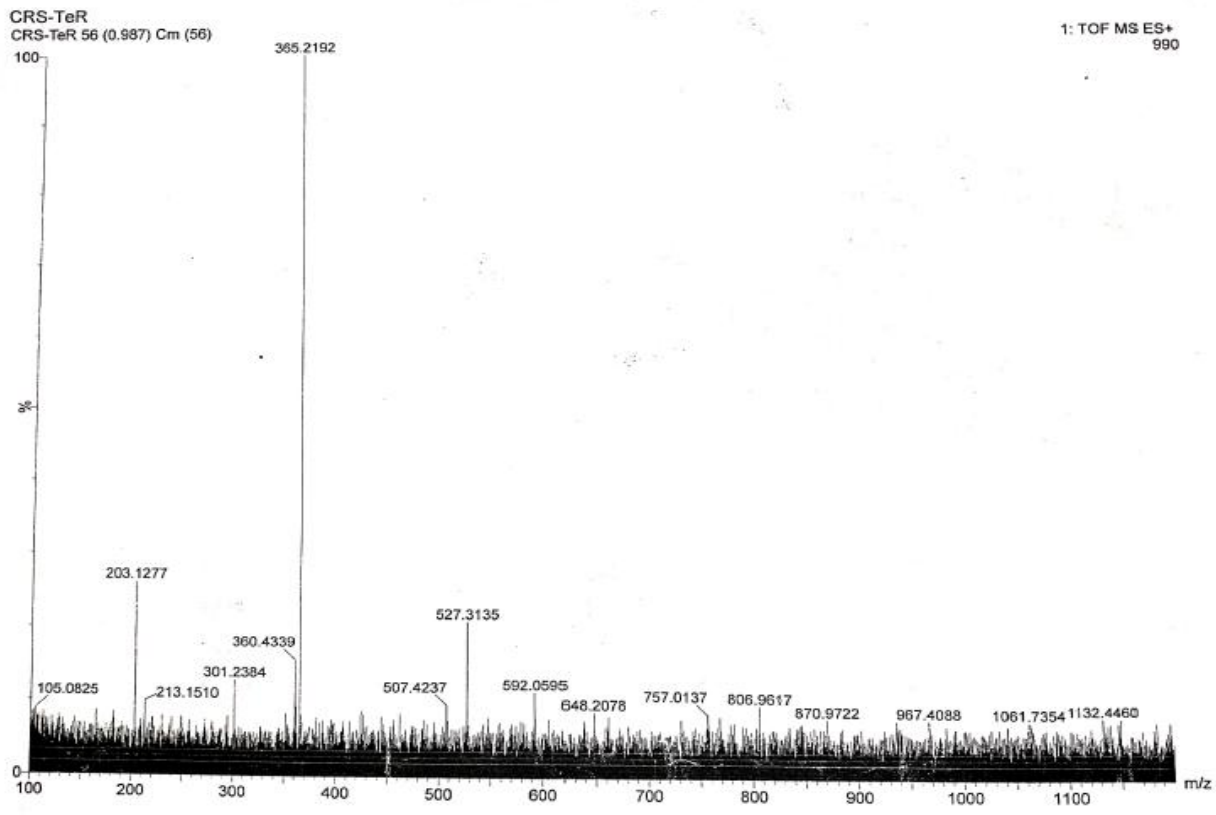


Figure 5: Mass spectroscopic data of the crude root extract of *Gymnadenia orchidis* Lindl.

Of course, using ESI-MS it was not possible to distinguish a particular compound. In this way, positive ESI-MS permitted to detect possibility of presence of eugenol compound. From the ESI– mass spectroscopy we have received an ion m/z peak 203 which indicates that eugenol compound may be present in the solution. But the FTIR spectroscopy confirms the presence of functional groups like -OH, -OCH_3, -CH_2, Phenol and alkanes in the root extracts indicating the presence of active compound eugenol because standard eugenol also contains phenolic group, ether and alcohol groups. Also UV-Vis spectrum provides a sharp peak at 280 which is very similar with the absorption spectra of standard eugenol compound. Thus it can be concluded that the crude extract may contains eugenol.

Acute oral toxicity study and selection of dose

In the acute toxicity study, the rats were treated with different concentration of 100, 200, 300, 500, 1000 mg of dry root powder/kg body weight/ day in the form of root Salep of *Gymnadenia orchidis* Lindl respectively for 10 days as oral supplementation. No adverse side effects like toxicity, behavioral changes, and mortality in the test groups of animals were observed in comparison to the control group animals. During this acute toxicity study, animals were healthy and alive even at maximum dose i.e. 1000 mg/kg b. wt. of root Salep throughout the 10 days of dosing which indicated that animals had tolerated the limit dose of 1000 mg/kg b.wt. of root Salep without showing any kind of acute toxicity. Also no mortality was observed at all the administered doses up to 1000 mg/kg b.wt. proved that the LD_{50} of the root Salep was higher than 1000 mg/kg b.wt. These results indicated that even at higher doses this root Salep was safe and edible. No significant changes were observed in the mean body weights of the treated animal in comparison to the control animal throughout the acute toxicity study (Table 4) and did not hamper the animal's growth thus indicating that crude extract did not possess any toxicity on the animal's growth.

Table 4: Body weight of the rats under toxicity test.

	Body weights		
Groups	**0 day**	**5 day**	**10 day**
Group 1	153 ± 6.6	157 ± 5.3	158 ± 4.1
Group 2	160 ± 7.4	164 ± 8.1	165 ± 7.8
Group 3	160 ± 8.9	162 ± 9.3	163 ± 9.1
Group 4	161 ± 8.6	165 ± 9.5	164 ± 9.8
Group 5	157 ± 7.2	155 ± 7.4	156 ± 7.1
Group 6	150 ± 6.3	152 ± 5.9	152 ± 6.5

n = 10 for each group. All values have been expressed as the mean ± S.D.

Table 5: Fasting blood sugar of the rats under toxicity test.

	Fasting Blood Sugar (mg/dl)	
Groups	**0^{th} Day of treatment**	**10^{th} Day of treatment**
Group 1	94.1 ± 7.8	99.9 ± 9.7
Group 2	99.0 ± 8.6	95.7 ± 10.3
Group 3	95.6 ± 10.1	88.6 ± 5.7
Group 4	94.7 ± 8.8	90.6 ± 10.4
Group 5	92.6 ± 11.2	91.7 ± 7.9
Group 6	95.5 ± 9.8	93.7 ± 8.9

n = 10 for each group. All values have been expressed as the mean ± S.D. Fastingblood glucose levels were found to be the minimum for the root Salep (200 mg/kg body wt.) supplemented animal in Group 3.

Effect of acute toxicity study on the biochemical parameters acid phosphatase, alkaline phosphatase, SGOT and SGPT

There was a trend of increase ACP (Figure 6A) levels (within normal range) in serum of root Salep supplemented rats in comparison to control rats. The levels of ALP (Figure 6B), SGOT (Figure 6C) and SGPT (Figure 6D) were initially decreased and then increased (within normal range) with the increased dose of Salep supplementation compared to the normal group. Urea and creatinine (Figure 7) both levels were produced similar results as seen in SGOT and SGPT. The most normal levels of blood glucose, liver function enzymes and kidney function parameters were observed to the animals belonging in the Group 3 suggesting that 200 mg/kg body weight dose of the Salep could be most suitable for the study of action against type 2 diabetes mellitus. This root Salep dose (200 mg/kg b. wt.) was taken as the effective dose and was supplemented to the diabetic animals to establish the ameliorative role of *Gymnadenia orchidis* Lindl root Salep against type 2 diabetes.

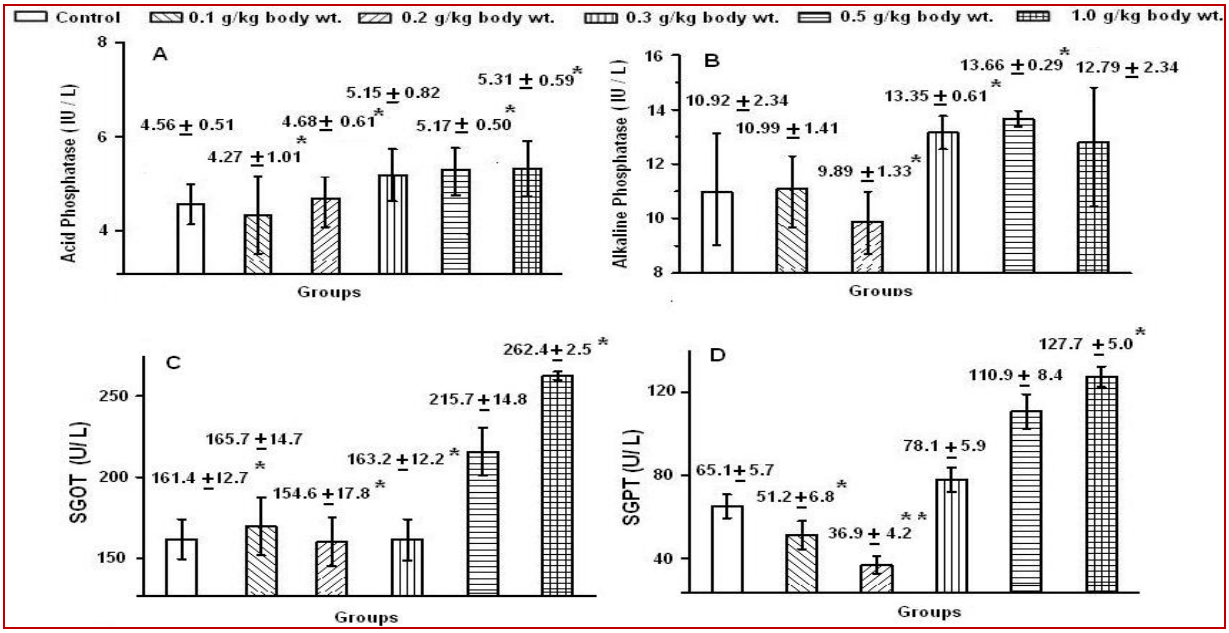


Figure 6: Acid phosphatase, Alkaline phosphatase, SGOT, SGPT level in animals treated with of different doses of root Salep during acute toxicity study.

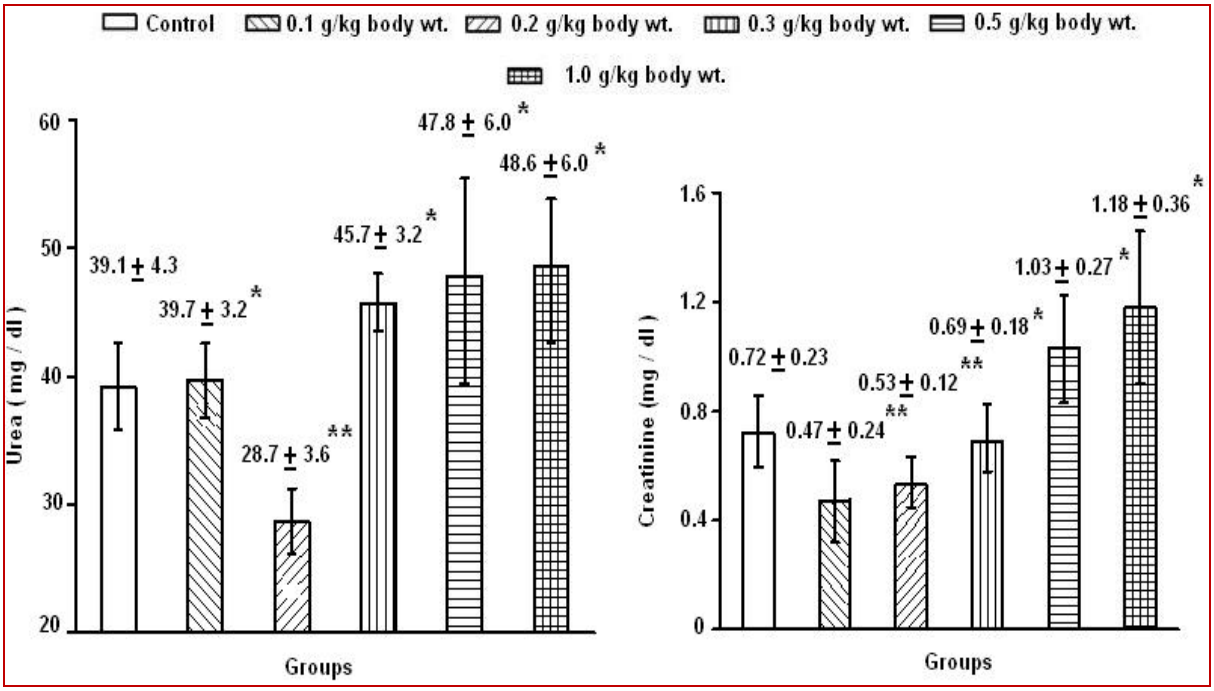


Figure 7: Urea and Creatinine level in animals treated with different doses of root Salep during acute toxicity study.

Effect of the root Salep for anti-diabetic study.

STZ-induced diabetic rats revealed decrease in body weight in comparison to the normal control rats due to induction of diabetes. Body weights of the root Salep treated rats were restored significantly ($P<0.01$) after receiving the root Salep supplementation (Figure 8).

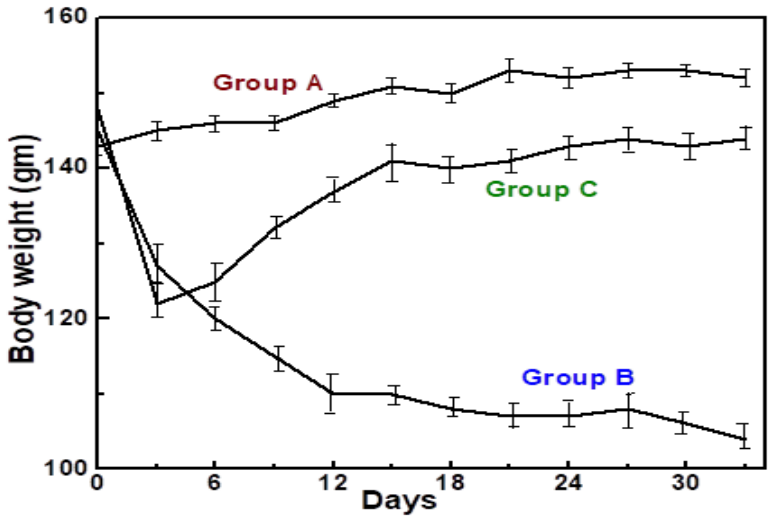


Figure 8: The body weights of the rats which received root Salep where, Group A: Normal control; Group B: Diabetic control; Group C: Root Salep treated group.

The blood glucose level of the STZ-induced diabetic group treated with root Salep (200 mg/kg body wt.) restored to normal levels after 3 doses of root Salep (Figure 9 A). Increment observed in the glycosylated haemoglobin percentage (about 30.5%) in STZ induced diabetic rats compared to normal control rats (Figure 9 B).

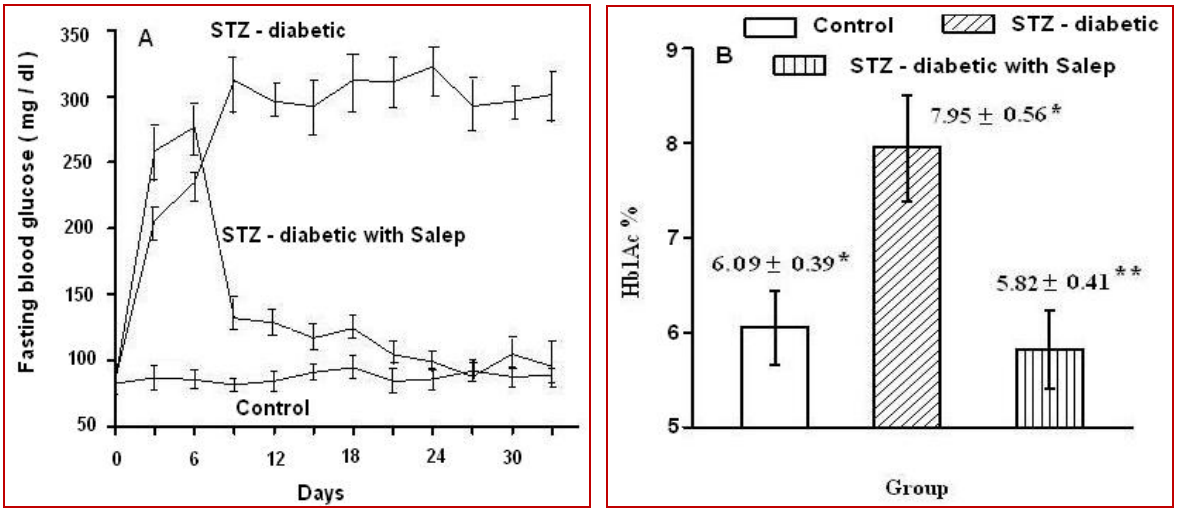


Figure 9: Effect of root Salep (0.2 g/kg body wt.) on diabetic rat. Where A: Blood glucose; B: Hb1Ac. Data were averaged and presented as mean ± S.D. (N = 10), where * means significant (P < 0.01) and ** implies more significant (P < 0.001).

More significant (P<0.001) reduction of glycosylated haemoglobin percentage was observed on the Salep supplemented diabetic group (about 26.8% lower than non-treateddiabetic group).

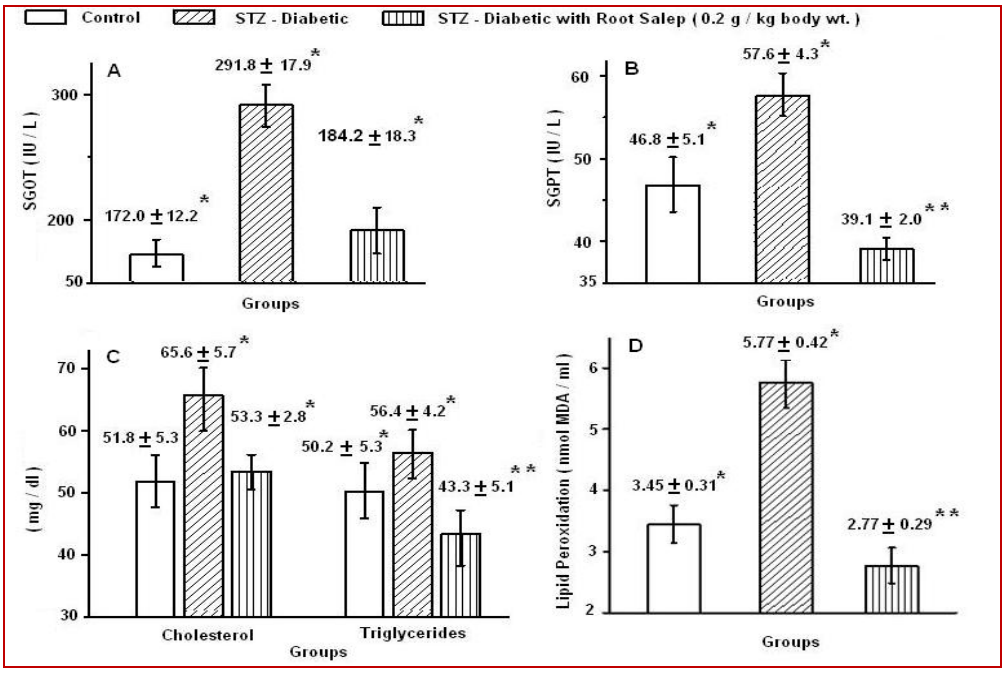


Figure 10: Effect of root Salep (0.2 g/kg body wt.) on diabetic rat. Where A: SGOT; B: SGPT; C: Lipid levels; D: Lipid peroxidation. Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant (P < 0.01) and ** implies more significant (P < 0.001).

The activity of liver function enzymes were elevated in case of diabetic rats. SGOT level of diabetic rats was increased by 69.6% (Figure 10 A) and SGPT level of diabetic animals was increased by 23.1% (Figure 10 B) with respect to their normal control rats.The levels of SGOT (about 19.8%) and SGPT (about 32.1%) both were decreased significantly ($P< 0.01$) in the Salep supplemented diabetic groups as compared to the non-treated diabetic group. Diabetes induction causes increment in both the cholesterol (about 26.6%) and triglycerides (12.4%) levels compared to the normal control rats (Figure 10 C). Salep supplementation to the diabetic rats reduced the total cholesterol level (about 18.8%) significantly ($P<0.01$) and triglycerides level (about 23.2%) more significantly ($P < 0.001$) compared to the diabetic induced rats. Lipid peroxidation also more significantly ($P < 0.001$) decreased in the Salep supplemented group with respect to the diabetic induced group (Figure 10 D). It was also observed that the levels of antioxidant enzymes such as SOD, GSH and GPx were lowered for diabetic rats but restored by

the treatment of root Salep (Figure 11).

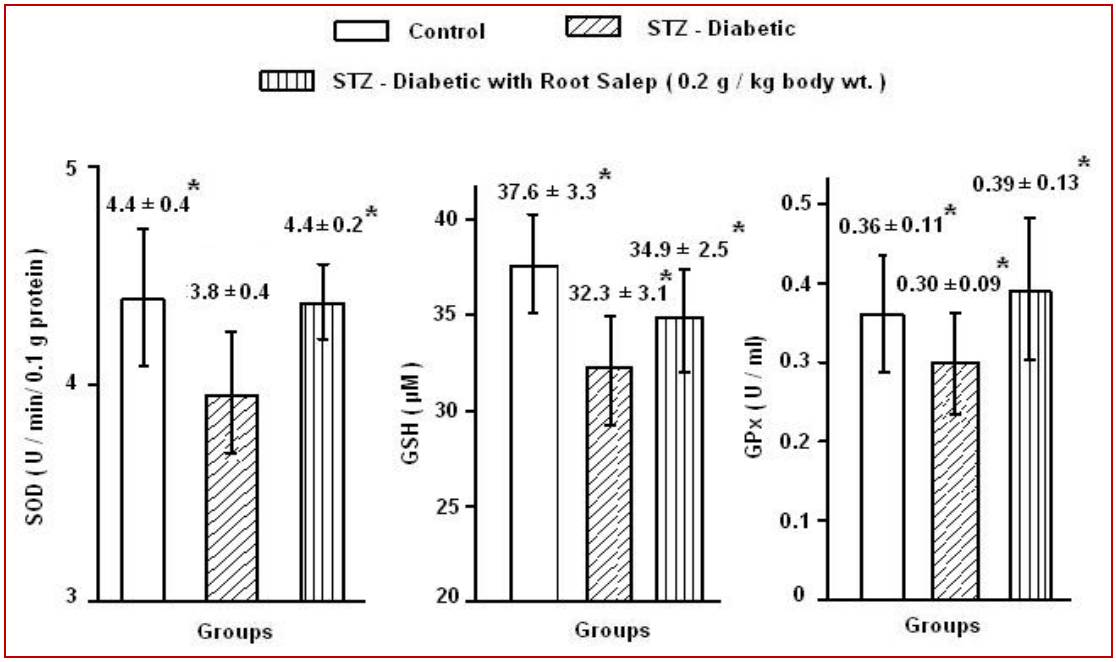


Figure 11: Effect of root Salep (0.2 g/kg body wt.) on antioxidant enzymes of diabetic rat. Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant ($P < 0.01$) and ** implies more significant ($P < 0.001$).

Effect of extracted terpenoids (as a trial dose) from *Gymnadenia orchidis* Lindl roots for anti-diabetic study.

In STZ-induced diabetic animals (DC) the blood glucose level was significantly higher in comparison to normal control (NC). Diabetic animals treated with terpenoid (DT) showed decrease in blood glucose level from 1stday (180.4 ± 25.3*) to 5thday (108.4 ± 9.7**) and were found to be normal (< 110 mg/dl) as seen from Table 6. Diabetic group treated with root Salep devoid of terpenoid (DWT) showed no effect in their blood glucose level and their glucose level was significantly increased from 1st day (215.0 ±25.5*) to 5th day (264.2 ± 47.2*). Diabetic group treated with metformin was also statistically significant (decrease) in blood glucose level from 1st day (157.8± 6.4*) to 5th (125.0 ± 9.7*) day (Table 6).

Table 6: Fasting blood glucose levels of the rat under different treatment.

Group	Glucose levels	
	1st day	5th day
Normal Control(NC)	98.2 ± 7.7*	102.2 ± 16.7*
Diabetic Control (DC)	196.2± 23.0*	206.0 ± 50.4*
Diabetic +Terpenoid(DT)	180.4 ± 25.3*	108.4 ± 9.7**
Diabetic – Terpenoid(DWT)	215.0 ±25.5*	264.2 ± 47.2*
Diabetic + Metformin(DM)	157.8 ± 6.4*	125.0 ± 9.7*

n = 10 for each group. All values are expressed as the Mean ± S.D. with Statistical significance as *, P < 0.01 (significant); **, P < 0.001 (more significant).

Liver glycogen content of the diabetic animals supplemented with terpenoid (DT) also gets increased in comparison to the diabetic control group. Animals treated with Salep lacking terpenoid (DWT), diabetic control animal group showed reduction in liver glycogen content (Figure 12).

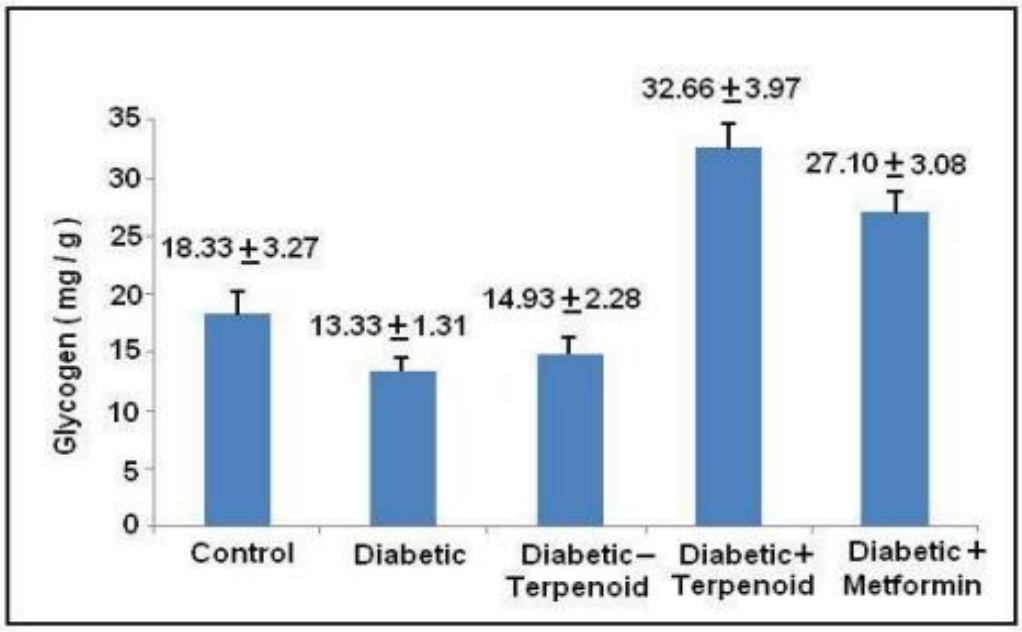


Figure 12: Liver glycogen content of diabetic rats under different treatment conditions.

Effect of root Salep with terpenoid, root Salep devoid of terpenoid, metformin (standard drug) on the hepatic enzymes of STZ-induced diabetic rats and histological analysis.

The diabetic rats showed increased level of ACP by 55.4%, ALP by 28.8%, AST by 42.34% and ALT by 45.65 % as compared to normal control group (NC). The levels of ACP, ALP, AST and ALT were decreased significantly ($P<0.001$) in the terpenoid supplemented diabetic rats (DT) as compared to the diabetic control rats.

However the levels of these enzymes did not decrease in diabetic group treated with root Salep without terpenoid (DWT). Diabetic rats treated with metformin also showed significant decrease in the levels of these hepatic enzymes (Figures 13 A, 13 B, 13 C and 13 D).

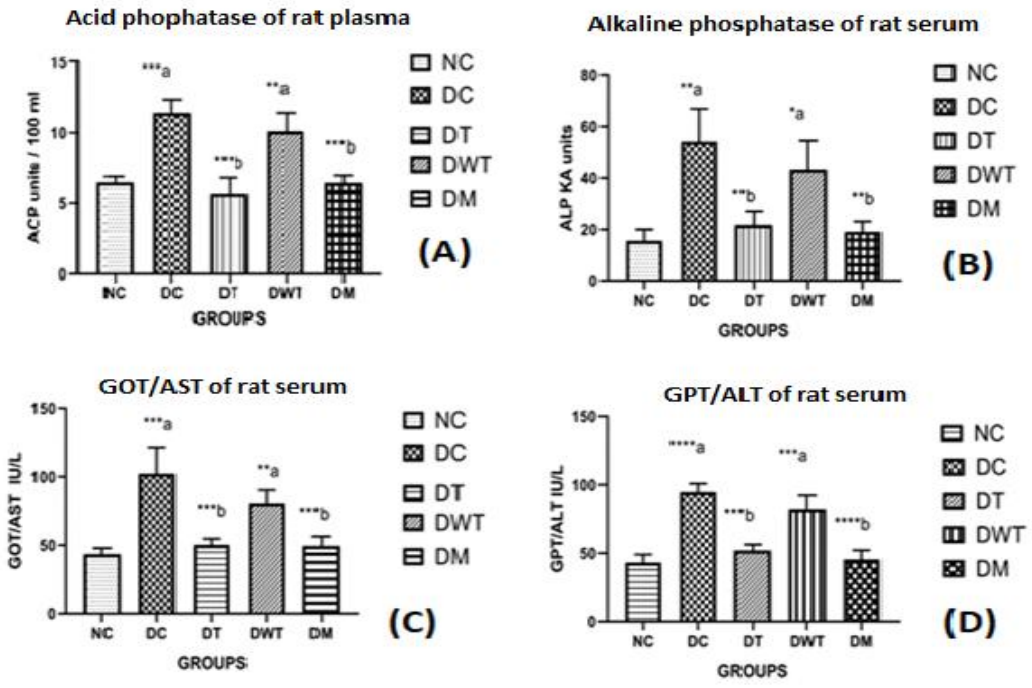


Figure 13: Effect of root Salep (containing terpenoid) on hepatic enzymes of rat plasma at different groups: NC - normal control; DC - diabetic control; D - diabetic terpenoid; DWT - diabetic without terpenoid; where inset A: ACP; B: ALP; C: SGOT; D: SGPT.

*Data were averaged and presented as mean ± S.D. (N = 5). *a significant (*P ≤ 0.01) different (increase) from normal control group; **a more significant (**P ≤ 0.001) different (increase) from normal control group; ***a highly significant (***P ≤ 0.0001) different (increase) from normal control group; **b more significant (**P ≤ 0.001) different (decrease) from diabetic control; ***b and ****b highly significant (***P≤0.0001) different (decrease) from diabetic control.*

Urea, creatinine and BUN levels were significantly elevated in diabetic rats ($p<0.001$) as compared to normal control animals. The levels of urea, creatinine and BUN were increased by 49.07%, 63.55% and 53.4% respectively in diabetic rats in comparison to the normal control groups. However terpenoid rich root Salep when supplemented to treated diabetic animals the levels of urea, creatinine and BUN decreased significantly by 57.7%, 64.18% and 59.97% respectively as compared to the diabetic control animals (Figure 14 A,14 B,14 C).

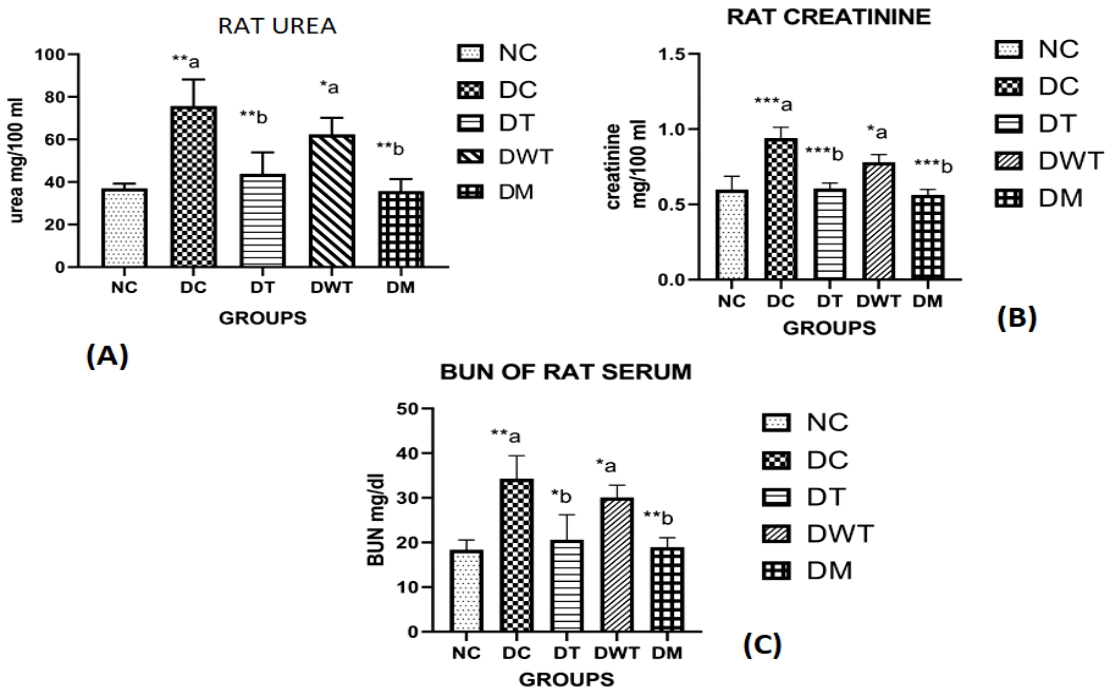


Figure 14: Effect of root Salep on renal parameters at different group of rats. Here A: Urea; B: Creatinine; C: BUN.

*Data were averaged and presented as mean ± S.D. (N = 5). *a significant (*P ≤ 0.01) different (increase) from normal control group; **a more significant (**P ≤ 0.001) different (increase) from normal control group; ***ahighly significant (***P ≤ 0.0001) different (increase) from normal control group; *b significant (*P ≤ 0.01) different (decrease) from diabetic control group. **b more significant (**P ≤ 0.001) different (decrease) from diabetic control; ***b and ****b highly significant (***P≤0.0001) different (decrease) from diabetic control.*

Lipid peroxidation levels were measured by the thiobarbituric acid (TBA) reaction with malondialdehyde (MDA) forming Thiobarbituric acid reactive substances (TBARS). In diabetic rats S.O.D decreases by 52.36% and TBARS level increases by 42.49%, 38.67% respectively (Figure 15 A, B and C).

However in treated group of animals the S.O.D activity increases by 66.04% and TBARS levels decreases significantly by 42.03%, 47.39% respectively (Figure 15 A, 15 B & 15 C).

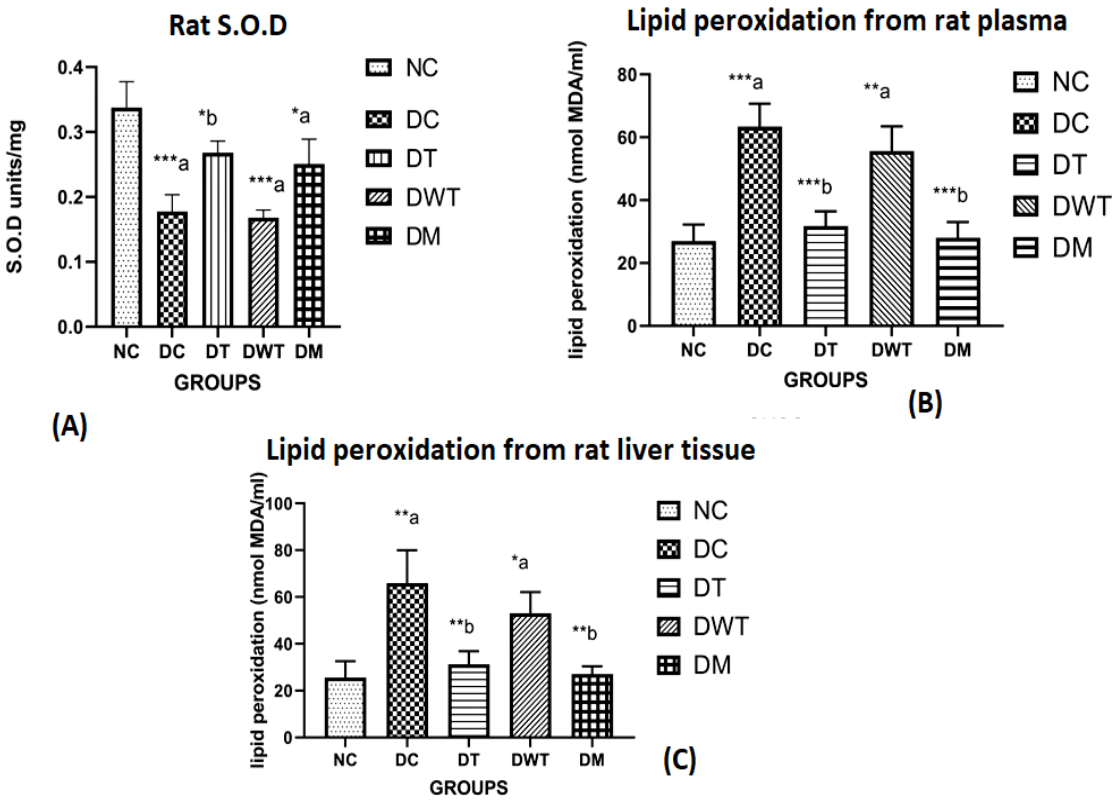


Figure 15: Effect of root Salep on antioxidant enzyme at different group of rats. Here A: S.O.D; B: lipid peroxidation from plasma; C: lipid peroxidation from liver tissue.

*Data were averaged and presented as mean ± S.D. (N = 5). In case of lipid peroxidation from rat plasma and rat liver tissue *a significant (*P ≤ 0.01) different (increase) from normal control group; **a more significant (**P ≤ 0.001) different (increase) in comparison to normal control group; ***a highly significant (***P ≤ 0.0001) different (increase) in comparison to normal control group.**b more significant (**P ≤ 0.001) different (decrease) from diabetic control group; ***b highly significant (***P≤0.0001) different (decrease) from diabetic control group. In case of S.O.D, DM group shows *a significant (*P ≤ 0.01) different i.e. almost nearer to normal control group; DC and DWT group shows ***a highly significant (***P ≤ 0.0001) different (decrease) in comparison to normal control group. Whereas DT group shows *b significant (*P ≤ 0.01)different (increase) in comparison to diabetic control group in case of S.O.D. level.*

Figure 16A, B, C, and D shows the levels of serum HDL, LDL cholesterol, TC (total cholesterol), TG in normal and experimental animals in each group. The diabetic untreated group (DC, DWT) had significant elevation of TC, TG, LDL and reduction in HDL-C levels as compared to the normal control rats. But significant reduction in TC, TG, LDL and increase in HDL-C levels were observed in diabetic rats treated with root Salep containing terpenoid.

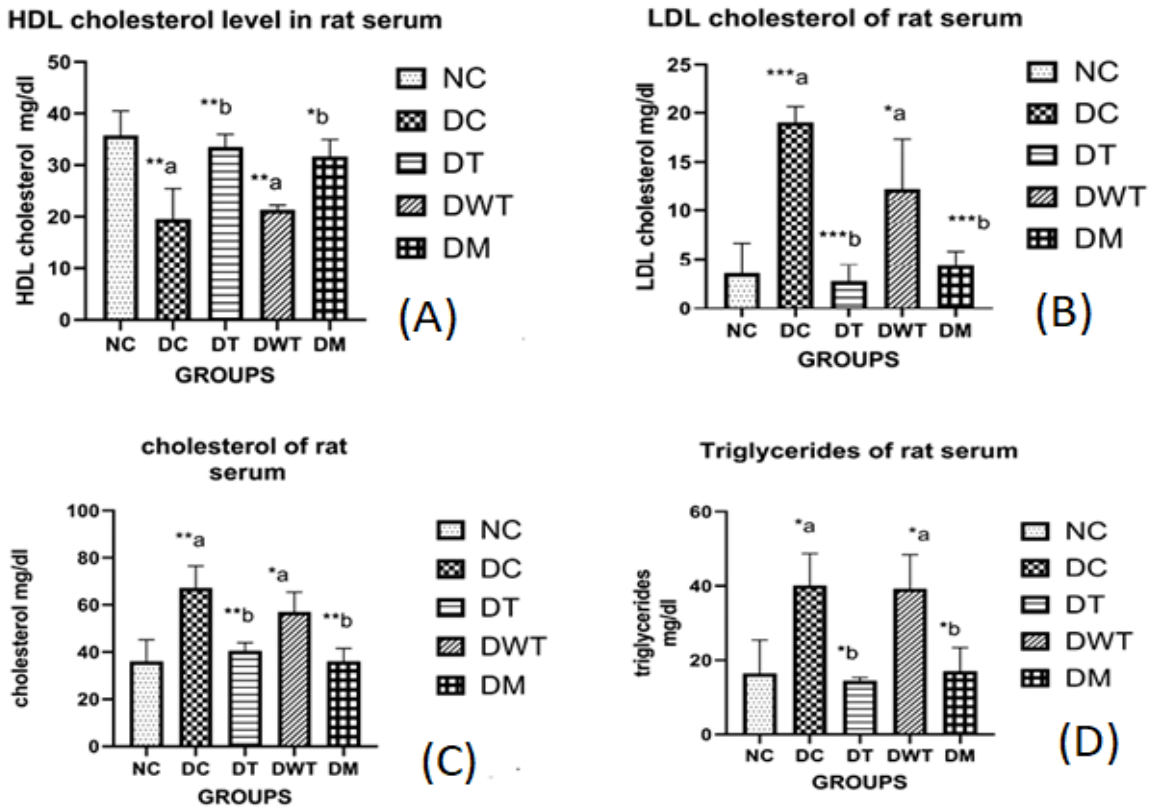


Figure 16: Effect of root Salep on lipid profiles at different group of rats where A: HDL; B: LDL; C: total cholesterol; D: triglycerides.

*Data were averaged and presented as mean ± S.D. (N = 5). DC group shows *a significant (**P ≤ 0.001) different (increase) from normal control group in triglyceride level; DC group shows **a more significant (**P ≤ 0.001) different (decrease) from normal control groupin HDL cholesterol level; DC group shows ***a highly significant (**P ≤ 0.001) different (increase) from normal control group in LDL cholesterol level; DC group shows **a more significant (**P ≤ 0.001) different (increase) from NC group in total cholesterol level. DM group shows *b significant (*P ≤ 0.01) different (increase) from diabetic control group in HDL cholesterol level. DT group and DM group shows *b significant (*P ≤ 0.001) different (decrease) from diabetic control group in triglyceride level. DT group shows **b significant (**P ≤ 0.001)different(increase) from diabetic control group in HDL cholesterol level. DT group and DM groups shows **b significant (**P ≤ 0.001) different (decrease) from diabetic control group in total cholesterol level. DT and DM groups shows ****b highly significant (***P≤0.0001) different (decrease) from diabetic control group in LDL cholesterol level.Whereas DWT group shows *a significant (*P ≤ 0.01) different (increase) from normal control group in LDL cholesterol level; total cholesterol level and triglycerides level.DWT group shows **a more significant (*P ≤ 0.01) different (decrease) from normal control group in HDL cholesterol level.*

Effect of root Salep on the histopathology of vital organs like pancreas, liver and kidney of rats.

Histological studies of pancreas, liver and kidney revealed normal histological features in normal control rats (Figure 17). But in diabetic control group, the pancreatic islet architectures exhibited shrunken islets with necrosis. The pancreatic islets in diabetic group treated with terpenoid exhibited more or less nearby normal architectures suggesting the protective role of root Salep containing terpenoid on the pancreas of diabetic rats by restoring the necrotic and fibrotic changes. Metformin treated group also revealed almost normal pancreatic islets architectures similar to normo-glycemic rat pancreas. Figure 17 (H&E StainingX100) shown a photomicrograph of the pancreas after 5 days of treatment.

Histopathology of rat pancreas

Figure 17A reveals that the pancreatic islets of Langerhans of normal control rats (NC) shows tightly arranged normal architectures. Throughout the cytoplasm of pancreatic acini, round and elongated islets are seen uniformly distributed with arranged lobules and prominent nuclei lightly stained than the surrounding acinar cells. No signs of inflammation or necrosis were observed. Normal control rats shows densely packed cells in the islets of Langerhans which seems like dense cord of islets cells. The rounded islets are surrounded by acinar cells and interlobular ducts. Alpha cells are located at the boundary and β cells in the centre with intervening capillaries (Figure 17A). The pancreatic architectures of STZ-induced diabetic control rats shows damaged islets in the pancreas with clogged blood vessels and signs of patchy necrosis. This degeneration and necrotic changes are present in both exocrine and endocrine portion (Figure 17B). In diabetic group necrosis is followed by fibrosis. Shrunken pancreatic islets with abundant vacuoles are observed where reduced number of beta cells located centrally and large numbers of alpha cells at the margin of islets.

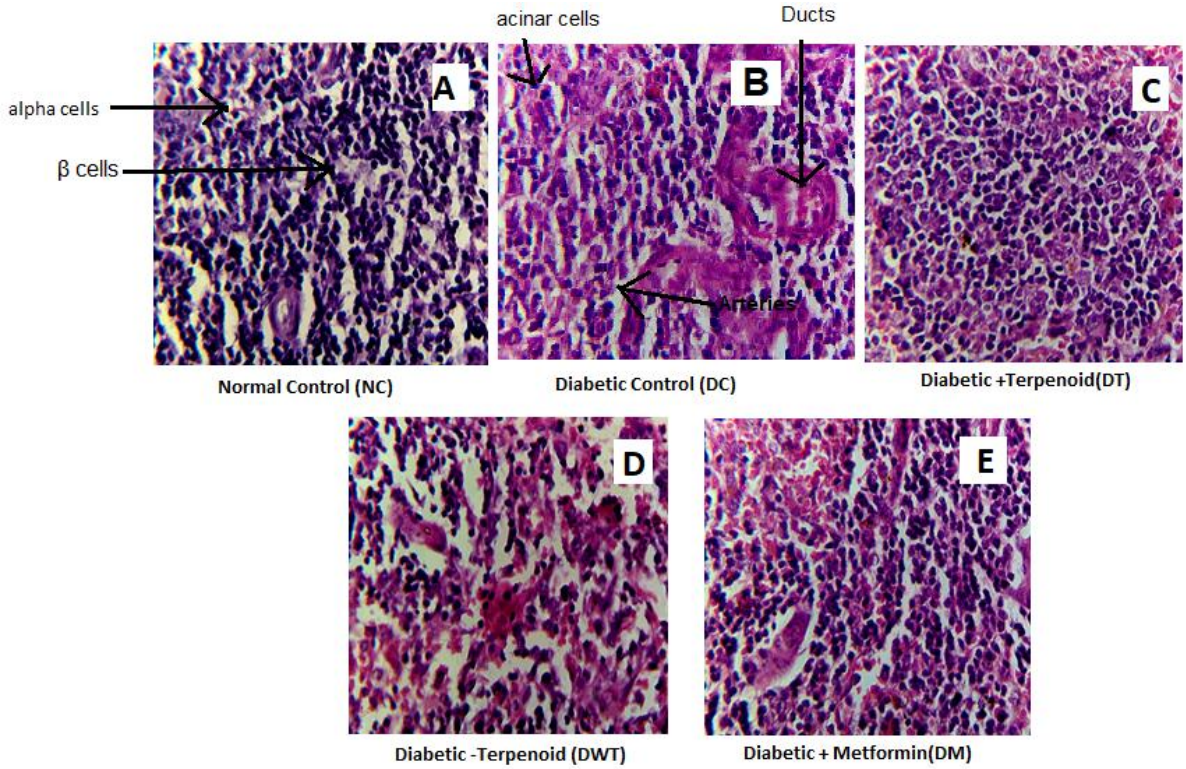


Figure 17: Photomicrographs of histological changes of rat pancreatic section with islets of Langerhans. (H&E: X100): A (Control group) - islet seems normal; B (Diabetic group)- islets are shrunken due to necrosis; C (terpenoid treated group)-islets shows regeneration in islets with increase cellular density; D (diabetic without terpenoid group)- islets appear as irregular and dispersed; E (diabetic +metformin/DM)- islets showing regeneration in islet cells and increased cellular density.

The pancreatic architectures of terpenoid treated diabetic rat's shows resemblance closely to normal control group islet architectures. Increased number of β-cells, pancreatic tissue with lobules, and proliferation of granulated normal β-cells in size with more symmetrical vacuoles were seen in this group (Figure 17 C) when compared to normal rats.

The pancreatic architectures of diabetic rats treated with root salep without terpenoid (DWT) resemblance the damages like diabetic control group. Here pancreas also reveals degeneration and patchy necrotic changes with clogged blood vessels followed by fibrosis (Figure 17 D). Here also shrunken pancreatic islets with degenerated acinar cells due to vascularisation and infiltration of lymphocytes were observed. Reduced number and size of

beta cells in the islets of Langerhans were also observed (Figure 17 D). In Metformin treated group pancreatic lobular architecture appears normal like normal control group (Figure 17E).

Histopathology of rat liver

The therapeutic efficiency of root Salep containing terpenoid was estimated by conducting histopathology of hepatic tissues under STZ-induced conditions and morphology of rat liver was examined through H & E staining. Figure 18 &19 (H & E StainingX100) shown a photomicrograph of the liver after 5 days of treatment. The histopathological changes in the STZ-induced diabetic rats revealed fat deposition with large hepatocytes. But root Salep containing terpenoid treated diabetic rat showed improvement in their liver cells.

Normal control groups reveal normal architecture of liver tissue comprising of hepatic lobules with no barriers separated, polyhedral shaped hepatocytes (Hc) containing one distinct central nucleus (N) or two round nuclei with sinusoidal spaces extends radially from the central vein to the boundaries of portal areas (Figure 18 A1& 19 A2). Clear lumen of central vein, vacuoles and blood sinusoids (S) lining the endothelial cells containing Kupffer cells (K) were observed (Figure 18 A1 & 19 A2). These kupffer cells (K) act as a barrier against pathogens and also function as a sieve which helps in the purification of substances passing from blood to the hepatocytes.

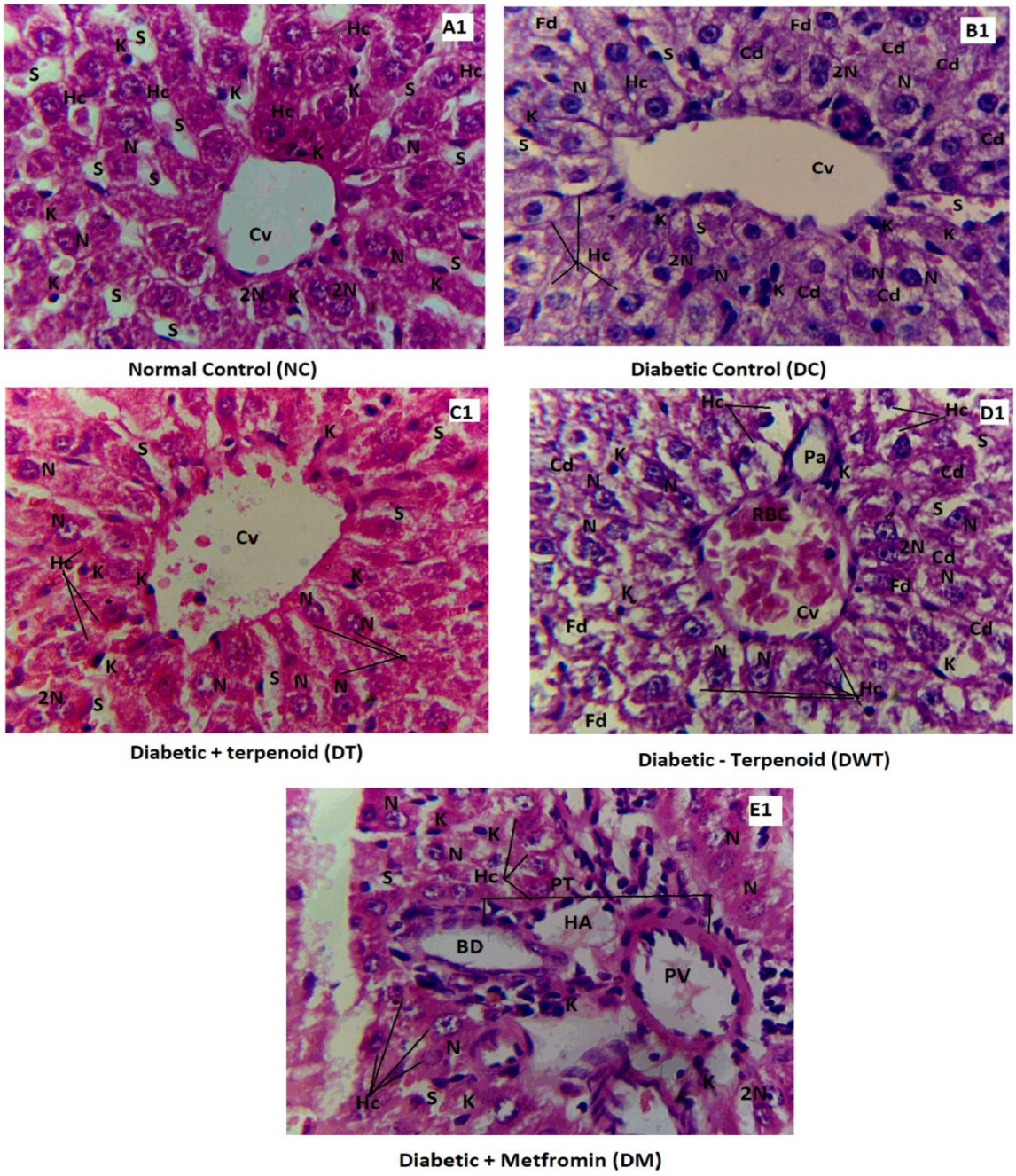


Figure 18: Photomicrographs of histological changes of rat liver section showing central vein region. (H&E: X100) A1-Group A Normal Control/NC rats;B1- Group B Diabetic control (DC) rats; C1- Group C diabetic rats treated with terpenoid (DT); D1- Group D Diabetic rats treated with root Salep devoid of terpenoid (DWT); E1- Group E Diabetic rats treated with Metformin (DM).Here Cv- Central vein; K- kupffer cells; N- Nucleus; Hc - Hepatocytes; Pa - Portal areas; S-sinusoidal space; 2N- two nucleus; Cd- cytoplasmic degeneration; Fd- fat droplets.

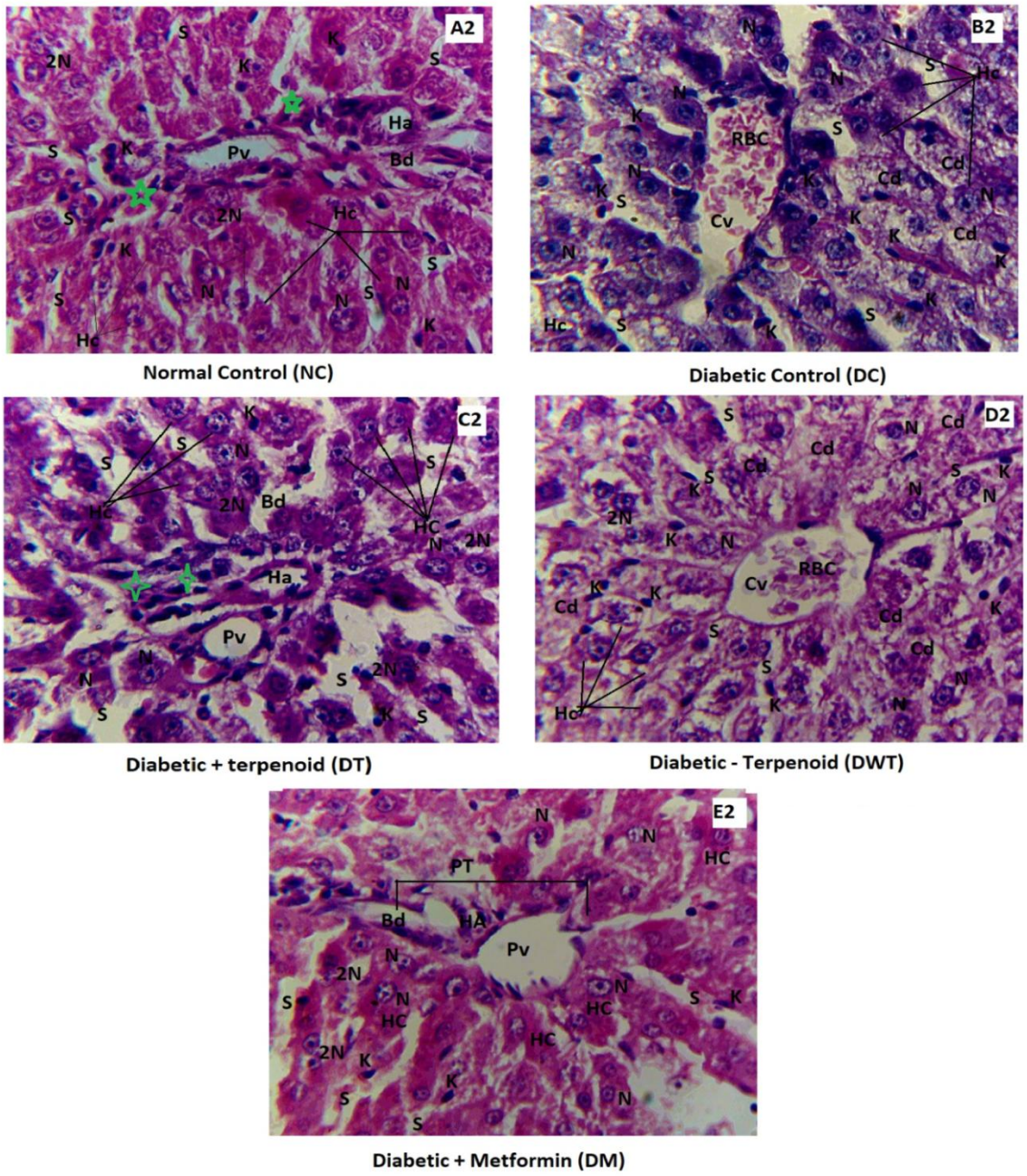


Figure 19: Photomicrographs of histological changes of rat liver section showing the portal triad. (H&E: X100) A2-Group A Normal Control/NC rats;B2- Group B Diabetic control (DC) rats; C2- Group C diabetic rats treated with terpenoid (DT); D2- Group D Diabetic rats treated with root Salep devoid of terpenoid (DWt); E2- Group E Diabetic rats treated with Metformin (DM). Here Cv- Central vein; K- kupffer cells; N- Nucleus; Hc-Hepatocytes; Bd-Bile duct; Pv- Portal vein; Ha- Hepatic artery; PT- portal triad; S-sinusoidal space; 2N- two nucleus; Cd- cytoplasmic degeneration; star marks- connective tissues.

Also no signs of lesion were observed. Portal triad with portal vein (Pv), hepatic artery (Ha), bile duct (Bd) appears normal with normal hepatic parenchyma and sinusoids (Figure 19 A2).

Liver tissue of STZ-induced diabetic rats (DC group) revealed degeneration (necrosis)/acute inflammation of hepatocytes (irregular shaped) with vacuolisation of cytoplasm, loss of glycogen granule, cloudy swelling and congestion of central vein, dilatation of sinusoidal space, accumulation of fat droplets and macro droplet of fat occupying large area of hepatocytes were seen in comparison to the normal control group (Figure 18 B1 &19 B2).

Root Salep treated STZ-diabetic animals (DT group) showed regeneration in the liver tissue where distinct hepatocytes looked like hepatic bands with prominent nucleus (N) or two-nucleus (2N) with reduced sinusoidal space, mild inflammation and decreased central vein congestion were observed (Figure 18 C1 &19 C2). The arrangement of the liver cells appears like the normal control group. Here in the treated group apparently less fat cell accumulation, reduced proliferation of kupffer cells (K), minimal infiltration of inflammatory cells and normal granule of glycogen content were observed in comparison to the diabetic control group.

Liver of STZ-induced diabetic rats treated with root Salep without terpenoid (DWT group) showed degeneration/necrosis of hepatocytes (irregular shaped) with vacuolisation of cytoplasm, loss of glycogen granule, fat droplets (Fd) accumulation, proliferation of kupffer cells (K), cloudy swelling of hepatocytes, congestion of central vein (Cv) with blood, dilatation of sinusoidal space(S) in comparison to the normal control group. Here hepatocytes were scattered with infiltration of inflammatory cells in the portal triad (Figure 18 D1 &19 D2). Also necrotic features were observed around the region of portal triad with collagen

deposits. Cytoplasmic degeneration (Cd) was prominent in the liver tissue of these DWT group (Figures 18 D1 & 19 D2).

Metformin treated rats (DM group) showed improvement in the hepatic architecture nearer to normal control group with regeneration in hepatocytes and reduction in these pathological changes (Figures 18E1 &19E2). Also fat accumulation gets reduced with decreased central vein (Cv) congestion and reduced sinusoidal (S) dilatation. Metformin treated groups show normal granule of glycogen contents in the hepatocytes thus revealing intact architecture of liver. The liver of this group also did not reveal any kind of infiltration of inflammatory cells in the portal triad (Pa) region and deposition of collagen in the hepatocytes thus ruling out any kind of fatty damages (Figures 18 E1 &19 E2).

Histopathology of rat kidney

The therapeutic efficiency of root Salep containing terpenoid along with standard drug Metformin was estimated by conducting histopathology of kidney under STZ-induced conditions and morphology of rat kidney was examined through H & E staining. Figure 20(H&E StainingX100) shown a photomicrograph of the kidney after 5 days of treatment. The histopathological features of normal control rats revealed normal prominent glomerulus, tubules, collecting ducts as shown in the Figure 20 A. The control groups (DC) did not reveal any kind of damages in the morphology of kidney in H&E. The morphological features of the kidney in this group showed normal prominent glomeruli surrounded by intact Bowman's capsule, proximal convoluted and distal convoluted tubules without any inflammations. And the thickness of glomerular basement membrane was uniform in the kidneys of normal control group (Figure 20 A). Kidney of STZ-induced diabetic rat (DC) revealed changes in size of the glomerulus, shrunken or completely lost glomeruli (sclerosis of glomerulus), loss of glomerular lobulation, thickening of bowman capsules, mesangial matrix expansion, intratubular blood congestion, tubular cytoplasmic vacuolation, inflammation in blood

vessels and some pyknotic nuclei. Mild vacuolar degeneration in the tubular epithelial cells with infiltration of lymphocytes was also observed (Figure 20 B). Also fat depositions, severe cystic dilatation of tubules (degeneration), interstitial inflammatory cell infiltration were detected in this diabetic group (Figure 20 B).

But diabetic rats treated with terpenoid (DT) showed regeneration in the architecture of kidney tissue with less inflammatory blood vessels, less fat deposition as compared to diabetic control.The treated group (DT) revealed regeneration in the tissue of kidney and improvement in the glomerular morphology in comparison to the diabetic control group (Figure 20 C). In this treated group, reduced inflammatory cell infiltration, reduced deposition of fat, reduced tubular necrosis, normal Bowman's space with glomerulus, normal basement membrane and capillaries were detected thus maintaining the normal architecture of kidney in comparison to diabetic control group (Figure 20 C).

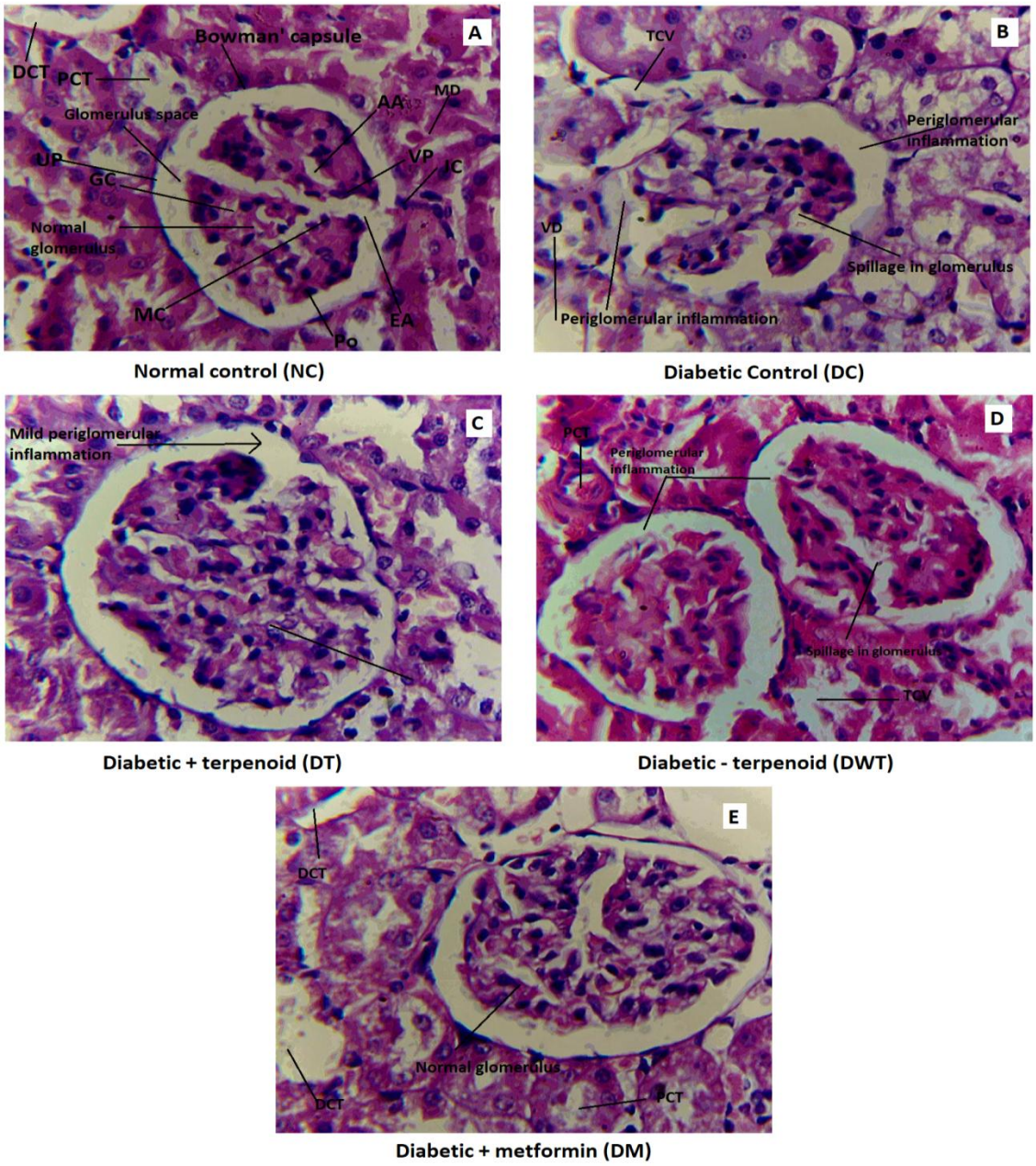


Figure 20: Photomicrographs of histological changes of rat kidney section. (H&E: X100) A2-Group A Normal Control/NC rats;B2- Group B Diabetic control (DC) rats; C2- Group C diabetic rats treated with terpenoid (DT); D2- Group D Diabetic rats treated with root Salep devoid of terpenoid (DWT); E2- Group E Diabetic rats treated with Metformin (DM). Here EA- Efferent arteriole; AA- Afferent arteriole; MD- Macula densa; VP- Vascular pole; JC- Juxta-glomerular cells; Po- Podocytes; MC- Mesangial cells; GC- Glomerular capillaries; UP- Urinary pole; PCT- Proximal convulated tubule; DCT- Distal convulated tubule; TCV-Tubular cytoplasmic vacuolisation; VD- Vacuolar degeneration.

The diabetic group treated with root Salep devoid of terpenoid (DWT) showed degenerative features of the kidney tissue like diabetic control group (Figure 20 D). Here inflammation in the periglomerular space was prominent with spillage in the glomerulus (degeneration).The basement membrane of the glomerulus appeared was much thickened and also expansion in the Bowman's space observed. Fat deposition, inflammation in blood vessels, severe cystic dilatation of tubules (degeneration) and interstitial inflammatory cell infiltration were observed in this group (Figure 20 D). The diabetic group treated with standard drug Metformin (DM) also revealed regeneration in the ultra-structure of kidney. And the changes in the glomerular morphology were similar like the normal control group. Metformin treated group showed normal size glomerulus with normal Bowman's capsule space, no infiltration of lymphocytes, improved structure of tubules and lesser tubular lipid deposition (Figure 20E).

Effect of extracted terpenoid (crude) on STZ induced diabetic mice.

The anti-diabetic efficacy of crude terpenoid was revealed by supplementing extracted crude terpenoid to STZ-induced mice for 5 days period. There was an increased level of serum acid phosphatase, GPT, GOT and S.O.D activity in diabetic control group (DC) as compared to the normal control animals (Figure 21). The serum acid phosphatase level, SGPT, SGOT activity increased by 40.31%, 45.21% and 52.89% respectively in diabetic control animal with respect to their normal control group (Figures 21 A, 21 B and 21 C). But in the terpenoid treated group (DT) there was significant decline in the level of serum acid phosphatase (ACP), SGPT and SGOT by 36.28%, 44.21% and 47.15% respectively.The activity of S.O.D was decreased significantly in the diabetic group (DC) by 55.86% in comparison to their normal control group whereas in the treated (DT) group there was significant increment (by 67.28%) in the S.O.D activity (Figure 21 D) in comparison to their diabetic control.

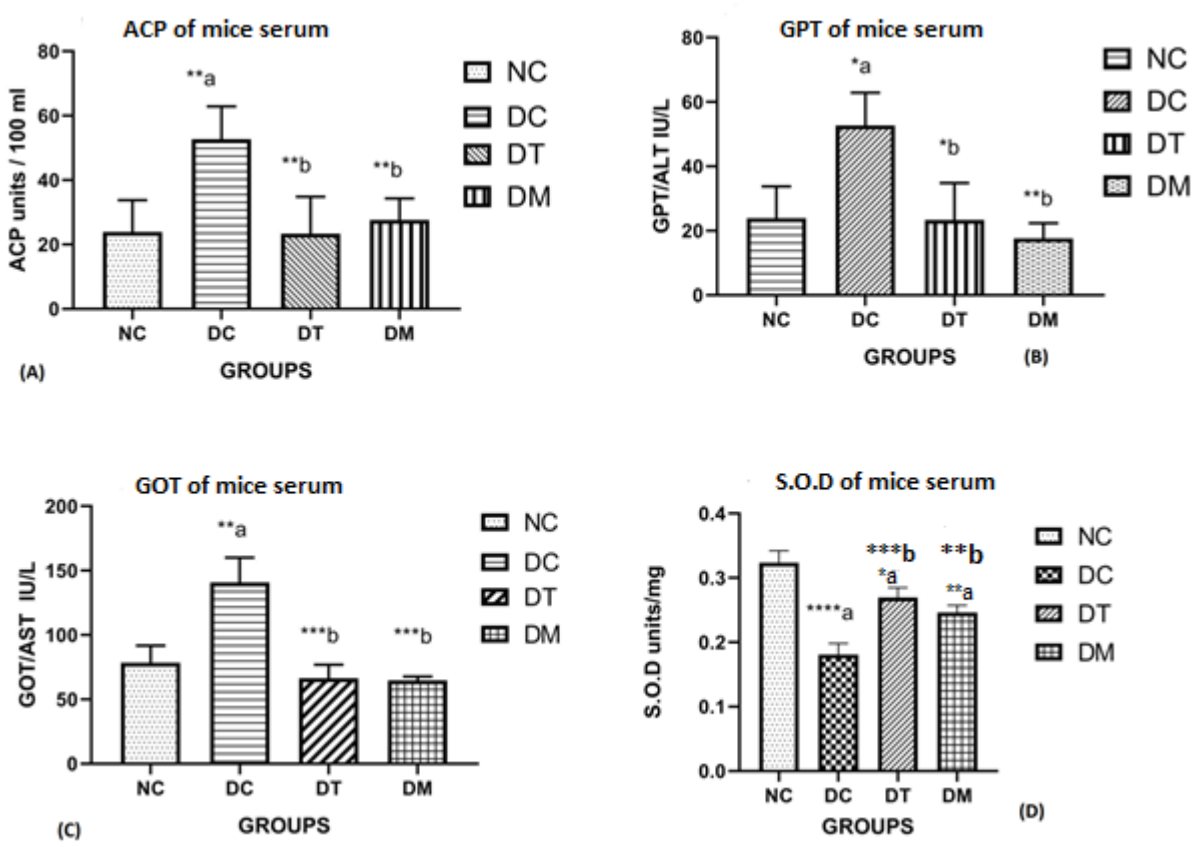


Figure 21: Effect of extracted crude terpenoid on hepatic enzymes (mice serum) at different groups where A: ACP; B: SGPT/ GPT; C: SGOT/GOT.

*Data were averaged and presented as mean ± S.D. (N = 5); *a significant (*P ≤ 0.01) different (increase) from normal control group; **a more significant (**P ≤ 0.001) different (increase) from normal control group; *b are significant (*P ≤ 0.01) different (decrease) compared with diabetic control; **b are more significant (**P ≤ 0.001) different (decrease) compared with diabetic control; ***b highly significant (***P≤0.0001) different (decrease) compared with diabetic control.*

The urea, creatinine and BUN level increased significantly in the diabetic control group (DC) by 54.45%, 69.59% and 54.47% respectively when compared with their normal control group (NC). In the treated group (DT) these levels decreased significantly($p<0.001$) by 56.15%, 60.35% and 56.18% respectively as compared to their diabetic untreated group (Figures 22 A, 22B and 22C).

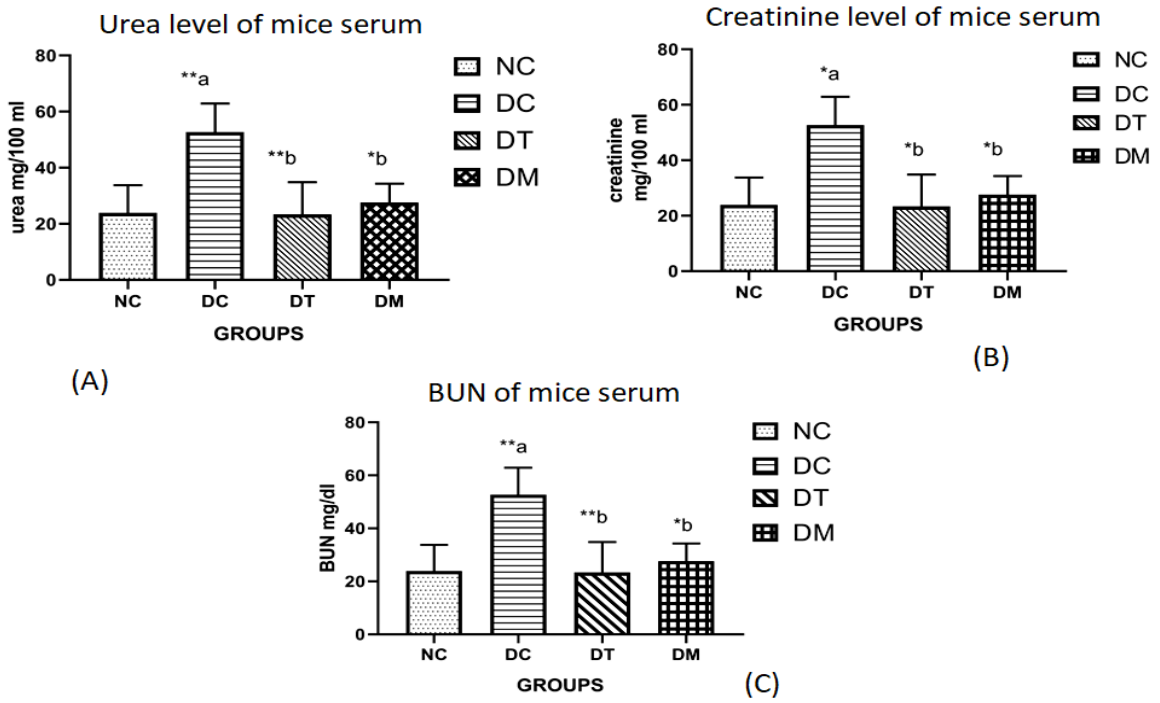


Figure 22: Effect of extracted crude terpenoid on renal parameters at different group of mice where A: Urea; B: Creatinine; C: BUN.

*Data were averaged and presented as mean ± S.D. (N = 5); *a means significant (*P ≤ 0.01) different (increase) compared to normal group; **a implies more significant (**P ≤ 0.001) different (increase) compared to normal group; *b means significant (*P ≤ 0.01) different (decrease) from diabetic control; **b means more significant (**P ≤ 0.001) different (decrease) compared with diabetic control.*

The level of cholesterol and Triglycerides in the serum of diabetic animal also increased significantly by 57.66% and 45.31% respectively as compared to the normal control group. When they were supplemented with Salep containing terpenoid the level of cholesterol ($p<0.0001$) and triglyceride ($p<0.001$) in the serum of treated group decreased significantly in comparison to the diabetic control group by 46.94% and 45.67% respectively (Figure 23 A & B).

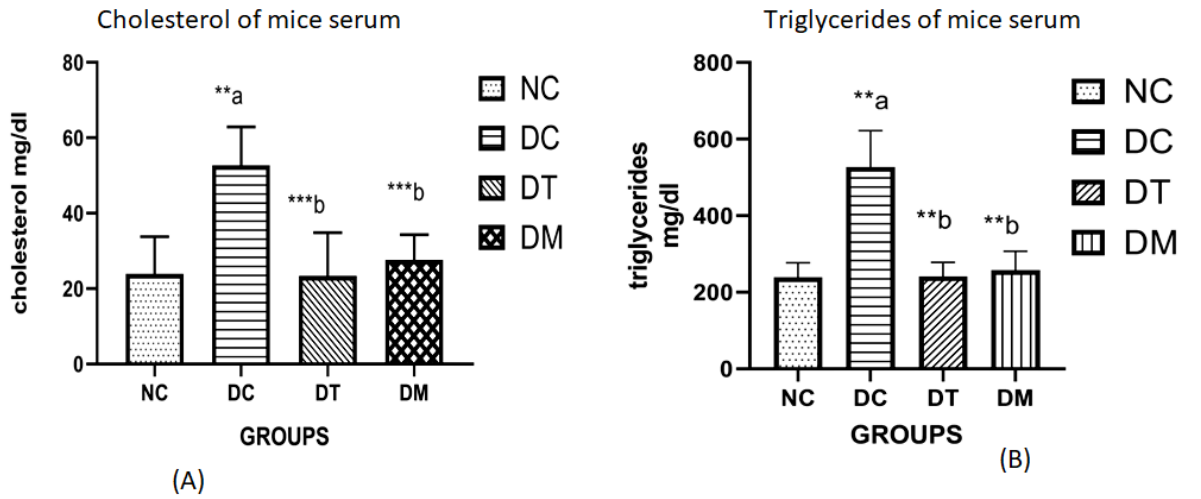


Figure 23: Effect of extracted crude terpenoid on lipid profile (serum) at different group of mice where A: Cholesterol; B: Triglycerides.

*Data were averaged and presented as mean ± S.D. (N = 5); **a implies more significant (**P ≤ 0.001) different (increase) compared to normal group; **b means more significant (**P ≤ 0.001) different (decrease) compared with diabetic control; ***b means highly significant (***P≤0.0001) different (decrease) compared with diabetic control.*

Effect of root Salep on the histopathology of vital organs like pancreas, liver and kidney of mice.

Histological studies of pancreas, liver and kidney revealed normal histological features in normal control (NC) mice (Figure 24A).

Histopathology of mice pancreas

Figure 24 (H&E Staining x 100) shown a photomicrograph of the mice pancreas after 5 days of treatment. The pancreatic sections of the normal control group (NC) clearly revealed distinct granulated, dark β-cells stained with H&E (Figure 24A). Pancreatic Islets of Langerhans in the pancreatic tissue appeared as normal, large and circular shaped with clusters of islet cells, with distinct cell lining, prominent nucleus. The acinar cells was properly organised (Figure 24A).

But in diabetic control group (DC) the pancreatic islet architectures exhibited shrinkage islets in size, reduction in islets cells number, reduction in their mean diameter,

vacuolation, atrophy and delicate incursion of connective tissues in parenchyma of pancreatic islets were observed (Figure 24 B). Pancreatic section of diabetic mice stained with H&E also revealed that diabetes caused necrotic damages of pancreatic islets (β-cells) especially in the islets centre.

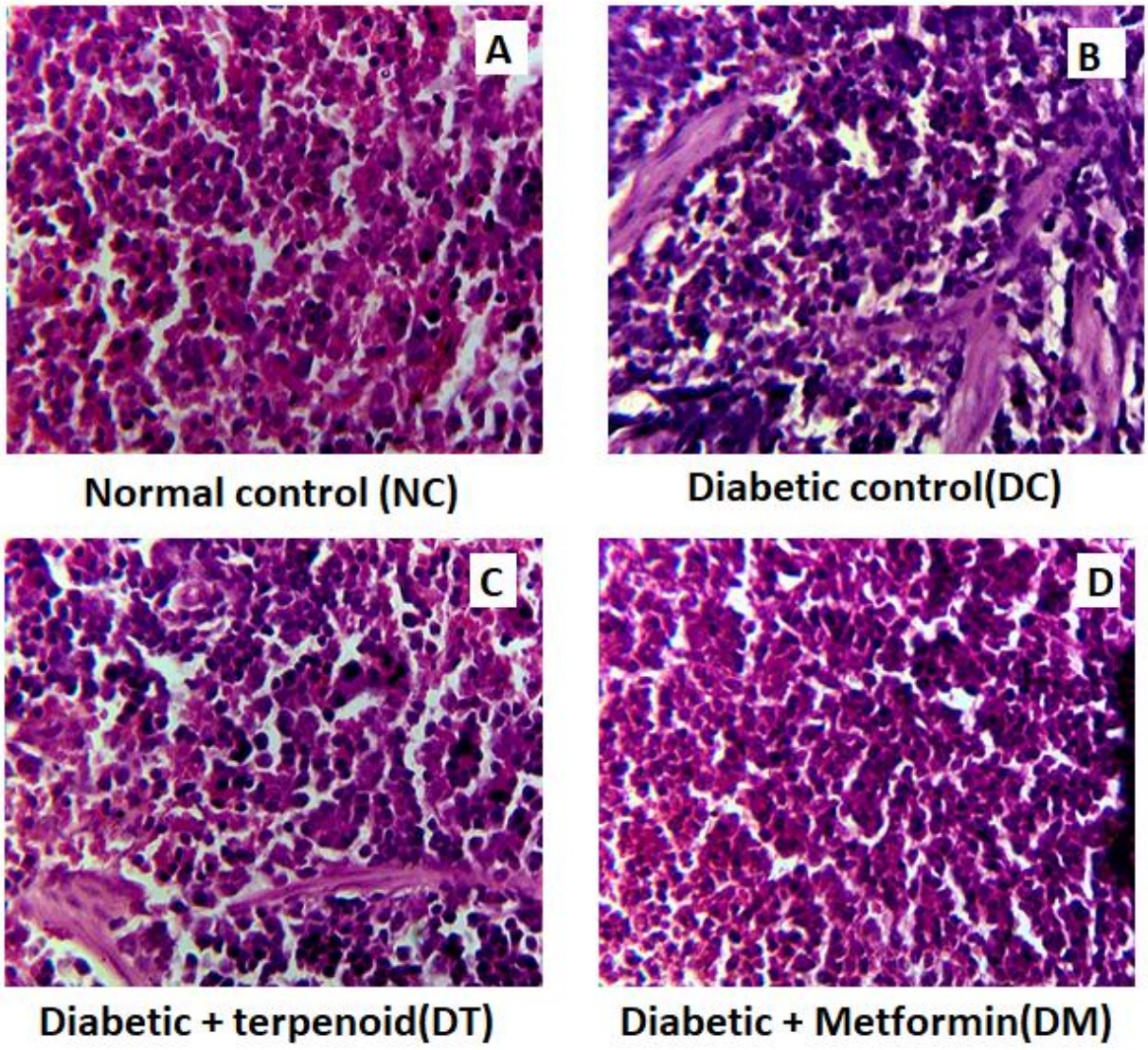


Figure 24: Photomicrographs of histological changes of mice pancreatic section. (H&E: X100) A (Normal Control group/NC) - islet seems normal; B (Diabetic control group/ DC) - islets are shrunken due to necrosis; C (terpenoid treated group/ DT)-islets shows regeneration in islets with increase cellular density; D (diabetic +metformin/DM)-islets showing regeneration in islet cells and increased cellular density.

In the diabetic group (DT) shrunken & small shaped acinar cells, degenerated β-cells were observed. Lymphocytic infiltration, hypochromatosis (karyolysis) and vanishing of pancreatic islets cell borders and nucleus were observed. In some cases two islets get joined together to form large islets with red blood cells infiltration indicating the damage area. Acinar parts of pancreas also revealed severe atropy (Figure 24B).

The pancreatic islets in diabetic group treated with terpenoid (DT) exhibited similar architectures like normal control group (NC) with no changes in the β-cells suggesting the protective role of terpenoid on the regeneration of β-cells by repairing necrotic and fibrotic changes. Also the acinar cells appeared normal with abundance of β-cells (Figure 24 C). Metformin treated group also preserved the architecture of islet cells similar to normoglycemic mice pancreas by reducing the pathological changes. Cytoplasmic vacuolisation and less degranulation were observed in the β-cells of this group (Figure 24D).

Histopathology of mice liver

Figure 25 (H&E Staining x 100) shown a photomicrograph of the liver after 5 days of treatment. Normal control mice exhibited normal hepatic architecture where morphology of hepatocyte was normal with properly arranged hepatic cell cords (Figure 25A).Polyhedral shaped hepatocytes were arranged radially around the central vein. These hepatocytes comprised of central distinctly rounded one or two nuclei. Well preserved acidophilic cytoplasm with prominent cell borders, normal rounded central vesicular euchromatic nuclei with well-defined nucleoli were observed. Central veins were observed in the hepatocytes of this normal control group (NC) (Figure 25A). Presence of thin-walled blood sinusoids lined by flat endothelial cells and prominent nuclei of von Kupffer cells were clearly visible in the normal control group.

The STZ-induced diabetic control mice (DC) revealed severe hypertrophy of hepatocytes (cloudy swelling), infiltration of lymphocytes, congestion in central vein, lipid

droplet accumulation, mussy hepatic cords, fatty degeneration, hypochromatosis, disappearance of cell borders and focal necrosis which ultimately resulted in liver damages (Figure25B). Deterioration of the polyhedral shape of hepatocytes and changes in size due to inflammatory effects were detected in the liver tissues of diabetic mice. Dilatation in the blood vessels of sinusoids, increased number of vacuoles and pyknotic nuclei were seen in the liver tissue of diabetic mice (Figure 25B).

The extracted terpenoid from root Salep showed ameliorating effect on the hepatic architecture of terpenoid treated group (DT) and reversing the pathological changes in the hepatic tissue of mice. The morphology was much similar to normal control group. Degeneration of the hepatocytes resulting to vacuolation also gets reduced (Figure 25C). Terpenoid helps in the alleviation of the symptoms of focal necrosis and congestion of central veins, reduction in the inflammatory cell infiltration and lipid droplet accumulation thus altering the damages in the liver. Also no such remarkable anomalies were observed in the histopathology of diabetic mice treated with terpenoid (Figure 25C).

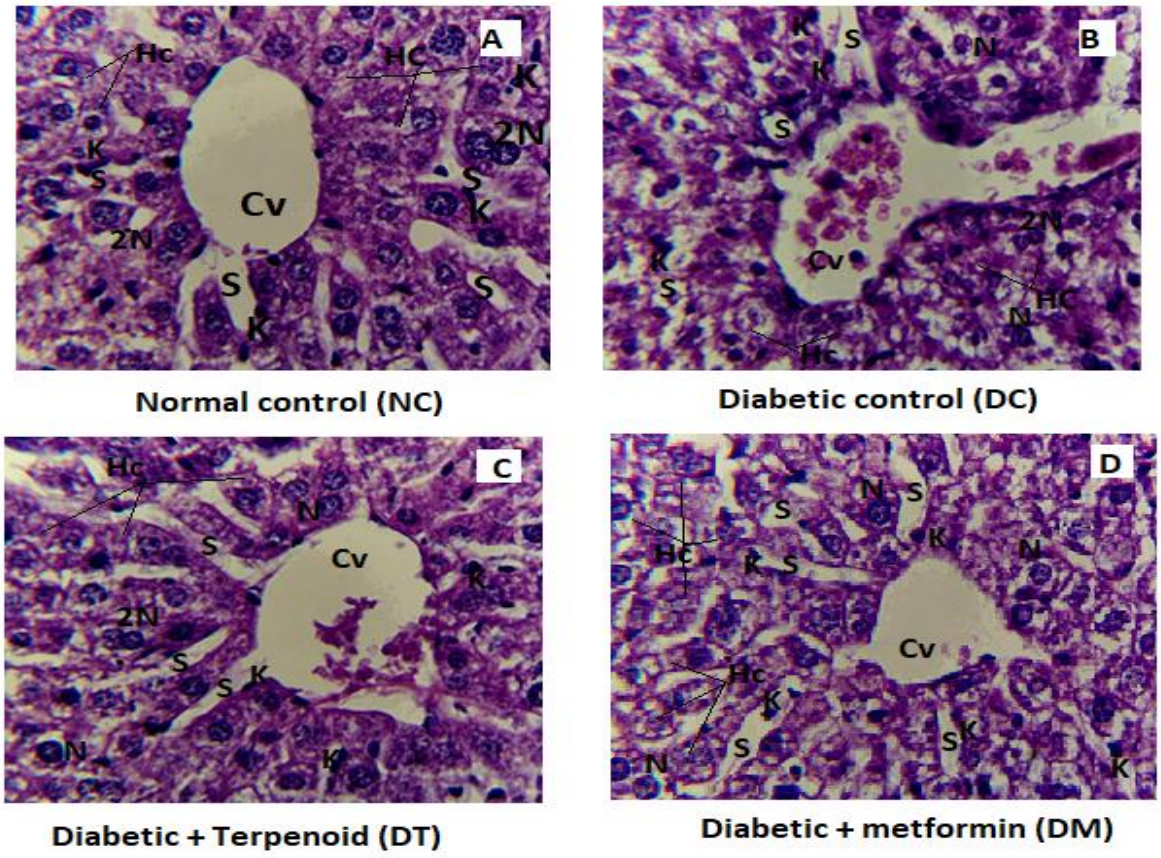


Figure 25: Photomicrographs of histological changes of mice liver section. (H&E: X100) A-Group A Normal Control/NC mice; B- Group B STZ-induced diabetic control (DC) mice; C- Group C diabetic mice treated with extracted crude terpenoid (DT); D-Group D Diabetic mice treated with Metformin (DM). Here Cv- Central vein; K-kupffer cells; N-Nucleus; Hc-Hepatocytes; artery; S- sinusoidal space; 2N- two nuclei.

Metformin treated diabetic mice (DM) also alleviated the symptoms of focal necrosis and congestion of central veins thus restoring the normal architecture of liver similar to normal control group. Radially arranged hepatocytes around the central vein and blood sinusoids with von Kupffer cells were observed in the liver section of diabetic metformin treated group (DM) and this morphology was similar to the normal control group (Figure 25D).

Histopathology of mice kidney

The therapeutic efficiency of extracted crude terpenoid from the roots of *Gymnadenia orchidis* Lindl along with standard drug Metformin was estimated by conducting histopathology of kidney under STZ-induced conditions and morphology of mice kidney was examined through H & E staining. Figure 26 (H&E Staining x 100) showed a photomicrograph of the kidney after 5 days of treatment.

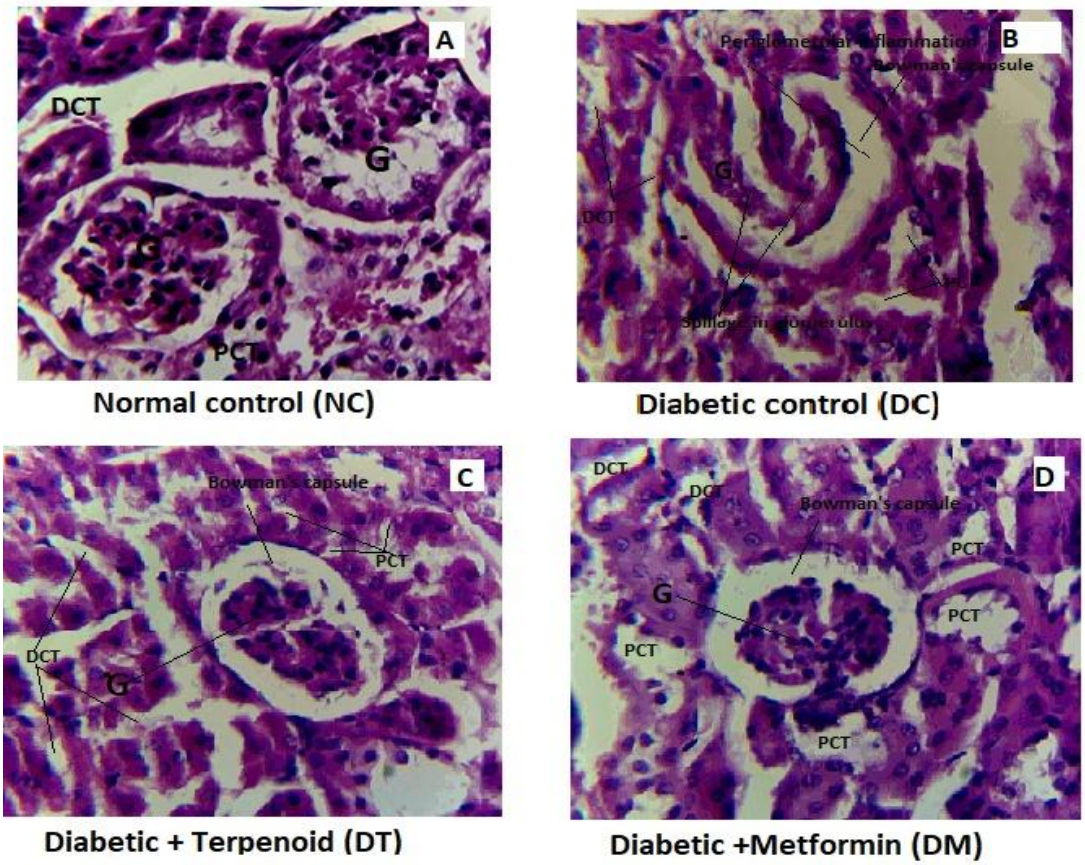


Figure 26: Photomicrographs of histological changes of mice kidney section. (H&E: X100) A-Group A Normal Control/NC mice showing normal architecture of kidney; B- Group B STZ-induced diabetic control (DC) mice showing periglomerular inflammation in the bowman capsule space, thickening of the basement membrane of glomeruli dilatation of the tubules; C- Group C diabetic mice treated with terpenoid (DT) showing regeneration in the medulla and cortex region, thinning of the tubules; D- Group D Diabetic mice treated with Metformin (DM) also showing restoration of kidney structure like normal control group. Here G-Glomerulus; PCT- Proximal convulated tubule; DCT- Distal convulated tubule.

Renal histopathology of normal control (NC) mice revealed the clear architecture of both cortex and medulla. Intact shape of glomeruli, renal tubule and collecting duct were illustrated clearly. Inflammatory cell infiltration, congestion of the tubules was not noticed in the interstitial part (Figure 26A). Arrangement of medulla was seen as properly ordered and packed in the kidney tissue of normal control mice.

In case of STZ-induced diabetic mice (DC) degenerated structure of renal cortex and medulla were observed. Irregular distribution of cortex and medulla, renal structure abnormality, thickening of the basement membrane (periglomerular inflammation) of the glomeruli, expansion of mesangial cells and sclerosis of glomerulus were prominent in this group (Figure 26B). Also reduction in the number of glomeruli, dilatation of kidney tubules and increased inflammatory cell infiltration in the glomeruli were prominent in diabetic group (Figure 26B).In terpenoid treated diabetic mice group (DT) degeneration of the glomeruli was alleviated to many degrees but still infiltration of inflammatory cells in the glomeruli was present in less extent. Restoration of the medulla structure was clear in this treated group (Figure 26C) suggesting the regenerative property of terpenoid on kidney structure restoration. In Metformin treated group (DM) also the architecture of kidney was restored near about normal like the normal control group. All the pathological features were restored (Figure 26D).

Effect of extracted terpenoid (crude) in two different doses on STZ-induced diabetic mice.

To see the efficacy of the extracted terpenoid (crude) on STZ-induced diabetic mice, two different doses of crude terpenoid were supplemented to diabetic mice for 5 days by using two different doses of the desired drug twice a day with 8 hour interval for nine doses of 5 days.

The diabetic control (DC) animals showed a significant decrease ($p<0.0001$) by 68.86% in the levels of haemoglobin concentration and a significant increase (by 69.13%) in the level of glycosylated haemoglobin (HbA1c) as compared to normal group. In the treated group (T2&T4) haemoglobin concentration increased significantly by 80.29% & 68.54% respectively and level of glycosylated haemoglobin in T2 and T4 group decreased significantly by 85.50% &74.09% respectively as compared to the diabetic control animals. Here two different doses (2 mg/kg and 4 mg/kg b.wt.) of crude terpenoid were supplemented to the diabetic animals, and T4 (4mg/kg b.wt) group showed more effective result (Figures 27A and 27B).

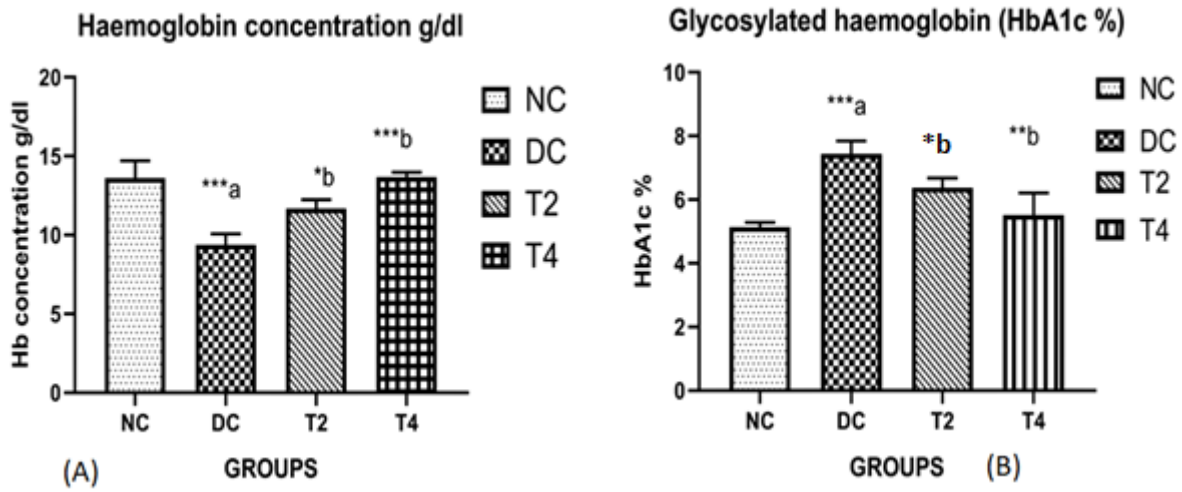


Figure 27: Effect of two different doses of crude terpenoid on haemoglobin and glycosylated haemoglobin. where A: Haemoglobin concentration; B: Glycosylated haemoglobin.

*Data were averaged and presented as mean ± S.D. (N = 5); In DC group ***a implies highly significant (***P ≤ 0.0001) different (decrease) compared to normal group for haemoglobin concentration; in glycosylated haemoglobin percentage DC group shows ***a highly significant (***P ≤ 0.0001) different (increase) compared to normal control group. In T2 group *b implies significant (*P ≤ 0.01) different (increase) in comparison to diabetic control group for Haemoglobin concentration level; for glycosylated haemoglobin percentage T2 group shows *b significant (*P ≤ 0.01) different (decrease) compared to diabetic control group. T4 group reveals **b more significant (**P ≤ 0.001) different (decrease) in comparison to diabetic control group for glycosylated haemoglobin percentage; T4 group shows ***b highly significant (***P≤0.0001) different (increase) compared with diabetic control in haemoglobin concentration.*

The G6PDH activities in the serum of diabetic animals (DC) decreased significantly by 69.73% and 32.21% respectively in comparison to their normal control animals (Figures 28 A &28 B). In the treated group (T2 and T4) the activity of G6PDH (U/10^{12}RBC) increased significantly by 69.58% & 54.96% respectively and the activity of G6PDH (U/g Hb) in treated group (T2 & T4) increased significantly by 32.70% and 30.71% respectively as compared to the diabetic control animals (Figures 28A & 28B). The serum insulin concentration decreased significantly by 82.94% in the diabetic control group as compared to the normal control animals. But in the treated group (T2 & T4) the insulin concentration increased significantly by 79.71% & 71.81% respectively as compared to the diabetic control group (Figure 28 C).

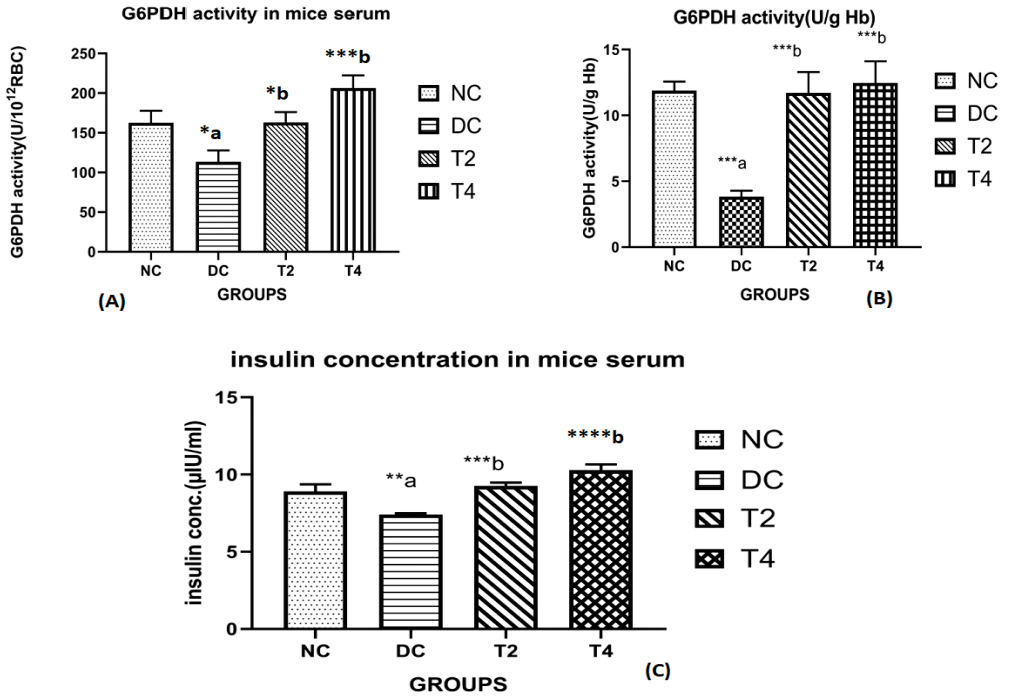


Figure 28: Effect of two different doses of crude terpenoid on G6PDH and Insulin Concentration where A & B: G6PDH activity; C: Insulin concentration.

*Data were averaged and presented as mean ± S.D. (N = 5); *a means significant (*P ≤ 0.01) different (decrease) in comparison to normal control; **a means more significant (**P ≤ 0.001) different (decrease) incomparison to normal control; ***a implies highly significant (***P ≤ 0.0001) different (decrease) compared to normal group. *b are significant (*P ≤ 0.01) different (increase) in comparison to diabetic control; ***b are more significant (**P ≤ 0.001) different (increase)in comparison to diabetic control; ****b are highly significant different (***P≤0.0001) compared with diabetic control.*

CHAPTER 5:
Discussions and Conclusion

Discussions

Diabetes mellitus is a chronic metabolic disorder affecting a major proportion of population in the world. It is characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. Inefficiency of the pancreatic β-cells releasing sufficient insulin in the blood is either inherited or acquired and this results in hyperglycemia.Diabetes leads to diabetic complications, cellular tissue damage etc. which are associated with oxidative stress persuaded by free radical generations [258]. Diabetes is a multifunctional disease associated with chronic hyperglycemia and lipoprotein abnormalities causing long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels resulting in increment of mortality and morbidity rate. Diabetes increases cell damage, abnormal production of reactive oxygen species (ROS) which ultimately causes diabetic complications resulting increase in the morbidity rate [25]. Plant kingdom always has been a source of natural medicine from ancient times and utilized in the treatment for different ailments and diseases. Medicinal plant-derived compounds like alkaloids, flavonoids, terpenoids, saponins, vitamins, carbohydrates and amino acids etc. have gain importancefor clinical use, better patient tolerance and acceptance. These natural products are relatively safe and have been proved to be efficient in comparison to the synthetic drugs without having any harsh side-effects and which are tested for biological, antimicrobial and hyperglycaemic activity. Although herbal/natural plant derived products plays an important role in the modern medicinal world but most of the plant derived compounds are still undiscovered due to their structural diversity in drug discovery.

Nowadays, people are turning towards herbal medicines to get some relief from the adverse side-effects of modern synthetic medicine. The herbal medicine acts as both an alternative and adjunct to modern synthetic chemical drugs.Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically as anti-diabetic and anti-hyperlipidemic remedies [25,64]. The ethno-botanical information reports that around 800 plant species may possess anti-diabetic potential. *Orchidaceae*/ orchid family which is the largest flowering plant kingdom comprises of about 20,000 to 35,000 species in the world. It is not only famous for itsexotic beauty but also serves as a valuable resource for herbal medicines [259]. Phytochemical constituents of orchids like alkaloids, flavonoids, glycosides etc. have increased the medicinal value of orchids [260].

The present research investigation was carried out to assess the phytochemical and pharmacological investigation of medicinal plant (perennial herb) *Gymnadenia orchidis* Lindl used by the local Bhutia community for the treatment of diabetes in form of roots Salep. A thorough literature study reveals that till yet no such scientific studies were carried out to establish the anti-diabetic potential of *Gymnadenia orchidis* Lindl. Also the exact molecular mechanism of action of this plant species active compound terpenoid (particularly eugenol) is unknown. Therefore my study has been designed to assess the anti-diabetic potential of this herb *Gymnadenia orchidis* Lindl.

The qualitative phytochemical investigation of the plant root aqueous extract by HPLC method reveals the presence of active constituent’s like terpenoids, steroids, cardiac glycosides, tannins, polyphenols, vitamin C, carbohydrate(s) and proteins in the root extracts of*Gymnadeniaorchidis*Lindl.The root powder of *Gymnadeniaorchidis*Lindl macerated with ethanol and subjected to differential fractionation method using petroleum ether and distilled waterpartitioned in a separating funnel to extract the crude terpenoid.Thin Layer Chromatography (TLC) confirms the presence of terpenoid which is known to be potent

antioxidant present in the roots of thisplantstudied here.Therefore we can say that polyphenols, vitamin C and tannins present in the root Salep also possesses some antioxidant property due to which root Salep can counteract the diabetic damages [261,262].

Characterization of phyto-contituents, e.g., terpenoid was confirmed by performing spectroscopic analysis like UV –Vis Spectroscopy, FTIR and Mass spectroscopywhich provided the valuable information about the qualitative and quantitative formulation of the plant species and their pattern of recognition by chemometry. The UV–Vis spectroscopy is a simple technique used in the identification of the main phytochemicals, discriminating between the lyophilic and hydrophilic molecules in relation to polarity. Together UV–Vis, FTIR methods or each one separately can be performed to confirm the different constituents present in the extracts.The UV-VIS spectra for terpenoid compound typically lie in the range of 280 nm. The presence of terpenoid was confirmed by the result of UV-VIS spectroscopic analysis in the ethanolic extract of *Gymnadenia orchidis* Lindl.

Fourier Transform Infrared Spectroscopy (FTIR) is a high resolution analytical technique useful in the identification of chemical constituent and in determination of the structural compound [263]. Therefore, in our present study, FTIR technique was conducted to determine the IR fingerprints of *Gymnadenia orchidis* Lindl. The results were shown in the Table 3. The results revealed the presence of terpenoids due to the presence of O-H stretching. The FTIR spectrum confirmed the presence ofalcohols, phenols, alkanes, carboxylic acids/esters, aromatics compound, phenol/ tertiary alcohol, cyclic ethers and esters in *Gymnadenia orchidis* Lindl. All these phytochemicals belong to the secondary plant metabolite as per the studies [264]. The FTIR analysis predicted the presence of the groups O-H stretch, H-Bonded, C-H stretch, C=O stretch, C=C-C aromatic ring stretch, O-H bend, alcoholic group, C-O-C asymmetric stretch, ether group, O-C-C stretch of esters due to the

presence of alcohols, phenols, alkanes, carboxylic acids/esters, aromatics compound, phenol/ tertiary alcohol, cyclic ethers and esters in *Gymnadeniaorchidis*Lindl.

Mass spectral analysis revealed the presence of abundant ions of eugenol with m/z value 203.1277. This ions are related to Thymol methyl ether, Carvacrol methyl ether, 2-Phenylethyl acetate, 3,4-Dimethoxystyrene, Chavibetol (m-Eugenol), (E)-Anethol epoxide, Methyl perillate. Although using ESI-MS only, it was not possible to distinguish a particular compound but positive ESI-MS permits to detectthe possibility of presence of eugenol compound. However, FTIR spectroscopy confirms the presence of -OH, $-OCH_3$, $-CH_2$, Phenol and alkanes which are present in the standard eugenol compound. Also UV-Vis spectrum provides a sharp peak at 280 which is very similar with the UV-VIS spectra of eugenol compound lying in the same region. Thus it can be concluded that the crude extract of *Gymnadenia orchidis* Lindlroot Salep may contains eugenol.

Literature study reveals that eugenol possesses the potential of decreasing the hyperglycaemia. Eugenol improves the body weight and controls the desire for food and excess water intake in diabetic animal thus improving the glycemic index similar like diosmin activity [102,265]. It has been observed that when diabetic rats are orally supplemented with Eugenol, activity of pyruvate kinase (key glycolytic enzyme) is enhanced in liver tissue nearer to normal level resulting in reduction of glycolysis and gluconeogenesis thus causing enhancement of plasma insulin level [102]. Insulin deficiency leads to elevation of hepatic enzymes like AST, ALT, and ALP indicating hepatic damages [266]. Eugenol also prevents the leakage of functional enzymes i.e. AST, ALT and ALP from liver cytosol to the blood stream due to reduced blood insulin level in STZ induced diabetic animals. Insulin deficiency in diabetes leads to protein breakdown thus enhancing amino acids catabolism and provides substrates for gluconeogenesis. Eugenol reduces the level of these hepatic enzymes thus protecting hepatic damages [102,267]. It is reported that Eugenol significantly improves

the liver glycogen content of diabetic animals due to amplified insulin secretion or increased hepatic hexokinase activity which ultimately increases the glucose utilisation for energy production. Thus improvement in hepatic glycogen level takes place. These findings were in accordance with the results of caffeic acid supplementation on glucose in db/db mice where blood glucose level reduce and glycogen content increases [268]. Eugenol extracted from *Syzygium aromaticum* (clove) exhibited Metformin like mode of action by increasing insulin sensitivity, peripheral glucose uptake, fatty acid oxidation thereby reducing glucose level and nephropathic complications when administered alone or in combination with Metformin [269]. Hayden and Tyagi have showed in their investigation that when diabetic rats were orally administered with eugenol causes reduction in the increased levels of blood urea and improved the CK activities in diabetic rats. Thus their findings suggest that eugenol has the potential to attenuate renal and cardiac injury caused by hyperglycemic state [270]. Eugenol improves the activity of Glucose-6-phosphate dehydrogenase (rate limiting enzyme of the pentose phosphate pathway) thereby increasing NADPH formation resulting in deceased oxidative stress and diabetic complication and also increasing lipogenesis (formation of fats from carbohydrates) and plasma glucose level decreases [102,236,271]. The levels of glucose-6-phosphatase and fructose-1,6-bisphosphatase increases in liver and kidney due to insulin deficiency in diabetic rats [272]. Srinivasan *et al.* (2014) have showed that eugenol reduced the higher levels of glucose-6-phosphatase and fructose-1,6-bisphosphatase in diabetic treated and normalized the glucose metabolismby augmentingglucose utilization and reducing hepatic glucose production through insulin secretion and this indicates the beneficial effects of eugenol in diabetes mellitus treatment [102]. Findings of Srinivasan *et al.* (2014) also matched with the results of Celik *et al*. (2009) who had confirmed that caffeic acid a type of phenolic compoundrecoversglucose metabolism by retarding gluconeogenesis in diabetic rats [273]. In diabetic condition accumulation of urea nitrogen increases due to enhanced

breakdown of liver and plasma proteins. Hepatic elimination of urea nitrogen and peripheral release of nitrogenous substances increases due to the modification in the nitrogen homeostasis thus disturbing the balance of nitrogen and causing changes in hepatocytes [274].Elevated levels of serum creatine kinase (CK) level are responsible for the cardiac muscular damages in diabetic condition [275]. Srinivasan *et al.* (2014) also proved that oral supplementation of eugenol improves CK activity and reduces the blood urea level in diabetic animals thus confirming the efficiency of eugenol to prevent renal and cardiac complication in hypoglycemic state[102]. Insulin plays the major role in the regulation of blood glucose homeostasis by enhancing hepatic glucose utilization.

Histopalthological studies also confirmed that terpenoid containing eugenol causes regeneration and helps in acquiring normal structural integrity of pancreatic β-cells in diabetic animals thus preventing fatty acid infiltration and shrinkage of islets cells in STZ induced diabetic rats [102].

Kang et al.(2009) also reported the regeneration property of dehydroabietic acid (DAA), a kind of diterpene on glucose and lipid metabolism of obese diabetic KK-Ay mice [276].Their experimental investigation confirmed that treatment with DAA on obese diabetic mice not only reduced the plasma glucose and plasma insulin levels but also reduced the levels of triglycerides (TG) in both plasma and hepatic tissue[276].The study of Nazaruk and Borzym-Kluczyk (2015) also showed that triterpines which are involved in the glucose metabolism also played an important role in inhibiting the development of insulin resistance thus normalising the plasma glucose and insulin levels [277]. Santos et al. (2012) also investigated the antihyperglycaemic and antihyperlipidemic effects of α, β-amyrin, a triterpenoid mixture from *Protium heptaphyllum* in mice [278]. Terpenoids is an important class of natural products and there are several reported terpenoids which act as anti-diabetic agents. Whereas, some of them are undergoing various stages of pre-clinical and clinical

evaluation to formulate them as the anti-diabetic agents. helps in the inhibition of those enzymes that are responsible for the development of insulin resistance. Terpenoids can also normalize the plasma glucose and insulin levels thus regulating glucose metabolism. Today, terpenoids are considered as an effective agent in the treatment of diabetic retinopathy, nephropathy, and neuropathy. It also helps in healing of wound by blocking several pathways of diabetes and its related complications [279]. Murali *et al.* (2012) had shown in their experimental study that d-limonene, a kind of cyclic monoterpene has the ability to reduce hyperglycemia and also improved the body weight of STZ induced diabetic rats [280]. This d-limonene also significantly reduced the fasting plasma glucose levels in diabetic rats and might be potentialto stimulate the insulin secretion from the pancreatic β-cells and thus helping in the glucoseutilization by the tissues.D-limonene causes significant reduction of glycosylated haemoglobin level, reduction in the activities of glucose 6-phosphatase and fructose 1, 6-bisphosphatase in the diabetic rats thusreducing gluconeogenesis and endogenous production of glucose and therefore establishing the anti-hyperglycemic capability of d-limoneme [280].

Acute toxicity studies are important in determining the effective dosage of drug for treating diabetes mellitus which limits any drug toxicity and mortality. Toxicological investigation on rats has revealed that the root Salep is toxicologically safe and can be used in edible form. The effects of toxicity and mortality of the terpenoid containing root Salep of *Gymnadenia orchidis* Lindl on the animal were also determined by observing the parameters like body weight, organ weight, physical characteristics, biochemical, haematology and histopathology. It was found to be very safe when used up to 1000mg/kg body weight by acute toxicity model study as per the OECD guidelines 423. No toxicity or mortality was evaluated in any of the drug dosages ranging from 100 mg/kg, 200 mg/kg, 300 mg/kg, 500

mg/kg, and 1000 mg/kg intoxicity studies. Based on different clinical observations, the optimum and effective dose for root Salep was selected as 200 mg/kg body weight.

The biochemical parameters like ACP, ALP, GPT, GOT, Urea and Creatinine etc.were analysed on the Salep supplemented ratsat higher doses (> 200 mg powder root) and were found to be high in comparison to the control level but still those values were within the accepted range and it can be concluded that the root Salep did not produce any deleterious effects on the normal functioning of liver (Figure 6), kidney (Figure 7) and on the body weight (Table 4) and on the fasting blood sugar (Table 5).The optimum dosage for the treatment of diabetes was thus standardized and confirmed as 200 mg/kg body weight per day.

Bacterium *Streptomycetes achromogenes* produces STZ and this STZ is considered as efficient model for inducing diabetes in rats and mice. STZ is capable of producing mild to severe diabetes according to the administered dose given to animals either by intravenous or intraperitoneal route. Our conducted experimental results showed that when the animals (rats) received a single dose(60 mg/ kg b.wt) injection of Streptozotocin (STZ), the fasting blood glucose level was significantly increased and body weight was significantly decreased as single dose injection of STZ causes severe type 2 diabetes to the animals [281].At low dose, STZ (60 mg/kg b.w) partially destructs the insulin producing β-cells which is taken up via the cell membrane GLUT2 glucose transporterof β-cells and causes DNA alkylation and eventually causes β cell death [282-284].

Our investigations clearly showed that when experimental STZ-induced diabetic rats were supplemented with root Salep, fasting blood glucose levels (Figure 9A, Table 6) were normalised and body weight were improvedin comparison to the normal group (Figure 8). Glycosylated haemoglobin (HbA1c) is a well-known marker for confirmation of diabetes in the patients. The presence of higher levels of HbA1c in people with persistently elevated

blood glucose clearly indicates diabetic condition.The International Diabetes Federation and American College of Endocrinology recommend HbA1c values below 6.5% is the reference range for non-diabetic patient, while American Diabetes Association recommends that the HbA1c above 7.0% for most patients [284]. Our experimental investigation had clearly confirmed that the HbA1c percentage in the STZ-induced diabetic rats were significantly higher (7.95) than the normal control group (Figure 9B). But when these STZ-induced diabetic rats were supplemented with root Salep, the percentage of HbA1c was more significantly ($P<0.001$) reduced in the induced diabetic animals (5.82) confirming the hypoglycaemic effect of the herb *Gymnadenia orchidis* Lindl against type 2 diabetes (Figure 9B). Further confirmation of the hypoglycaemic effect of *Gymnadenia orchidis* Lindl extract was done by observing the results (Figure 27 A&B) where STZ induced diabetic mice were treated with two different doses of extracted crude terpenoid T2 and T4 (2 mg/ kg and 4 mg/ kg body weight respectively). In our studies the percentage of haemoglobin concentration level was found to be significantly lower in the STZ-induced diabetic group in comparison with normal control group. But, the two different doses of crude terpenoid (T2&T4) helped in the significant improvement ($P<0.001$) of haemoglobin concentration when compared with diabetic control (Figure 27A) group. Also glycosylated haemoglobin (HbA1c) was higher (7.8) in the diabetic control mice group which was dramatically reduced when treated with crude terpenoid to the treated diabetic mice group (Figure 27B). The level of glycosylated haemoglobin significantly ($P<0.001$) decreased by the two different doses of crude terpenoid in comparison to the diabetic control animals (Figure 27B). The dose T4 (4mg/ kg b.wt.) showed more effective result. The level of total haemoglobin (levels) in diabetic group (mice) decreased mainly due to the increased formation of HbA1c. HbA1c increased in diabetic condition and the incrementwas observed as directly proportional to the fasting blood glucose level. During diabetes mellitus, the excess glucose present in the blood reacts with

haemoglobin to form HbA1c. HbA1c also serves as an important marker of estimating the degree of protein glycation in diabetes mellitus. When diabetic animals were treated with terpenoid then the level of glycosylated haemoglobin was decreased by virtue of its normoglycemic activity. This normalisation of glycosylated haemoglobin indicated the decreased glycation of proteins (Figure 27B). Normalisation of the enhanced blood glucose level by *Gymnadenia orchidis* Lindl root Salep indicates the effective role of the root Salep in the enhancement of glucose transport across the cell membranes and stimulation of glycogen synthesis or increase in glycolysis.

The master organ for carbohydrate metabolism is liver and it releases glucose according to metabolic needs. The complication of diabetes mellitus mainly increases due to liver damage and various studies suggest that mortality rate in diabetic patient is due to liver damage than cardiovascular disease [195]. Though several pathways have been identified for liver damages, insulin resistance is the main cause of liver damage because of oxidative stress and increased production of ROS [285]. Liver functioning key enzymes like serum glutamate oxaloacetate (SGOT)/aspartate transaminase (AST), serum glutamate pyruvate transaminase (SGPT)/alanine transaminase (ALT), acid phosphatase (ACP) and alkaline phosphatse (ALP) etc. are present in various tissues, including liver, cardiac muscle,skeletal muscle, kidney, brain, pancreas, lungs, leukocytes and erythrocytes. Due to diabetes, hepatic functions also deteriorated severely as a result of which enzymes of SGOT/ASTand SGPT/ALT levels were increased in STZ treated induced diabetic rats [286]. This increment in the activities of hepatic enzymes in blood and their leakage into the blood stream in diabetic condition is due to insulin deficiencies which are related with increased gluconeogenesis and ketogenesis. But oral supplementation of the root Salep in the treated diabetic animals helped in reduction of SGOT and SGPT level significantly ($P< 0.01$) as indicated in our experimental results (Fig.10A & 10B).The hepatotoxic effect of STZ on the liver of diabetic rats and mice was

ameliorated by the root Salep which contains terpenoid (Figure 13 & 21). Significant ($P<0.001$) reduction of ACP, ALP, AST and ALT concentrations in the serum were noted in the terpenoid supplemented diabetic rats (Figure 13) and significant ($P<0.001$) reduction of ACP, GOT & GPT concentrations were noted in the serum of diabetic mice treated with crude terpenoid (Figure 21). This was a clearly indication of the protective role of terpenoid (may be Eugenol) present in the root Salep against the hepatic damages. However, the levels of those enzymes did not decrease in diabetic animals without terpenoid group (DWT). Diabetic rats treated with standard drug Metformin also showed significant ($P<0.0001$) decrease in the levels of those hepatic enzymes (Figure 13) in comparison to the diabetic control group. The increased activity of these enzymes in the blood in diabetic condition was due to insulin deficiency which was related with increased gluconeogenesis and ketogenesis. The levels of ACP, ALP gets increased along with AST and ALT levels. Higher levels of these enzymes are associated with hepatic dysfunction and their leakage into the blood stream.

Disruption in the carbohydrate metabolism due to diabetes also results in the deregulation of lipid metabolism as both carbohydrates and lipid metabolism are interrelated to each other [283].Due to insulin deficiency or insulin resistance in diabetes mellitus, alteration in lipid metabolism and its regulatory mechanism occur which results in the accumulation of lipids.Abnormally higher concentration of serum lipids in diabetic condition is mainly due to enhanced mobilization of free fatty acids from the peripheral fat depots, as insulin plays the vital role of inhibiting the hormone-sensitive lipase enzyme. Therefore hyperlipidemia, the characteristic feature of diabetes is regarded as the result of unregulated activities of lipolytic hormones on the fat depots.STZ stimulates the production and release of excess of fatty acids in the plasma thus stimulating the conversion of hepatic fatty acids into phospholipids and cholesterol. Phospholipids and cholesterol, together with abundant amount

of triglycerides (TG) may get discharge into lipoproteins in the blood. Hypertriglyceridemia and hypercholesterolemia are the most common form of lipid abnormalities in diabetic condition. In the diabetic control groups, increased level of cholesterol and triglyceride level were observed indicating the deficiency of insulin and its failure to activate the lipase thereby causinghypercholesterolemia and hypertriglyceridemia. Diabetes is responsible for the severity of coronary diseases. STZ-induced diabetes also developed hyperlipidemia. Therefore increased concentrations of cholesterol and triglycerides levels (Figure 10 C) within the blood and the serum (Figure 16 C&D) of diabetic rats were observed. Also increased levels of cholesterol and triglycerides in induced diabetic mice (Figure 23A&B) were observed. Lower levels of high density lipoprotein level (HDL) and higher levels of low density lipoprotein levels (LDL) in the diabetic rats (Figure 16 A&B) were observed in our experimental results. But all these abnormalities were improved by the root Salep containing terpenoid and crude terpenoid extracted from *Gymnadenia orchidis* Lindl. Root Salep supplemented animals showed significant reduction of cholesterol ($P<0.001$) and triglycerides ($P < 0.01$) in the blood and serum of treated diabetic rats treated (Figure 10C, 16 C&D). Serum of terpenoid treated mice also revealed significant ($P < 0.001$) reduction of cholesterol and triglycerides levels (Figure 23A & 23B). Standard drug Metformin similarly showed significant increase in the HDL level whereas significant ($P < 0.001$) reduction in cholesterol, triglycerides and LDL levels.

HDL-cholesterol is involved in the transport of cholesterol from peripheral tissues to liver and therefore it acts as a protective factor. Thus we can say that terpenoid containing root Salep and extracted crude terpenoid from *Gymnadenia orchidis* Lindl is showing hypolipidemic effect in diabetic treated group and it increases the HDL-cholesterol level in diabetic treated group. The abnormality of insulin secretion results in enhanced metabolism of lipids from the adipose tissue to the plasma.Further, it has been reported that diabetic rats

treated with insulin shows normalised lipid levels [287]. Thus, by observing the reduced levels of cholesterol, triglyceride in the Salep supplemented rats, we can conclude that root Salep of *Gymnadenia orchidis* Lindl is very much effective in regulating the lipid profile. The terpenoid of *Gymnadenia orchidis* Lindl shows insulin-like action by virtue of its lipid lowering levels.

In the serum (Figure 10D) and plasma (Figure 15 B&C) of diabetic rats, higher level of lipid peroxidation was observed. Reduction of lipid peroxidation was similar with the findings of Smith *et al.* (2008) and Scoppola *et al.* (1995 288-289]. Significant ($P < 0.0001$) reduction of lipid peroxidation level in the plasma of root Salep treated diabetic rats indicated the antioxidant property of *Gymnadenia orchidis* Lindl. Metformin showed significant reduction in the lipid peroxidation level. In diabetes mellitus, oxygen free radicals (OFRs) are generated by stimulating H_2O_2 *in vitro*, as well as in β-cells [290]. OFR-scavenging enzymes responds according to the oxidative stress condition within the pancreas, a compensatory mechanisms that increases the enzyme activity in diabetic rats. In diabetic group animals as cell oxidative stress increases, the concentration of the lipid peroxidation increases due to increment in the generation of free radicals and absence of antioxidant scavenger systems. The present finding indicated the significant increment of lipid peroxidation inSTZ-induced diabetic rats and its attenuation by *Gymnadenia orchidis* Lindl treatment. This suggests that the protective role of *Gymnadenia orchidis* Lindl root extracts could be due to the anti-oxidative property of terpenoids present in the root, which in turn act as strong superoxide radicals and singlet oxygen quenchers.

Higher level of glucose in both blood plasma and tissues due to diabetes mellitus facilitates the abnormal production of reactive oxygen species (ROS) in diabetic condition. Increased amount of ROS causes cellular damage and oxidative stress [291]. Antioxidants enzymes like SOD, GSH and GPx which all together forms a defence mechanism against

ROS, get dramatically reduced in STZ-induced condition (Figure 11). Superoxide dismutase (SOD), one of the important scavenging enzymes, removes the toxic free radicals *in vivo*. It protects tissues against oxygen free radicals by catalyzing the removal of superoxide radical ($O2^{\cdot}$), which damages the membrane and biological structures. In diabetic condition the activities of S.O.D significantly decreased (Figure 15 A & 21D) in the diabetic control groups indicating deleterious effects due to the accumulation of superoxide radicals. These observations emphasised the critical importance of maintaining the antioxidant potential of the pancreatic β-cells in order to ensure both its survival and insulin secretion capacity during times of increased oxidative stress. The activity of SOD decreased in the liver and kidneys during diabetes mellitus might be due to the production of reactive oxygen free–radical that could themselves reduce the activity of these enzymes. Diabetic rats when treated with root Salep showed significant ($P<0.001$) increment in the SOD levels (15 A). Diabetic mice also when supplemented with crude terpenoid showed significant ($P<0.0001$) improvement in the S.O.D level (21D). Therefore our present study reveals that *Gymnadenia orchidis* Lindl root Salep containing terpenoids when supplemented to diabetic animals undergoing oxidative stress, can partially reduce the imbalances between the generation of reactive oxygen species (ROS) and the scavenging enzyme activity. Standard drug Metformin also showed significant ($P<0.01$) improvement in the S.O.D level (Figure 15A).

Glutathione peroxidase (GPx) is another kind of cystolic antioxidant enzyme whose main function is to protect the cell from oxidative damage. GPx catalyzes the reaction of lipid hydroperoxides with GSH to form glutathione disulfide (GSSG) and the reduced product of the hydroperoxides [292]. Activities of these antioxidant enzymes in the serum of diabetic animals were seen to diminish which suggested the inhibition of defence mechanism by these enzymes in counteracting the lipid peroxide damages. Glutathione plays an important role in intracellular defensive mechanisms against various harmful stimuli especially against

oxidative stress. It reacts directly with ROS and electrophilic metabolites, for several enzymes [293]. Hepatic GSH which combats the deleterious effect of oxidative stress by scavenging the free radicals (ROS) in the body also helps in the detoxification of drugs [292]. Reduction in the GSH activity in diabetic condition severely impairs the cellular defence mechanism along with enhanced oxidative stress and tissue damage (Figure 11). In our study it has been confirmed that activity of SOD, GSH and GPx increases significantly ($P < 0.01$) due to the healing properties of root Salep of *Gymnadenia orchidis* Lindl in diabetic induced rats.

Glycogen acts as the primary intracellular storable form of glucosepresent in higher concentration in various tissues, skeletal muscle etc. Glycogen plays a critical role in glucose homeostasis as muscle glycogen serves as a source of glucose-6-phosphate in the ATP generation by rapid anaerobic glycolysis. Liver glycogen serving as a source of glucose is released into the blood stream during diabetic condition to prevent hypoglycaemia. Utilization of glucose in the hepatic cell is stimulated by insulin thereby maintaining blood glucose homeostasis [294]. Pancreatic β-cells severely damaged by STZ are unable to secrete sufficient insulin thus causing reduction of glycogen level in hepatic tissues. Deficiency in insulin secretion thus inhibits the input of glucose in liver [295]. Glycogen has direct relation with insulin activity as insulin promotes intracellular deposition of glycogen by inhibiting glycogen phosphorylase and stimulating glycogen synthase. But in diabetic rats, glycogen synthesis literally reduced in the liver and skeletal muscle [296].This reduction in the hepatic glycogen in diabetic condition is may be due to deficiency of insulin and ultimately inactivation of glycogen synthesize system.The levels of liver glycogen content of diabetic induced rat when supplemented with root Salep (Figure 12) were seen to increase. In this study we have observed a significant decrease in blood glucose level in diabetic treated groups (rats)with root Salep when compared with diabetic control rats (Table 6) after 5 days

of treatment given (9 doses). The hypoglycemic activity of *Gymnadenia orchidis* Lindl may be through potentiating of pancreatic secretion of insulin from β - cell of islets or due to enhanced transport of blood glucose to the peripheral tissue [297]. Increased glycogen content in the liver of treated diabetic rats indicated that *Gymnadenia orchidis* Lindl modifies the activities of glycogen metabolizing enzymes such as glycogen synthase and glycogen phosphorylase thus resulting in effective glucose utilization and thus improving insulin sensitivity.

Similarly urea, creatinine and BUN levels was significantly elevated in STZ-induced diabetic rats (Figure 14) and treated diabetic mice (Figure 22) suggesting that renal functions of the diabetic animals had been impaired due to abnormal glucose regulation, including elevated glucose and glycosylated protein tissue levels, haemodynamic changes within the kidney tissue, and increased oxidative stress [298]. Negative nitrogen balance with enhanced tissue proteolysis and decreased protein synthesis can contribute to increased serum urea and creatinine levels, indicating impaired renal functions in diabetic animals [299]. After treatment with terpenoid containing root Salep to the diabetic animals (Figure 14 &22), serum urea, creatinine and BUN level were decreased significantly ($P<0.001$). Thus renal damage in these treated animals was prevented.

In this study, insulin level gets significantly ($P<0.01$) increased in diabetic mice treated with crude terpenoid (Figure 28C) in comparison to the diabetic control group. The observed increment in the serum insulin level of terpenoid treated group indicates that terpenoid (Eugenol) extracted from *Gymnadenia orchidis* Lindl stimulates the insulin secretion from the remnant beta cells (β-cells) or from regenerated beta (β) cells of pancreas. In the diabetic control group the concentration of serum insulin significantly decreases due to the damage of pancreatic β- cells, which were unable to produce insulin due to DNA alkylation arising from the production of carbonium ions [300]. In this context, a number of

other plants have also been reported to have hypoglycaemic and insulin release stimulatory effects [301-302].

Liver is an insulin-sensitive tissue and plays a major role in glucose metabolism by regulating the interaction between glucose utilization and gluconeogenesis. A partial or total deficiency of insulin causes derangement in glucose metabolism that decreases the activities of hexokinase, pyruvate kinase and glucose-6-phosphate dehydrogenase, causing impaired peripheral glucose utilization and improved hepatic glucose production [102]. Glucose-6-phosphate dehydrogenase is the first rate limiting enzyme of the pentose phosphate pathway, a metabolic pathway that results in the production of ribose-5-phosphate and supplies reducing energy to cells (e.g. erythrocytes) by maintaining the level of the co-enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) [236]. However when the activity of this enzyme decreases in diabetic condition, the concentration of NADPH diminishes along with increment of oxidative stress which leads to diabetic complications [303]. In our study the activity of glucose -6- phosphate dehydrogenase (G6PD) was significantly lowered in diabetic control animal (mice) compared to treated diabetic and normal mice (Figure 28A & 28B). The activity of this enzyme increased significantly ($P<0.001$) on administration of crude terpenoid in the treated group indicating G6PD supplies hydrogen,which binds with $NADP^+$ and produces NADPH and thus facilitating the synthesis of fats from carbohydrates i.e., lipogensis, and finally the plasma glucose levels were decreased. Thus treatment with extracted crude terpenoid in diabetic treated group causes recovery of G6PD enzyme and glucose is directed mainly toward the pentose phosphate pathway for NADPH production.

In our investigation, the effects of terpenoid present in *Gymnadenia orchidis* Lindl and synthetic drug Metformin on pancreas, liver and kidney tissues of diabetes-treated animals were studied for histopathological changes to evaluate tissue damage which is responsible mainly for the changes in biochemical and physiological status.

Histological analysis of pancreas of normal control rats and mice revealed normal lobules, blood vessels, and arteries with normal islet number and morphology. But in the case of diabetic rats and mice degeneration of islets and acinar cells, karyolysis, and degranulation of β-cellswere observed mainly due to vascularization and infiltration of lymphocytes which causes in reduction of size and number of islets (Figure 17B & 24 B).The pancreatic architectures of terpenoid treated diabetic rats and mice showed resemblance closely to normal control group islet architectures. It suggested the protective role of root Salep (terpenoid) and crude terpenoid on the pancreas of STZ-induced treated diabetic rats and mice (DT) by repairing necrotic and fibrotic changes (Figure 17C & 24C). In the treated groups, pancreas also revealed increased number of β-cells and proliferated granular normal β-cells in size with more symmetrical vacuoles when compared to normal rats and mice. Here pancreatic tissue with lobules comprising acinar cells were lined by serous cells thus suggesting the efficacy of plant extracts reversing the degeneration of STZ induced damage in the islets of Langerhans. Thus dose of root Salep (200mg/kg b. wt.) containing terpenoid helped in the regeneration of the pancreatic islet cells in number and size (Figure 17C&24C). Diabetic animals treated with root Salep devoid of terpenoid (DWT) also showed degeneration in the islets structure strongly confirmed that absence of terpenoid alleviates the diabetic damages (Figure 17D). Near normal round shaped islets with reduced number of vacuoles and increased cellular density indicated the recovery of pancreas due to action of Metformin. Wider interlobular duct, regeneration of β-cells in the centre and α-cells at the periphery were also noted in Metformin treated group indicatingreduced necrosis (Figure 17 E& 24D).

Histopathological analysis of hepatic tissue of normal control rats and mice demonstrated intact architecture of central vein, normal portal triad and normal hepatocytes radiating from the central vein in form ribbon like structure (Figure 18A1, 19A2 and 25A). STZ induced diabetic rats and mice revealed proliferation of Kupffer cells (K), cytoplasmic

degeneration (Cd), infiltration of inflammatory cells, irregularity in nuclear size, pyknosis and karyolysis, degeneration of the portal veins (Pv), proliferation of bile duct (Bd), fibrosis of hepatic artery (Ha) and separation of endothelial cells due to damaging effects of STZ causing diabetes. This hepatic damage was mainly due to hepatocellular injury by STZ (Figures 18 B1 & 19 B2 and 25B). This diabetic control group showed marked reduction of glycogen granules in the hepatocytes (Figures 18 B1, 19 B2 & 25B). Treated diabetic group i.e. DT group (rats and mice) showed regeneration in the hepatocellular architectures with less infiltration of inflammatory cells in central vein, intact hepatocytes, less proliferation of Kupffer cells and reduced cytoplasmic degeneration etc. In other words we can say that these damages were being reversed by terpenoid containing root Salep in the diabetic treated group/ DT (Figures 18 C1, 19 C2 & 25 C). Diabetic animals treated with root Salep devoid of terpenoid (DWT) also showed damaged hepatic architecture.

Histopathological analysis of kidney of normal control group (rats and mice) revealed normal glomeruli with normal tubules (Figure20A&26A). But kidney of STZ induced diabetic rats and mice revealed degenerated structure of renal cortex and medulla with irregular distribution of cortex and medulla, renal structure abnormality, diffuse glomerular basement membrane thickening, expansion of mesangial cells and sclerosis of glomerulus (Figure 20B & 26B). In contrast the terpenoid treated animals (rats and mice) revealed regeneration in renal architecture comprising of normal tubule with improved vasculature and normal structure of glomeruli without any infiltration of lymphocytes (Figure 20 C&26C) strongly suggesting the ameliorative property of terpenoid of *Gymnadenia orchidis* Lindl in reversing the deleterious effect of diabetes. Metformin treated groups also showed regeneration in the renal architecture (Figure 20 E and 26 D). Diabetic animals treated with root Salep devoid of terpenoid (DWT) also strongly confirmed the fact that terpenoid plays a protective role in curbing the renal damages and in this group damages were prominent due to the absence of terpenoid in the root Salep (Figure 20D).

Conclusion

In this study scientific perceptions are gathered by observing the results accumulated from the pharmacological study of *Gymnadenia orchidis* Lindl. The conclusions obtained are emphasized here and the additional visions gathered in this scientific analysis are also explained.

The present study concluded that this medicinal plant viz. *Gymnadenia orchidis* Lindl is a promising source of various phytochemicals and may be efficient as antidiabetic agents in the management of diabetes. Root extracts of *Gymnadenia orchidis* Lindl have antioxidant, antihyperglycemic, antihyperlipidemic effect and can be used as alternative remedy for the treatment of diabetes mellitus. Identification of the plant by Dr. D. K. Agarwal and authentication by Dr. M. Gangopadhayay respectively in Botanical Survey of India, Sikkim Himalayan Regional Centre, Gangtok, India as *Gymnadenia orchidis* Lindl of the *Orchidaceae* family (Accession No.: 0046 dated 26.09.2014. V. No. SHRC - 5/02/2012 - Tech. - 195) also confirms this perennial herb as an orchid. Qualitative phytochemicals screening confirms the presence of important phytocontituents like flavonoids, alkaloids, terpenoids, steroids, cardiac glycosides, saponin, tannin, polyphenols, vitamin C, carbohydrate(s), protein(s) and free amino acid etc. The active compound terpenoid responsible for showing anti hyperglycemic activity was further confirmed by TLC. Characterization of the terpenoid by spectral analysis like UV-VIS spectroscopy, FTIR and Mass spectroscopy also validates the chance of presence of eugenol in the extracted terpenoid. Toxicity study revealed the nontoxic nature and protective activity of the root Salep without showing any mortality even at dose 1000mg/kg body weight. Standardised effective dose of root Salep i.e. 200 mg/kg bwt. showed the anti hyperglycemic activity in STZ-induced diabetic rats. Improved results of glycemic profiles like fasting blood glucose, plasma insulin, total haemoglobin and glycosylated haemoglobin (HbA1c) in treated diabetic

animals confirmed the insulin stimulatory and /or the insulin-mimetic properties of *Gymnadenia orchidis* Lindl root extracts. Potentiality of the anti hyperglycemic activity of root extracts containing terpenoid was due to the stimulation of pancreatic β-cells insulin secretion or increment of glucose uptake by muscle cells thus resulting in augmentation of glucose utilization by peripheral tissues either by promoting glucose uptake and metabolism, or by inhibiting hepatic gluconeogenesis in diabetic animals. Reduction in the activities of hepatic key enzymes such as Acid phosphatase, Alkaline Phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT) and reduction in levels of protein, urea, uric acid and creatinine in the treated diabetic animals strongly validate the safe, tissue-protective and renoprotective effects of the *Gymnadenia orchidis* Lindl root extracts. Improvement in the HDL level and reduction in the cholesterol, triglycerides and LDL levels of treated diabetic animals revealed the antihyperlipidemic activity of the *Gymnadenia orchidis* Lindl root extracts. *Gymnadenia orchidis* Lindl root extracts also helps in improving the glycogen content of liver in the experimental animals by moderating the activities of glycogen metabolizing enzymes such as glycogen synthase and glycogen phosphorylase thus resulting in effective glucose utilisation and improving insulin sensitivity. Activity of carbohydrate metabolising enzymes i.e. glucose-6-phosphate dehydrogenase also improves due to the regulatory role of *Gymnadenia orchidis* Lindl root extracts in the preservation of carbohydrate metabolism. The antioxidant defence capability of root extracts of *Gymnadenia orchidis* Lindl can be concluded from the results where increased activity of oxidative stress marker i.e. lipid peroxidation and decreased activities of enzymatic and non-enzymatic antioxidants like S.O.D, GPx and GSH were improved to near normalcy levels after treatment with *Gymnadenia orchidis* Lindl root extracts in the treated diabetic animals. Thus *Gymnadenia orchidis* Lindl has the capability to rejuvenate the antioxidant defence mechanism in the treated diabetic animals due to the presence of phytochemicals in the root

extract. The histopathological investigations revealed the substantial alterations in pancreas, liver and kidney of STZ - induced diabetic animals (rats &mice) where pancreatic sections with destroyed islets cells, liver tissue with fatty irregular shaped hepatocytes, inflammatory cells infiltrations in the central vein and kidney tissues with thickened glomerulus basement membrane, messangial capillary proliferation etc in diabetic animal. Modification of these pathological changes to near normal architecture in the treated diabetic animals comfirmed the fact that root extracts of *Gymnadenia orchidis* Lindl has the capability to repair the damages due to diabetes. Metformin treated animals also showed improvement in the histopathological changes.

Limitation

- Due to infrastructure unavailability in the laboratory, proper isolation and detection of the active components responsible for antidiabetic activity could not be carried out.
- As *Gymnadenia orchidis* Lindl is found in the Himalayan region, identification and collection of the species was difficult.
- Large scale extraction of the active compound was not possible because of the scarcity of the root sample.
- Our research work was restricted only on rats and mice model. Due to unavailability of proper fund in our laboratory we could not able to perform the investigation on rabbit model etc.

Future scope of the study

The future aspect of this investigation on antihyperglycemic activity of medicinal plant *Gynadenia orchidis* Lindl are as follows:

- To isolate, purify and identify the various active components of roots extracts of *Gymnadenia orchidis* Lindl which are responsible for the antihyperglycemic activity so that it can pave the way for establishing the drug as a promising antidiabetic agent for diabetes mellitus treatment.
- To find the structure of the active compounds by applying modern techniques like X-Ray Diffraction, Nuclear Magnetic Resonance, Circular Dichroism, Scanning Electron Microscopy and Atomic Force Microscopy etc.
- To investigate respective phytochemicals with their molecular mechanism and target(s) of action in combination with the conventional synthetic drugs.
- To explore the molecular mechanism particularly responsible for the antihyperglycemic effect and establishing its safety as a drug for proper clinical trials.
- To detect other mechanism of actions besides antihyperglycemic, antihyperlipidemic actitivity. The root extracts of *Gymnadenia orchidis* Lindl can be verified whether it is an insulin secretagogues or not; whether it is retarding hepatic glucose output or if it regulating carbohydrate metabolizing enzymes like hexokinase, glucose-6-phosphatase, lactic dehydrogenase etc.
- To perform *in vitro* study of anti-diabetic activity like alpha-glucosidase inhibition, DPPIV inhibition etc.
- To investigate the interaction like Drug-DNA interaction, Drug-Protein interaction.

CHAPTER 6
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Therapeutic Implications of *Gymnadenia Orchidis* Lindl Root Salep Against Induced-Diabetes

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ABSTRACT

Diabetes, the world largest metabolic disorder has become a serious threat to public health. The management of diabetes by synthetic drugs causes many unwanted complications. Hence this study was designed to explore the root Salep of *Gymnadenia orchidis* Lindl against type-2 diabetes to achieve a complications free herbal treatment for the disease. The Streptozotocin (STZ) induced-diabetic rats were supplemented with root Salep orally daily at an effective dose (200 mg/ g of body weight). The body weights and fasting blood glucose levels were measured periodically for 32 days. After treatment period, the animals were sacrificed and glycosylated haemoglobin, lipid profiles, antioxidant enzymes levels, liver function enzymes etc. were determined. Phytochemically determined terpenoids was extracted from the root and orally supplemented (4 mg/g body weight) to the induced-diabetic animals. Normalization of fasting blood glucose levels, significant ($P < 0.001$) decrement of glycosylated haemoglobin percentage, liver enzymes activities and increase body weights and anti-oxidants levels were noted for the Salep supplemented diabetic rats. Terpenoids played the key role in such observations. The root Salep of *Gymnadenia orchidis* Lindl or its terpenoids may be used as potentially herbal therapeutic agent for long term and effective solution against type-2 diabetes mellitus.

Keywords: Anti-oxidants, Diabetes, *Gymnadenia orchidis* Lindl, Lipid peroxidation, Toxicity.

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INTRODUCTION

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces. It is the fastest growing metabolic disorder that causes a serious threat to public health. The number of diabetic patients has risen from 108 million in 1980 to 422 million in 2014.[1] The global prevalence of diabetes among adults over 18 years of age has risen from 4.7% to 8.5% within this period. About 1.5 million deaths were caused directly by diabetes and another 2.2 million deaths were due to high blood glucose in 2012. Also WHO projects that diabetes will be the 7th leading cause of death within 2030.[2] In India, the number of people suffering from diabetes is about 35 million, which is a gross estimate as many people are unaware of whether they are carrying the disease or not.[3] These alarming figures show that diabetes could be more severe in India in the near future.

Diabetes is a major cause of blindness, kidney failure; heart attacks, stroke and lower limb amputation etc. Adults with diabetes have a 2-3-fold increased risk of heart attacks and strokes. [4] Combined with reduced blood flow, neuropathy (nerve damage) in the feet increases the chance of foot ulcers, infection and eventual need for limb amputation. Diabetic retinopathy is a vital cause of blindness, and occurs as a result of long-term accumulated damage to the small blood vessels in the retina. About 2.6% of global blindness can be attributed to diabetes.[5] Diabetes is among the leading causes of kidney failure.[6]

The general trend for the treatment of diabetes is to use synthetic drugs and insulin therapy. But synthetic drugs or insulin therapy for the management of diabetes mellitus causes numerous drawbacks, like insulin resistance, [7-8] anorexia nervosa, brain atrophy, fatty liver [9] and so on. Also this type of management for diabetes is not only causing many side effects but also very costly and furthermore, those synthetic drugs alone will not be able to fulfill the needs of the growing number of diabetic patients in the near future. Hence the alternative solutions to this problem need to be explored from abundance of natural medicinal plants which have shown the ability to control diabetes. [10-12] However, these medicinal plants are mostly used only locally and very little scientific work has been done on these life saving plants to identify their active compounds responsible for the management of blood glucose level.

One of such medicinal plants that could have the ability to reduce hyperglycemia is *Gymnadenia orchidis* Lindl. The plant belongs to the family *Orchidaceae* and is found in the Himalayan region from Pakistan to South–East Tibet at an altitude range of 2400 – 4000 m. This perennial herb has a tuberous root which is divided into 2 or 3 lobes. The roots of this plant when grinded and mixed

with water form a thick 'Salep' which is traditionally used by the people of Bhootia community to get some relief against diabetes.[13] Our study has established that the *Gymnadenia orchidis* Lindl, root Salep is a compatible, safe and complication free agent against type-2 diabetes whose terpenoids component plays the active role against the disease.

MATERIALS AND METHOD

Materials and chemicals

The fibrous roots of *Gymnadenia orchidis* Lindl was collected from the local market in Darjeeling, West Bengal, India. The freshly picked roots were immediately transferred into a container with dry ice and were brought to the laboratory and stored in a -20 ^{o}C refrigerator until used. Streptozotocin was purchased from Sigma Chemical Company Inc. USA. All other analytical grade chemicals were purchased from standard chemical companies. Glycosylated haemoglobin kit was provided by Biosystems, Barcelona, Spain. Cholesterol and Acid phosphatase (ACP) kits were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. Urea, Alkaline phosphatase (ALP), Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) kits were supplied by Piramal Healthcare Limited, Mumbai, India. Triglyceride and creatinine were measured by using the kits provided by Merckotest®, Merck, Goa, India.

Authentication of the plant and phytochemical analysis of the root Salep

The plant whose root used in the study was submitted to Botanical Survey of India, Sikkim Himalayan Regional Centre, Gangtok, India for phytochemical analysis. Dr. D. K. Agarwal identified the root and Dr. M. Gangopadhayay authenticated the plant as *Gymnadenia orchidis* Lindl of the Orchidaceae family (Accession No.: 0046 dated 26.09.2014. V. No. SHRC- 5/02/2012 - Tech. - 195).

The method described by Harbone (1973), [14] and also by Trease and Evans (1983), [15] was used for identification of different phytochemicals in the root Salep of *Gymnadenia orchidis* Lindl. Aqueous extract of the dried roots was made for three different concentrations (1 mg/ml, 10 mg/ml and 25 mg/ml) and analyzed in triplicate for each concentration in HPLC to get statistical average. The presence of flavonoids, alkaloids, terpenoids, steroids, cardiac glycosides, saponin, tannin, polyphenols, vitamin C, carbohydrate(s), protein(s) and free amino acid etc. were determined by the standard laboratory methods.

Toxicity study of the root Salep

Dust root powder was prepared from the tuberous root by grinding the dry roots in a mortar placed in ice bath and different concentrations of Salep as per requirement was freshly prepared with sterile deionized water to the powder root before use.

Adult female albino rats (30 in number) of Wistar strain having standard body weights (130 – 150 g) and age (4 – 5 months) were procured at a time from the animal housing facility of Jadavpur University. The animals were acclimatized under standard conditions of temperature and humidity with 12 h light/dark cycles. They were maintained in accordance with the guidelines of the rule of Institutional Animal Ethics Committee of Jadavpur University, Kolkata, India (constituted as per the "Gazette of India "notification part II Sec. 3 (ii) 17 of the Ministry of Environment & Forestry, Government of India, dated 8th September 1998 for the "Prevention to cruelty to animal 1968"). They were housed to polypropylene cages and fed normal protein diet (18% casein, 70% carbohydrate, 7% fat, 4 % salt mixture and 1% vitamin mixture).[16] The animals were divided into 6 groups having 5 rats in each for toxicological study as follows.

Group 1 – The animals in this group were maintained with normal protein diet but not supplemented with root Salep. This group served as control group.

Group 2 to Group 6 – The animals in these groups maintained with normal protein diet and received graded dose of root Salep containing 0.1 g, 0.2 g, 0.3 g, 0.5 g and 1 g of dry root powder/ kg body weight/day respectively for 10 days. After oral administration of the root Salep, the gross activity, posture and tone, eye ball movement, reaction and reflexes of the animals were noted in every day. On the 11th day the rats were sacrificed with mild anesthesia and the blood was collected from the heart. Liver function enzymes such as acid phosphatase (ACP), alkaline phosphatase (ALP), Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) and kidney function test (urea and creatinine) were determined from serum by using the supplied kits for toxicological analysis of the Salep treated animals.

Extraction of terpenoids from root Salep

A 40 g of root sample was taken and crushed in mortar and pestle and kept soaked in 200 ml of ethanol for 6-8 days. Then it was filtered in filter paper and the filtrate was taken in a conical flask. The ethanol was removed completely by heating the sample over water bath. The sample was suspended in 20 ml of distilled water and subsequently 100 ml of petroleum ether in a separating funnel and shaken vigorously for 1 min and kept for standing time about 10 min for separation of two layers. The expected terpenoids that came to the pet ether layer was eluted. Again some amount of pet ether was mixed with the rest of the solution, shaken vigorously and kept for some time until two layers separated. In this way by repeating the process 10-12 times, terpenoid was

eluted in the pet ether layer. The pet ether was removed from the sample by distillation process. Finally crude terpenoid obtained was kept in 4 ºC for further analysis. The presence of terpenoid was confirmed by thin layer chromatographic process with two known standards menthol and limoneme. Approximately 30 mg crude terpenoid was obtained from 40 g dry root.

Experimental design on diabetic rats by using root Salep

Female albino rats of Wistar strain (40 in number and having fasting blood sugar levels < 110 mg/dl) were procured similarly from animal housing facility. Out of 40 animals, 10 were taken in the control group. The rest of the animals were intraperitonialy injected with streptozotocin (STZ) (60 mg/kg body weight dissolved in 0.1M citrate buffer at pH 4.5) to induce diabetes (Daisy et al., 2006). The induced diabetes was confirmed by measuring the fasting blood glucose level after 3 days. Those induced animals were then clustered into two experimental groups (STZ-induced diabetic group and STZ-induced diabetic with Salep supplemented group) having 10 rats in each whose fasting blood sugar levels were > 150 mg/dl. The experiment was thus conducted on those 3 groups of rats as follows:

Control group – Animals having fasting blood glucose level < 110 mg/dl and not given any treatment.

STZ-induced diabetic group – Animals having blood glucose level > 150 mg/dl and were not supplemented with root Salep.

STZ-induced diabetic with Salep supplemented group – Animals in this group having blood glucose level > 150 mg/dl and supplemented with root Salep (200 mg powder root/ kg body weight for 32 days).

The blood glucose level in 12 hour fasting condition of each rat was measured by making a small incision at the tip of the tail by using digital Breeze2 glucometer supplied by Bayer Healthcare LLC, USA. This measurement was repeated in every 3 days till the completion of the experiment. Body weight and general health conditions were also monitored every day in the fasting state. After the treatment period (32 days) all the rats were sacrificed after mild anesthesia and the blood samples were collected from the heart and stored in normal BD Vacutainer and EDTA (5.4 mM/3ml blood) containing BD Vacutainer for further analysis. Serum was isolated from the blood stored in normal BD Vacutainer.

Biochemical analysis of serum

Glycosylated haemoglobin (Hb1Ac) was measured using the kit and procedure provided by Biosystems, Barcelona, Spain (Bisse and Abraham, 1985). SGOT and SGPT were determined by the kits supplied by Piramal Healthcare Limited, Mumbai, India. Total serum protein was

measured using the method given by Lowry et al. (1951). Total Cholesterol was measured using the kit provided by Accurex Biomedical Pvt. Ltd., Mumbai, India. Triglyceride was measured using kit provided by Merckotest®, Merck, Goa, India. Lipid peroxidation was determined from the Thiobarbuturic Acid test (TBA test) with modification by Kumar and Das. [17] Super oxide dismutase (SOD) activity was assayed by the method based on the reduction of nitroblue tetrazolium (NBT) to blue pharmazone by superoxides, produced phytochemically in the reaction system.[18] Reduced glutathione (GSH) was determined by using the method of Davila et al. [19] and glutathione peroxidase (GPx) was estimated by using the method of Levander et al. [20]

Experiments on diabetic rats by using extracted terpenoids

In this experiment, 5 groups of female albino rats of Wistar strain having 5 animals in each were taken and maintained as described earlier. Diabetes to the animals was induced by STZ. The groups were as follows:

Control – The fasting blood glucose levels of all the animals was < 110 mg/dl and no treatment was given.

Diabetic – The fasting blood glucose levels of all the animals was > 150 mg/dl and no treatment was given.

Terpenoid (+) – The fasting blood glucose levels of all the animals was > 150 mg/dl and terpenoid (4 mg/ kg bogy weight as a trial dose) extracted from the root of *Gymnadenia orchidis* Lindl was supplemented orally.

Terpenoid (-) – The fasting blood glucose levels of all the animals was > 150 mg/dl. After extraction of terpenoid from the powder root of *Gymnadenia orchidis* Lindl, the powder was washed repeatedly by deionized distilled water, air dried and the supplemented (200 mg/ kg body weight) to the diabetic animals orally.

Metformin – The fasting blood glucose levels of all the animals was > 150 mg/dl and Metformin (100 mg/ kg bogy weight) was supplemented orally.

Different groups of animals were treated with drugs twice a day with 8 h interval for nine doses of 5 days schedule as described by Chakrabarti et al. [21] Fasting blood glucose levels and liver glycogen estimation were done on the sacrificed animals after the treatment.

Statistical analysis

All experimental set up was repeated twice and data (n = 10 for toxicological test and n = 20 for biochemical analysis) were averaged and given mean ± SD. The statistical analysis of the data obtained from control, diabetic and aqueous roots Salep of *Gymnadenia orchidis Lindl* supplemented groups was performed by one way analysis of variance (ANOVA). The significant

levels of the observed data were determined at $P < 0.01$ (significant) and $P < 0.001$ (more or highly significant).

RESULTS AND DISCUSSION

Toxicity test

Toxicity results showed that the Salep did not produce any harmful effect on the animals as all the animals were remained alive and healthy even after 10 days of dosing. The body weight of the rats remained steady throughout the toxicity study (Table 1). The overall behaviors of the animals noted regularly were found to be normal after the oral supplementation of the root Salep. Fasting blood glucose levels were found to be the minimum for the Salep (200 mg/kg body wt.) supplemented animal in Group 3 (Table 1). It was observed that there was a trend of increase ACP (Fig.1A) levels (within normal range) in serum of Salep supplemented rats in comparison to control rats. The levels of ALP (Fig. 1B), SGOT (Fig. 1C) and SGPT (Fig. 1D) were initially decreased and then increased (within normal range) with the increased dose of Salep supplementation compared to the normal group. Urea and creatinine (Fig. 2) both levels were produced similar results as seen in SGOT and SGPT. The most normal levels of blood glucose, liver function enzymes and kidney function parameters were observed to the animals belonging in the Group 3 suggesting that 200 mg/kg body weight dose of the Salep could be most suitable for the study of the action against type 2 diabetes mellitus. This was taken as the effective dose of the Salep for treatment against diabetes.

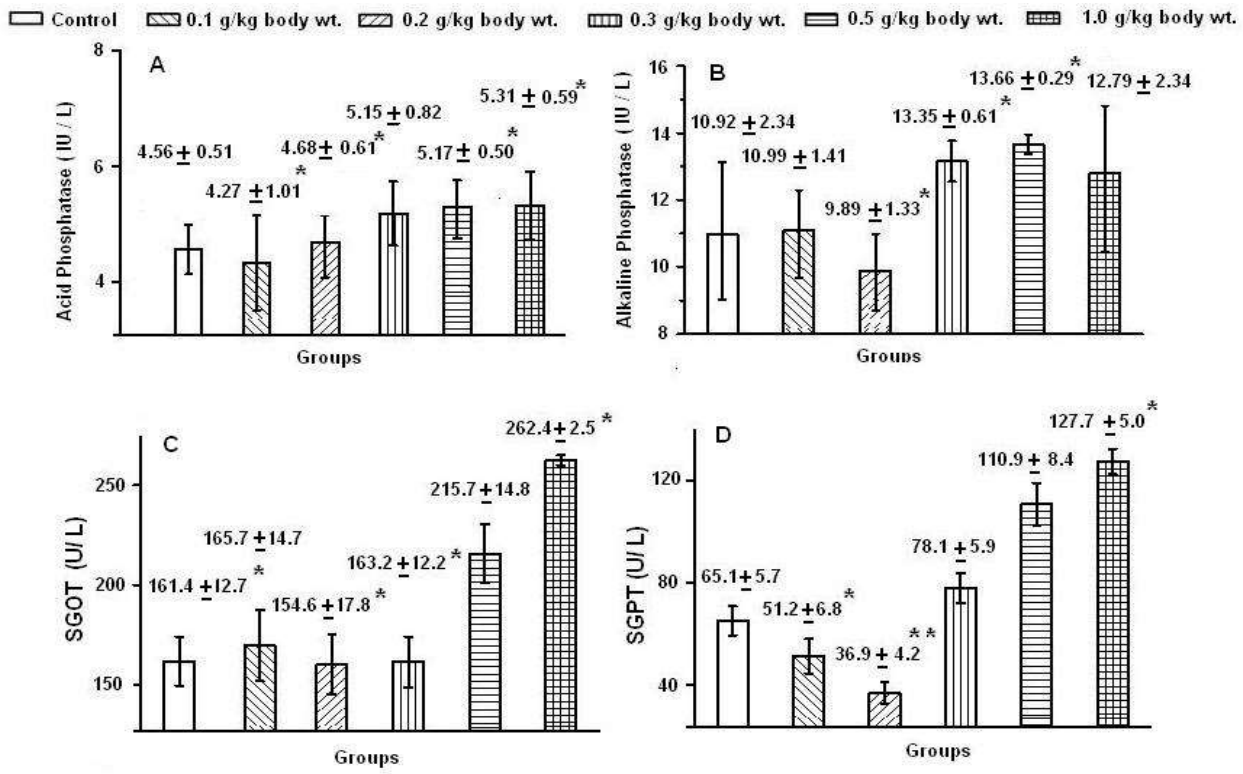

Figure 1: Effect of root Salep at different doses on hepatic enzymes of rat serum A: ACP; B: ALP; C: SGOT; D: SGPT

Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant ($P < 0.01$) and ** implies more significant ($P < 0.001$).

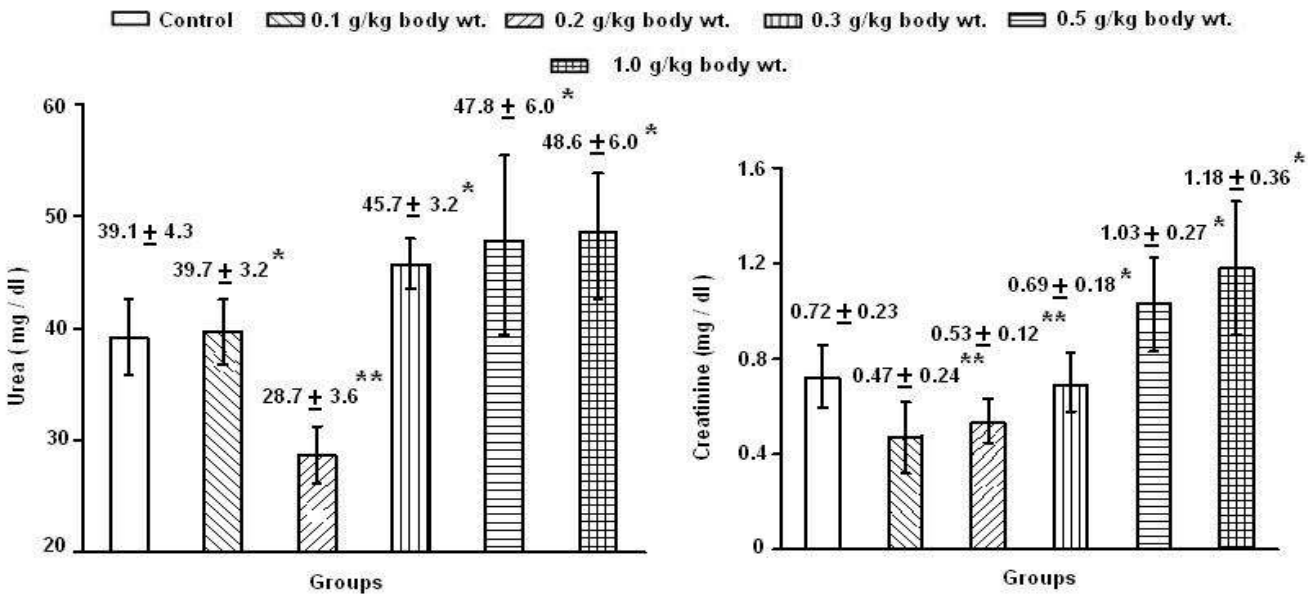

Figure 2: Effect of root Salep at different doses on renal parameters Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant ($P < 0.01$) and ** implies more significant ($P < 0.001$).

Phytochemical analysis

Phytochemical analysis showed that the root Salep of *Gymnadenia orchidis Lindl* contained adequate amount of terpenoids, carbohydrates and proteins and trace amount of tannins, polyphenols, steroids and vitamin C (Table 2). Flavonoids, alkaloids, saponin and free amino acids were not detected in the root Salep.

Effect of the Salep on Diabetic rats

The blood glucose level of the Salep treated (200 mg/kg body wt.) diabetic rats was restored to normal levels after 3 doses of Salep (Fig. 3A). It was noted from Fig. 3B that the percentage of Glycosylated haemoglobin was increased (about 30.5%) in STZ induced diabetic rats compared to control rats. More significant ($P < 0.001$) reduction of Glycosylated haemoglobin percentage was observed on the Salep supplemented diabetic group (about 26.8% lower than that of non-treated diabetic group). The body weights of the induced diabetic rats were decreased compare to the control rats due to the induction of the disease which were restored significantly ($P<0.01$) when the animals received Salep supplementation (Data not supplied).

The activity of liver function enzymes were elevated in case of diabetic rats. SGOT was increased by 69.6% (Fig. 4A) and SGPT was increased by 23.1% (Fig. 4B) with respect to their control rats. The levels of SGOT (about 19.8%) and SGPT (about 32.1%) both were decreased significantly ($P < 0.01$) to the Salep supplemented diabetic rats as compared to the non- treated diabetic rats. Diabetes induction increased both the cholesterol (about 26.6%) and triglycerides (12.4%) levels compared to the control rats (Fig. 4C). Salep supplementation to the diabetic rats reduced the total cholesterol level (about 18.8%) significantly ($P<0.01$) and triglycerides level (about 23.2%) more significantly ($P < 0.001$) compared to the diabetic induced rats. Lipid peroxidation also more significantly ($P < 0.001$) decreased to the animals belonging in the Salep supplemented group with respect to the animals of the diabetic induced group (Fig. 4D). It was also observed that the levels of antioxidant enzymes such as SOD, GSH and GP_x were lowered for diabetic rats but restored by the treatment of root Salep (Fig. 5).

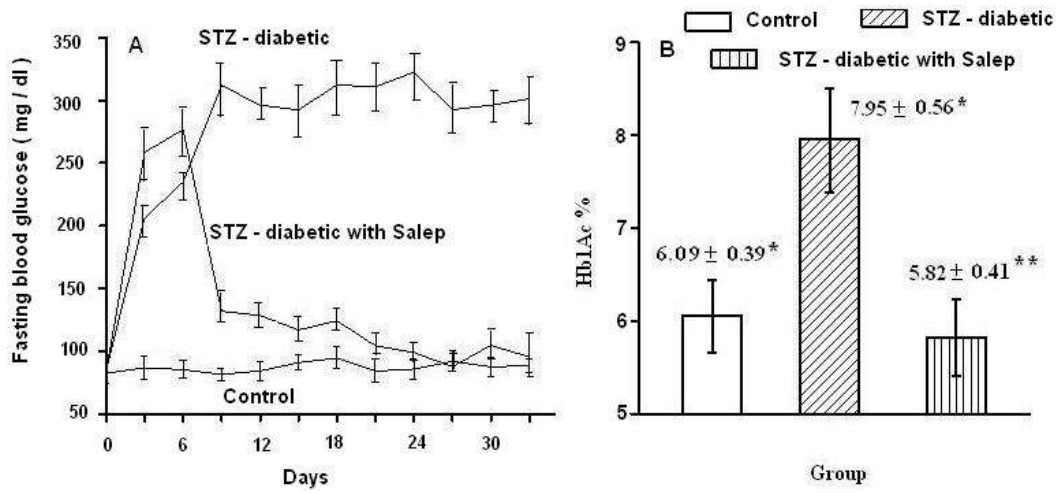

Figure 3: Effect of root Salep (0.2 g/kg body wt.) on diabetic rat A: Blood glucose; B: Hb1A$_c$

Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant

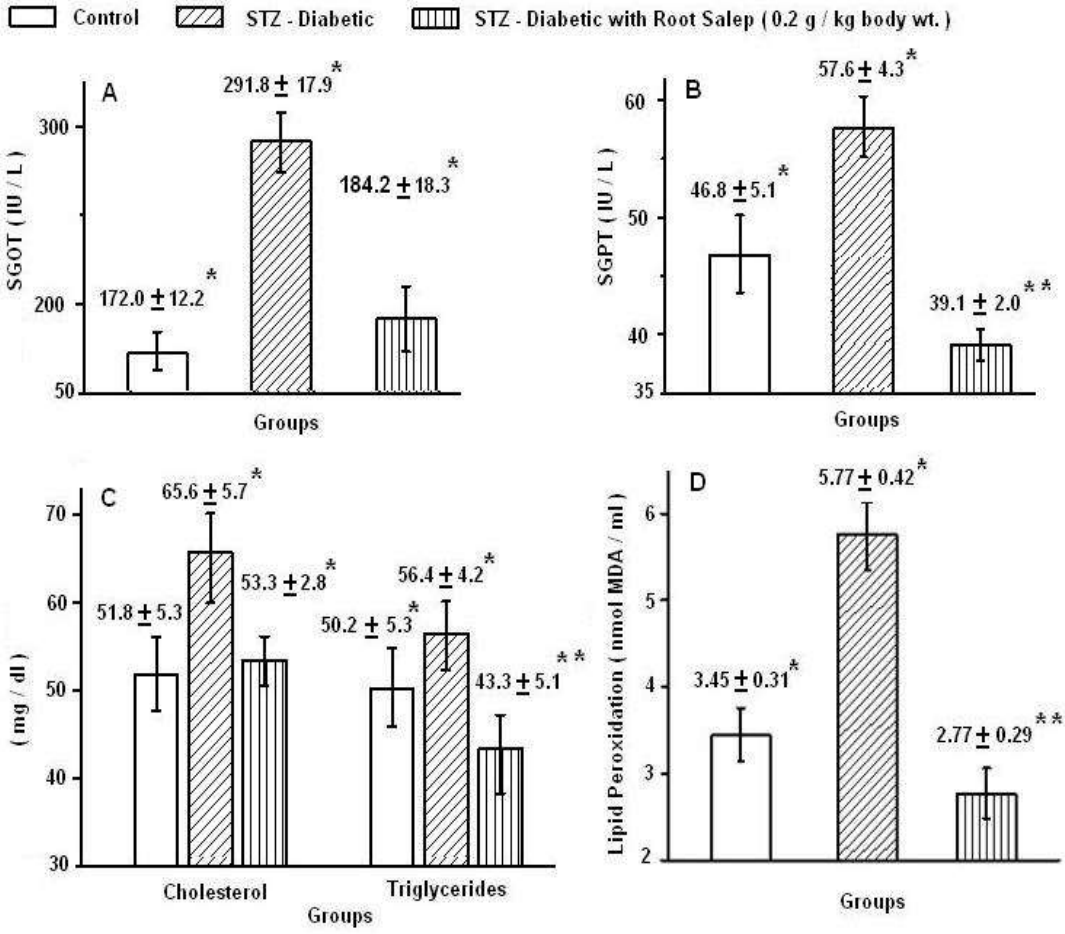

Figure 4: Effect of root Salep (0.2 g/kg body wt.) on diabetic rat A: SGOT; B: SGPT; C: Lipid levels; D: Lipid peroxidation

Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant ($P < 0.01$) and ** implies more significant ($P < 0.001$)

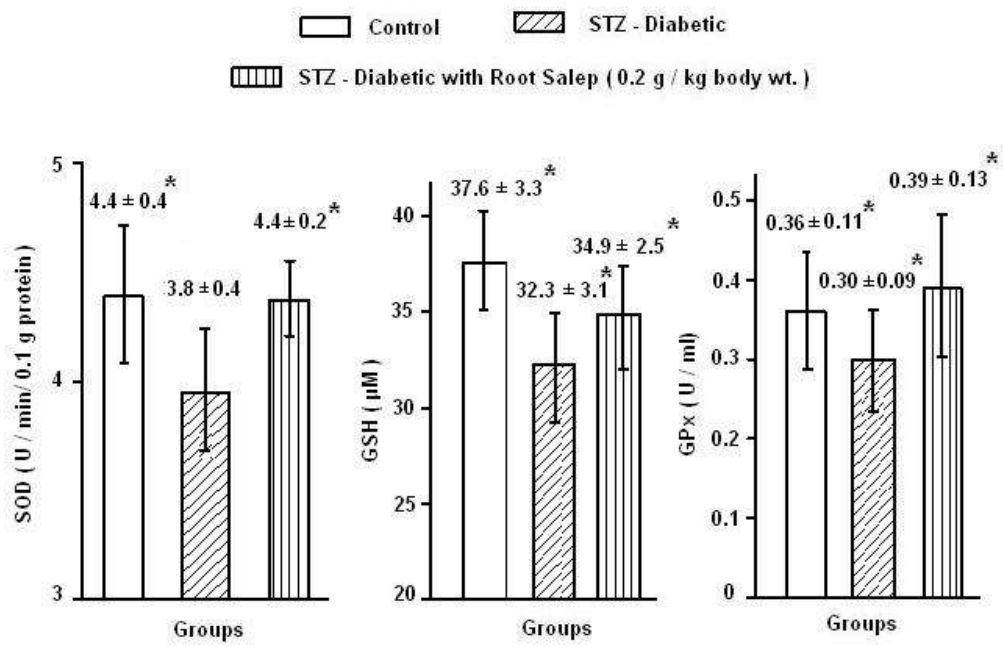

Figure 5:Effect of root Salep (0.2 g/kg body wt.) on antioxidant enzymes of diabetic rat Data were averaged and presented as mean ± S.D. (N = 10) where, * means significant (P < 0.01) and ** implies more significant (P < 0.001).

Effect of terpenoid on diabetic rats

The blood glucose levels of the STZ induced diabetic animals treated with terpenoid (extracted from the root) were found to be normal (< 110 mg / dl) as seen from Table 3. There was no effect on blood glucose level of the diabetic animals when supplemented root Salep devoid of terpenoid. Liver glycogen content of the diabetic animals also increased with the terpenoid supplementation compared to the control group but remained almost same when terpenoid lacking Salep treated diabetic animals (Fig. 6).

Table 3: Fasting blood glucose levels of the rat under different treatment

Group	Glucose levels	
	1st day	5th day
Control	98.2 ± 7.7*	102.2 ± 16.7*
Diabetic	196.2± 23.0*	206.0 ± 50.4*
Diabetic +Terpenoid	180.4 ± 25.3*	108.4 ± 9.7**
Diabetic – Terpenoid	215.0 ±25.5*	264.2 ± 47.2*
Diabetic + Metformin	157.8 ± 6.4*	125.0 ± 9.7*

n = 10 for each group. All values are expressed as the Mean ± S.D. with Statistical significance as P < 0.01 (significant); P < 0.001 (more significant)

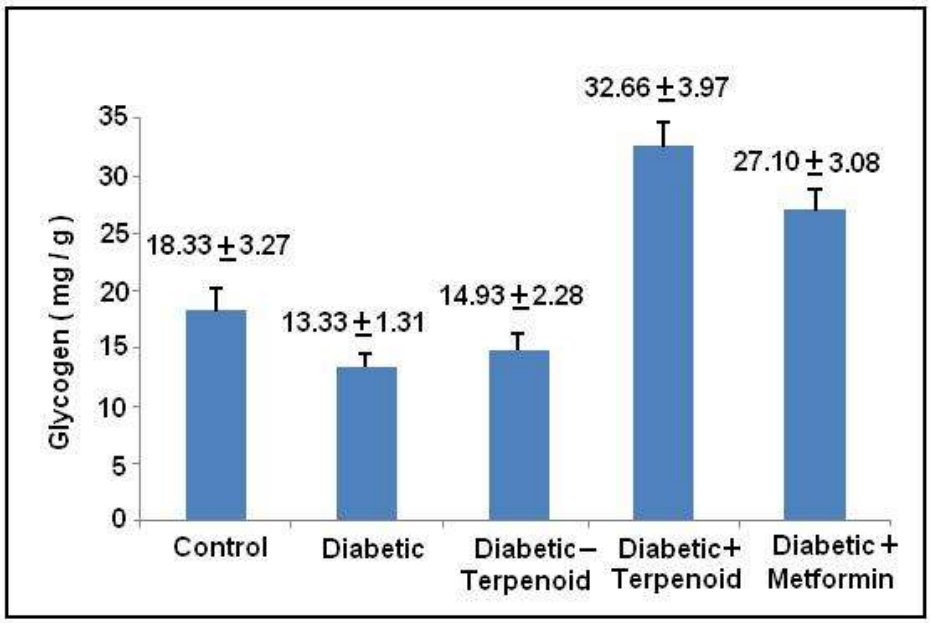

Figure 6: Liver glycogen content of diabetic rat under different treatment conditions

Data were averaged and presented as mean ± S.D. (N = 10)

DISCUSSION

The perennial herb *Gymnadenia orchidis* Lindl is mostly used in locally for the treatment of diabetes but no scientific study was done before on this life saving plant in this respect. This study furnishes to establish the protective role of the tuberous root of *Gymnadenia orchidis* Lindl against diabetes through proper scientific approaches. Toxicological investigation on rats has revealed that the Salep is toxicologically safe and can be used in edible form. The tested biochemical parameters (like ACP, ALP, GPT, GOT, Urea and Creatinine etc.) of the Salep supplemented rats at higher doses (> 200 mg powder root) were found to be slightly higher values with respect to control level but still the values were within the accepted range suggesting that the root Salep did not produce any harmful effects on the normal functions of liver (Fig. 1), kidney (Fig. 2) and general health conditions (Table 1). It was also evident from the studies that the most effective dose of Salep for the treatment of diabetic rats was 200 mg/kg body weight per day.

Table 1: Body weight and fasting blood glucose levels of the rat under toxicity test

Body weight (g)			**Blood glucose (mg/dl)**	
Group	**0 day**	**10 days**	**0 day**	**10 days**
Group 1	153 ± 6.6	158 ± 4.1	94.1 ± 7.8	**99.9 ± 9.7**
Group 2	160 ± 7.4	165 ± 7.8	99.0 ± 8.6	95.7 ± 10.3
Group 3	160 ± 8.9	163 ± 9.1	95.6 ± 10.1	**88.6 ± 5.7**
Group 4	161 ± 8.6	164 ± 9.8	94.7 ± 8.8	90.6 ±10.4
Group 5	157 ± 7.2	156 ± 7.1	92.6 ± 11.2	91.7 ± 7.9
Group 6	150 ± 6.3	152 ± 6.5	95.5 ± 9.8	**93.7 ± 8.9**

n = 10 for each group. All values are expressed as the Mean ± S.D

Our experimental results showed that the fasting blood glucose level was significantly increased and body weight was significantly decreased of the rats when the animals received a single dose injection of STZ. Single dose injection of STZ causes severe type 2 diabetes to the animals. [22] It is generally assumed that STZ is toxic to the insulin-producing β cells which is taken up via the cell membrane GLUT2 glucose transporter and causes DNA alkylation and eventually causes β cell death.[23-25] Our findings clearly showed that Salep supplementation to the diabetic induced rats normalized the fasting blood glucose levels (Fig. 3A) and increased the body weight (data not supplied) in comparison to the normal group. Glycosylated haemoglobin (HbA_{1c}) is a well known marker for confirmation of diabetes to the patients. Higher levels of HbA_{1c} are found in people with persistently elevated blood glucose, as in diabetes mellitus. The International Diabetes Federation and American College of Endocrinology recommend HbA_{1c} values below 6.5% is the reference range for non diabetic patient, while American Diabetes Association recommends that the HbA_{1c} be below 7.0% for most patients. [25] Our findings showed that the HbA_{1c} percentage of STZ treated induced diabetic rats were quite higher (7.95) than that of the control rats (6.09) as seen from Fig. 3B. The percentage of HbA_{1c} was more significantly ($P < 0.001$) reduced in Salep supplemented induced diabetic rats (5.82) confirming the protective role of the herb *Gymnadenia orchidis* Lindl against type 2 diabetes.

Diabetes caused dysfunction as well as damage of the liver cells, [26] due to which the liver function enzymes SGOT and SGPT levels were increased in STZ treated induced diabetic rats. Oral supplementation of the root Salep reduced significantly ($P < 0.01$) the levels of SGOT and SGPT levels as observed in experimental observations (Fig. 4A & 4B). Diabetes also causes disorder in carbohydrate metabolism that leads to disorder in lipid metabolism because carbohydrates and lipid metabolism are interrelated to each other.[27] Diabetes thus increased the concentrations of cholesterol and triglycerides levels (Fig. 4C) within the blood. Increment of serum lipid peroxidation (Fig. 4D) associated with diabetic induced rats was in agreement with the earlier findings. [27, 28] Lowered levels of cholesterol, triglyceride and lipid peroxidation of the Salep supplemented animals suggested that the lipid profile was very well regulated by the *Gymnadenia orchidis* Lindl root Salep.

In diabetes mellitus, increased formation of reactive oxygen species (ROS) due to high level of glucose in both blood plasma and tissues creates oxidative stress that damages the tissues. [29] Enzymatic antioxidants like SOD, GSH and GPx constitute a mutually supportive team of defense against ROS that were found to decrease in diabetic induced condition (Fig. 5). SOD, the

mitochondrial enzyme and usually found in plasma membrane, is a ubiquitous enzyme and protects aerobic cells against ROS.[30] GP_x is a seleno enzyme that catalyzes the reaction of hydroperoxides with GSH to form glutathione disulfide (GSSG) and the reduced product of the hydroperoxides.[31] Depletion in the activities of SOD, GSH and GPx in the serum of diabetic induced rats might be due to the increased utilization of these antioxidants to counter lipid peroxidation. Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. Glutathione reacts directly with ROS and electrophilic metabolites, for several enzymes.[30] Hepatic GSH plays a crucial role in both scavenging ROS and detoxification of drugs.[31] Thus, a decrease in GSH activity (Fig. 5) not only impairs cell defense against diabetes, but also results in enhanced oxidative stress and tissue damage. The root Salep of *Gymnadenia orchidis* Lindl increased the activity of SOD, GSH ($P < 0.01$) and GPx ($P < 0.01$) of diabetic induced rats and therefore played an important role in cellular defense against type 2 diabetes.

Phytochemical analysis showed that the root Salep contained various amount of terpenoids, tannins, polyphenols, vitamin-c, steroids and proteins (Table 2). The effects of dehydroabietic acid (DAA), a diterpene, on glucose and lipid metabolism were examined using obese diabetic KK-Ay mice by Kang et al.[32] They have shown that DAA treatment decreased not only plasma glucose and insulin levels but also plasma triglyceride (TG) and hepatic TG levels. Nazaruk and Borzym-Kluczyk,[33] have shown that triterpenes involved in glucose metabolism, prevent the development of insulin resistance and normalize plasma glucose and insulin levels. Santos et al.[34] demonstrated the antihyperglycemic and hypolipidemic effects of α,β-amyrin, a triterpenoid mixture from *Protium heptaphyllum* in mice.

Table 2: Photochemical compositions of the root Salep

Phytochemical	Present (+) / Absent (--)
Flavonoids	--
Alkaloids	--
Terpenoids	++
Steroids	+
Cardiac glycosides	+
Tannins	+
Saponins	--
Polyphenols	+
Vitamin C	+
Carbohydrates	++
Proteins	++
Free amino acids	--

++ Adequately present

The decrease of blood glucose and lipid profile levels and increase of liver glycogen content (Fig. 6) of the Salep supplemented rats may be thus explained on the basis of terpenoids present in the root Salep of *Gymnadenia orchidis* Lindl. The antioxidant potency of polyphenols,[35] vitamin C and tannins, [36] present in the root Salep may also take some definite role to combat against diabetes. The structural and functional properties of these compound could be lost during extraction of terpenoids from the root for which the therapeutic effect of those components could not be observed in our results.

CONCLUSION

Our results thus suggested that the root Salep could be an effective herbal therapeutic measure in controlling the blood glucose and regulating normal metabolism for patients having type 2 diabetes mellitus. Terpenoid present in the root Salep should have some definite role to counter the effect of diabetes on rats. Further studies will enable us for identification of highly effective key component(s) present in the complication free *Gymnadenia orchidis* Lindl for clinical trials to prevent or treat the life threatening diabetes disease over a long period of time.

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