Preclinical Evaluation and Molecular Mechanism of Flavonoid Enriched Fraction of *Campylandra aurantiaca* on Type-2 Diabetic Rat

Thesis submitted by

Mainak Chakraborty

Doctor of Philosophy (Pharmacy)

Division of Pharmacology

Department of Pharmaceutical Technology

Faculty Council of Engineering & Technology

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1. **Title of the thesis:** Preclinical Evaluation and Molecular Mechanism of Flavonoid Enriched Fraction of *Campylandra aurantiaca* on Type-2 Diabetic Rat2.

2. **Name, Designation & Institution of the Supervisor/s**

I**. Dr. Pallab Kanti Haldar**, Ph.D, F.I.C

Associate Professor,

Department of Pharmaceutical Technology,

Jadavpur University, Kolkata-700 032

&

II. **Dr. Asis Bala**, Ph.D.

Fellow (Post-Doctoral Research)

Department of Pharmacology,

Faculty of Health Science

University of the Free State

Bloemfontein - 9300, SA

3. List of Publications with first authorship:

1. **Chakraborty M,** Bala A, Haldar PK. Flavonoid enriched fraction of *Campylandra aurantiaca* attenuates carbon tetrachloride induced oxidative DNA damage in mouse peritoneal macrophages in animal model. Current Drug Discovery Technologies. 2017; DOI: 10.2174/1570163814666170504115137.

2. **Chakraborty M,** Bala A, Bhattacharya S, Haldar PK. 2018.Hypoglycemic effect of ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome on high fat diet and low dose streptozotocin induced diabetic rats. Pharmacognosy Magazine. 2019; DOI: 10.4103/pm.pm_191_17

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CERTIFICATE FROM THE SUPERVISOR/S

*This is to certify that the thesis entitled "**Preclinical Evaluation and Molecular Mechanism of Flavonoid Enriched Fraction of Campylandra aurantiaca on Type-2 Diabetic Rat**" submitted by Shri Mainak Chakraborty, who got his name registered on 16-12-2013 for the award of Ph.D. (Pharmacy) degree of Jadavpur University is absolutely based upon his own work under the supervisions of Dr. Pallab Kanti Haldar and Dr. Asis Balaand that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.*

1. ----------
Signature of the Supervisor and date with Office seal

2. ----------
Signature of the Supervisor and date with Office seal

Dedicated

to

My Family

&

My Guide

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Date:

Place: Jadavpur University *Mr. Mainak Chakraborty*

Preface

The present thesis entitled "***Preclinical Evaluation and Molecular Mechanism of Flavonoid Enriched Fraction of Campylandra aurantiaca on Type-2 Diabetic Rat***" comprises the work done by the author in Division of Pharmacology, Department of Pharmaceutical Technology, Jadavpur University, Kolkata for the degree of Doctor of Philosophy in Pharmacy.

To the existence of human civilization History of medicine dates back practically. Historically and scientifically, the majority of new drugs have been generated from natural products (secondary metabolites) and from compounds derived from natural products.

Humans have long history to used naturally occurring substances for medical purposes. Most cultures plants play an important role in medicinal field. Modernization of the science of chemistry at the beginning of the 19th century, plants began to be examined more closely to understand why they were medically useful.

Many of the currently available drugs have been derived directly or indirectly from plants sources. If we look into the relationship plants use as medicine, it can be traced back to the ancient days and they are as old as mankind itself. Natural product are historically been the major source of pharmaceutical agents which also have an important role in the development of modern medicinal system.

Traditional medicines (TMs) make use of natural products and are of getting importance day by day. Some common forms of medicine are traditional Chinese medicine (TCM), Ayurveda, Kampo, traditional Korean medicine (TKM), and Unani employ natural products and have been practiced all over the world for hundreds or even thousands of years, and they have blossomed into orderly-regulated systems of medicine. In their various forms, they may have certain defects, but they are still a valuable repository of human knowledge.

Nature stands as an inexhaustible source of novel chemotypes and pharmacophores, and has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs find their origin in natural products.

In the area of drug discovery Natural products, have played an invaluable role process. Recently, there has been a renewed interest in natural products research due to the failure of alternative

drug discovery methods to deliver many lead compounds in key therapeutic areas such as immunosuppressant, anti-infective, and metabolic diseases. However, continuing improvements in natural products research are needed to continue to be competitive with other drug discovery methods, and also to keep pace with the on going changes in the drug discovery process

Natural products have provided a important role to the pharmaceutical industry over the past half century, in the areas of infectious diseases and oncology have benefited much from numerous drug classes derived from the natural form and as templates for synthetic modification. About 40 new drugs launched on the market between 2000 and 2010, originating from terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates are reported (Brahmachari).

There is an urge to find out an efficient and economic way to manage diabetes and its complications. The scientists are coming up with new synthetic molecules, but they are having a range of limitations including their side effects and the total treatment cost. On this backdrop, the use of natural products is gaining popularity among the people(Kulkarni, 2014).On the basis of importance of herbal research, one plant have been selected which is well used traditionally used for various diseases and disorders in Sikkkim and Darjelling.

In the present study leave of *Campylandra aurantiaca* was selected for fractanation, pharmacological evaluation and molecular mechanism of antidiabetic activity has been done with highfat diet and low dose streptozotocin induced diabetic model.

Before the pharmacological evaluation, LD_{50} of extracts has been determined, to fix the dose levels for pharmacological activities. The plant fraction was also evaluated for its antidiabetic activity with the induction of diabetes through high fat diet and low dose streptozotocin.. Finally, antidiabetic activity of flavonoid enriched fraction of Campylandra aurantiaca was evaluated. Thus the thesis covered the above mentioned studies in a logical sequence with related references annexed to each chapter. In conclusion the detailed study has been linked up in a manner to justify the relation of the work to establish the antidiabetic actions especially in the induction through high fat diet and low dose streptozotocin induced diabetic model..

Mr. Mainak Chakraborty

Contents

CHAPTER-1

INTRODUCTION

Introduction

Diabetes

Diabetes mellitus, a metabolic disorder in which a person has high blood sugar, has become a matter of serious challenge because of its worldwide prevalence. This may be because of the body's inability to produce enough insulin, or because of lesser response of the cells to insulin that is being produced. Diabetes mellitus are of different types based on the causes. The majority of type 1 diabetes (IDDM) is related to immunity, where β cells are destroyed by T-cell mediated autoimmune attack. Type 2 diabetes (non-insulin-dependent diabetes mellitus or NIDDM) occurs from insulin resistance, and sometimes involves deficiency in insulin production. Gestational diabetes is another type which is found during pregnancy. This may lead to development of type 2 diabetes mellitus (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1997).

Epidemiology It is estimated that 366 million people had DM in 2011; by 2030 this would have risen to 552 million. The number of people with type 2 DM is increasing in every country with 80% of people with DM living in low- and middle-income countries. DM caused 4.6 million deaths in 2011. It is estimated that 439 million people would have type 2 DM by the year 2030. The incidence of type 2 DM varies substantially from one geographical region to the other as a result of environmental and lifestyle risk factors (Olokoba *et al*., 2012).

Several new epidemiological characteristics were found in relationship with T2DM. Firstly, diabetes keeps a steady increase in developed countries, such as United States and Japan. And it is worthy of note that T2DM has become a serious issue at an alarming rate in developing countries. It is predicted that T2DM will continue to increase in the next twenty years, and more than 70% of the patients will appear in developing countries, with the majority of them being 45-

64 years old. Even today, seven out of top ten countries with the largest number of diabetes patients are lower middle-income countries, including India, China, Russia, Brazil, Pakistan, Indonesia, and Bangladesh, among which the prevalence rates are 12.1% and 9.7% in India and China, respectively. Secondly, although advancing age is a risk factor for T2DM, rising rates of childhood obesity have resulted in T2DM becoming more common in children, teenagers and adolescents, which is a serious emerging of the epidemic and a new public health problem of significant proportions (Wu *et al*.,2014).

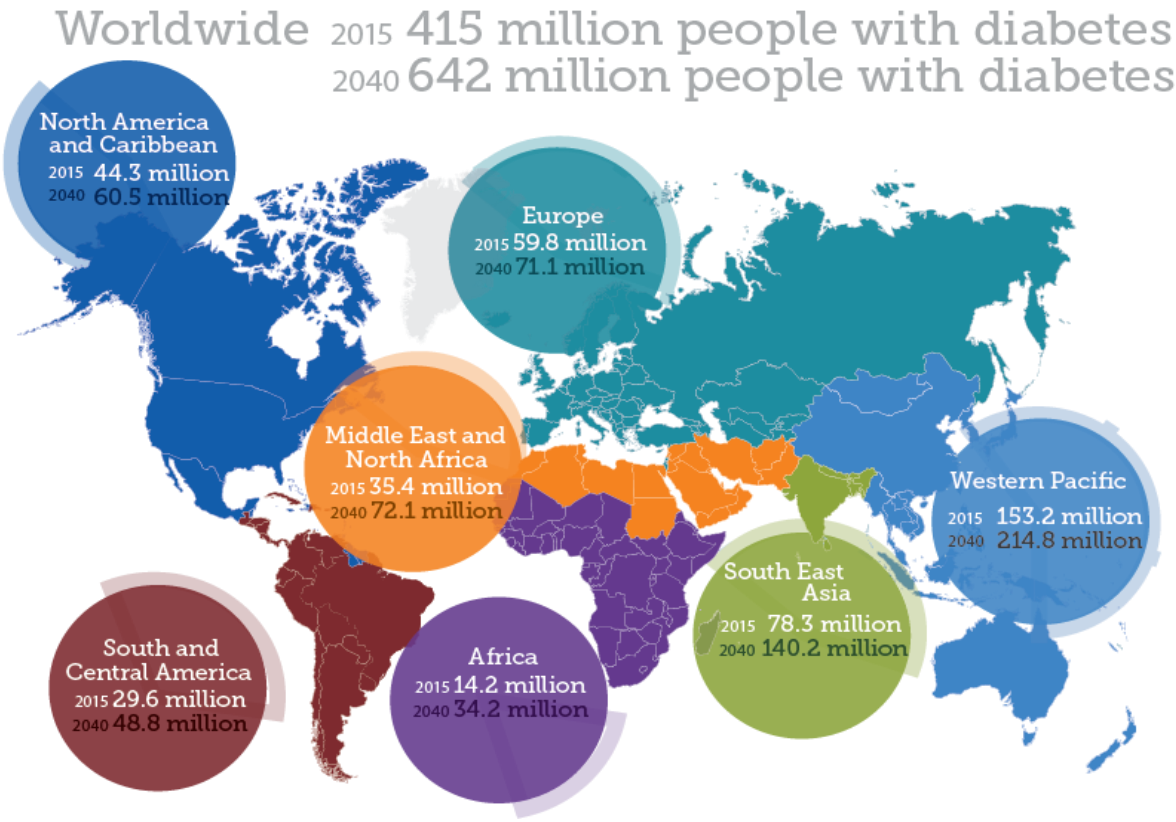


Table 1: List of countries with the highest numbers of estimated cases of diabetes for 2000 and 2030 (Wild *et al*., 2030).

Ranking	Country	People with Diabetes (2000) millions	Country	Estimated People with Diabetes(2030)
1	**India**	31.7	**India**	79.4
2	**China**	20.8	**China**	42.3
3	**United States**	17.7	**United States**	30.3
4	**Indonesia**	8.4	**Indonesia**	21.3
5	**Japan**	6.8	**Pakistan**	13.9
6	**Pakistan**	5.2	**Brazil**	11.3
7	**Russia**	4.6	**Bangladesh**	11.1
8	**Brazil**	4.6	**Japan**	8.9
9	**Italy**	4.3	**Philippines**	7.8
10	**Bangladesh**	3.2	**Egypt**	6.7

Role of pancreas in diabetes:

The pancreas is located in the upper abdomen behind the stomach. It's a most important part of the gastrointestinal system which are mainly responsible for secretes digestive enzymes into the intestine, and also an endocrine organ that makes and secretes hormones into the blood to control energy metabolism and storage throughout the body (Longnecker, 2014). Based on the function of pancreas it is divided into two categories; namely exocrine pancreas; the portion of the pancreas that makes and secretes digestive enzymes into the duodenum. This includes acinar and duct cells with associated connective tissue, vessels, and nerves. The exocrine components

comprise more than 95% of the pancreatic mass and endocrine pancreas; the portions of the pancreas (the islets) that make and secrete insulin, glucagon, somatostatin and pancreatic polypeptide into the blood. Islets comprise 1-2% of pancreatic mass (Longnecker, 2014).

Exocrine gland is made up of ***pancreatic acinar cells*** and ***duct cells*** and produce digestive enzymes and sodium bicarbonate, respectively; and the second part **endocrine gland** which made up of ***four islet cells***, namely *α-, β-, delta-, PP-*, and *ipsilon- cells* that produce glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin respectively. **Exocrine pancreas is made-up of** >80 % by volume and responsible for secrete digestive enzymes for our normal digestion, absorption and assimilation of nutrients, the **endocrine pancreas made up** <2% by volume and responsible for secrete islet peptide hormones for the maintenance of our glucose homeostasis and maintained the normal physiological function of body. The pancreatic functions are finely regulated by neurocrine, endocrine, paracrine and/or intracrine mechanisms. Thus, dysregulation of these pathways should have significant impacts on our health and disease (Chang and Leung, 2014).

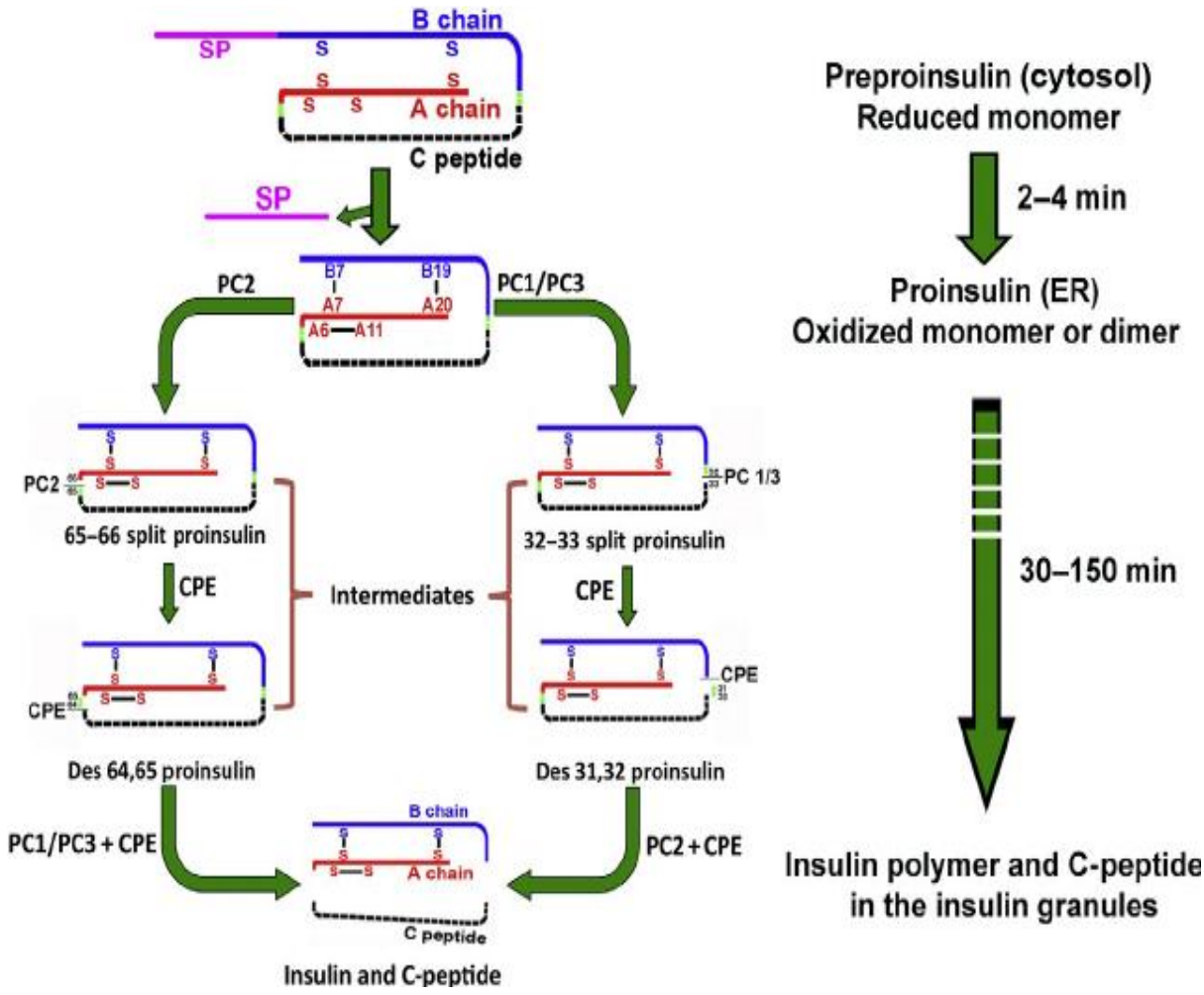
Insulin and its structure:

Insulin is a peptide hormone secreted from β cells of the pancreatic islets of Langerhans and maintains the normal blood glucose levels by facilitating cellular glucose uptake, regulating carbohydrate, lipid and protein metabolism and promoting cell division and growth through its mitogenic effects. Pancreas is the body's production house of insulin, and the hormone is secreted by its β cells, primarily in response to glucose (Wilcox, 2005).

Structure of Insulin Like most of the other hormones, in nature insulin is a protein consist of 2 polypeptide chains A (with 21 amino acid residues) and B (with 30 amino acid residues). Chains A and B are linked by disulphide bridges. In addition A-chain contains an intra-chain disulphide

bridge linking residue 6 and 11. C-chain, which connects A and B chains is liberated along with insulin after breakdown of proinsulin. Insulin monomers aggregate to form dimers and hexamers. Zn hexamer is composed of three insulin dimmers associated in threefold symmetrical pattern (Joshi *et al*., 2007).

Biosynthesis of Insulin:



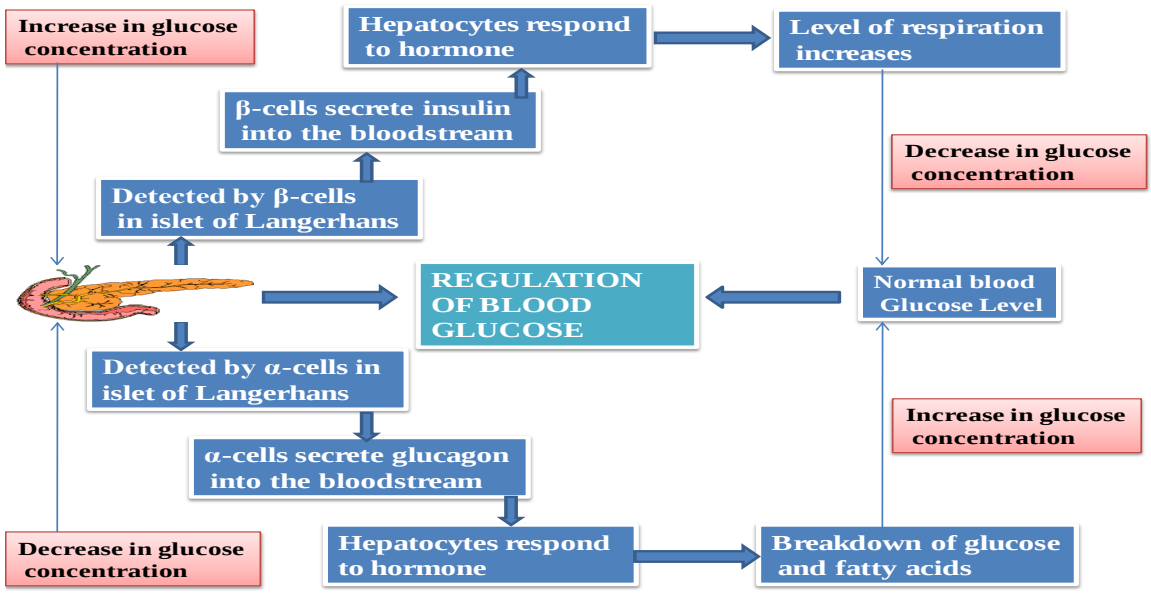
Insulin is biosynthesis from pancreatic β cells and the biosynthesis start with a precursor, preproinsulin, within the cytosol of the cells. Preproinsulin is comprised of sequentially as signal peptide (magenta), insulin B-chain (blue), C-peptide (black), and insulin A-chain (red). Newly synthesized preproinsulin undergoes co- and posttranslational translocation into the endoplasmic reticulum (ER), where it is cleaved by signal peptidase, forming proinsulin. The proinsulin folds in the ER, forming three evolutionarily conserved disulfide bonds, including two inter chain disulfide bonds B7–A7 and B19– A20 and one intra chain disulfide bond A6–A11. Properly folded proinsulin forms dimers and exits from the ER, trafficking through Golgi complex into secretory granules where prohormone convertases (PC1/3 and PC2) along with carboxypeptidase E (CPE) to process proinsulin to C-peptide and two-chain mature insulin stored in the insulin granules (Liu *et al*., 2014).

Insulin secretion

In response to various stimuli like glucose, arginine, sulphonylureas Insulin is secreted from the β cells of pancreas, but physiologically glucose is the major determinant. Various neural, endocrine and pharmacological agents are also exert stimulatory effect. Glucose is taken up by β cells through GLUT-2 receptors. After entering the β cell, glucose is oxidized by glucokinase, which acts as a glucose sensor. Glucose concentration below 90 mg/dl does not cause any insulin release. At such sub stimulatory glucose concentrations, K+ efflux through open KATP channels keeps the ß cell membrane at a negative potential at which voltage-gated Ca2+ channels are closed. As there is increase in plasma glucose, glucose uptake and metabolism by the β cell is enhanced. Rise in ATP concentration result in closure of KATP channels, leading to a membrane depolarization, opening of voltage-gated Ca2+ channels, Ca2+ influx, a rise in intracellular calcium concentration, and ultimately exocytosis of insulin granules. Structurally, the pancreatic

KATP channel consists of two unrelated subunits: a sulfonylurea receptor (the SUR1 isoform) and a potassium channel subunit (Kir6.2) that forms the central ion-conducting pathway. The mature KATP channel exists as an octamer of Kir6.2and SUR1 subunits in a 4:4 stoichiometry. A subunit specific site specific to pancreatic KATP channel, confers glimepiride an advantage over the other sulfonylurea secretagogues. Sulfonylurea and non-sulphonylurea drugs act as insulin secretogogues by closing KATP channels by passing the ß cell metabolism. **(**Joshi *et al*., 2007).

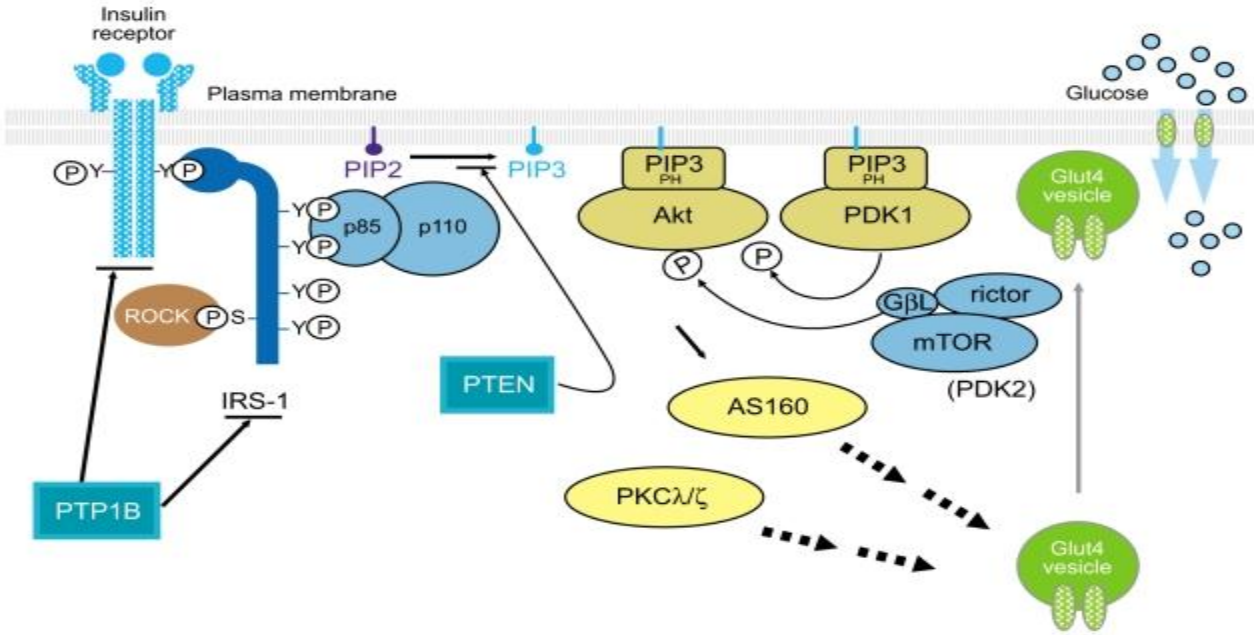
Regulation of normal blood glucose



Insulin, which is made by β cells, lowers elevated blood glucose levels. Glucagon, which is made by α cells, raises low blood glucose levels. When blood glucose levels rise after a meal, the pancreas releases insulin into the blood. Insulin helps muscle, fat, and liver cells absorb glucose from the bloodstream, lowering blood glucose levels. Insulin stimulates the liver and muscle tissue to store excess glucose. Insulin also lowers blood glucose levels by reducing glucose

production in the liver. When blood glucose levels drop overnight or due to a skipped meal or heavy exercise, the pancreas releases glucagon into the blood. Glucagon signals the liver and muscle tissue to break down glycogen into glucose, which enters the bloodstream and raises blood glucose levels. If the body needs more glucose, glucagon stimulates the liver to make glucose from amino acids (Grant *et al*., 2009).

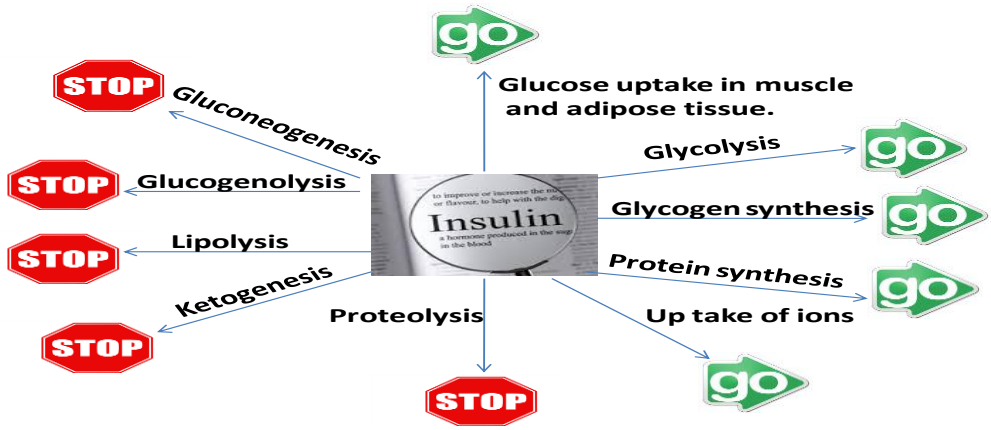
Insulin signaling



Insulin signaling involves a cascade of events initiated by insulin binding to its cell surface receptor, followed by receptor autophosphorylation, and activation of receptor tyrosine kinases, which result in tyrosine phosphorylation of insulin receptor substrates (IRSs) including IRS1, IRS2, IRS3, IRS4, Gab1, and Shc. Binding of IRSs to the regulatory subunit of phosphoinositide 3-kinase (PI3K) via Src homology 2 (SH2) domains results in activation of PI3K, which phosphorylates membrane phospholipids and phosphatidylinositol 4,5-bisphosphate (PIP2) on the 3' position. This complex activates the 3-phosphoinositide-dependent protein kinases (PDK-1

and PDK-2) resulting in activation of Akt/protein kinase B (PKB) and atypical protein kinase C λ and ζ, (PKCλ/ζ), each of which are serine/threonine kinases. Activated Akt phosphorylates its 160 kDa substrate (AS160), which stimulates the translocation of insulin-mediated Glut4 from intracellular vesicles to the plasma membrane. Moreover, activation of PKCλ/ζ is also involved in the regulation of Glut4 translocation in response to insulin. However, the insulin receptor (IR) is also dephosphorylated and inactivated by protein tyrosine phosphatases (PTPs), which comprise an extensive family of proteins that exert negative effects on insulin action and glucose metabolism. In addition, phosphatase and tension homologue deleted on chromosome 10 (PTEN), a lipid phosphatase, serves as an important negative modulator for the insulin signaling pathway by hydrolyzing phosphatidylinositol 3,4,5-triphosphate to PIP2, antagonizing the PI3K pathway . Thus, the physiological regulation of insulin action is controlled by the balance between phosphorylation and dephosphorylation (Choi K and Kim YB,2010).

Function of Insulin: (Qaid MM and Abdelrahman MM, 2016).



Biochemical background of diabetes mellitus

In the human body regular functioning of every cell an energy source is required. Glucose is the primary source of energy, which circulates in the blood as a mobilizable fuel source for cells. In human body pancreatic hormone insulin maintained the blood glucose level. The hormone binds to its receptor sites on peripheral site of the cell membranes. It helps the entry of glucose into respiring cells and tissues via requisite channels. Insulin converted the glucose into pyruvate through glycolysis via catabolism. It also up regulates glycogenesis from excessive cytosolic glucose and lipogenesis from excessive cytosolic acetyl-COA. The hormone glucagon is the antagonist of action of insulin. Below threshold level, glucose stays in the blood instead of entering the cells. The body attempts to arrest hyperglycaemia, by drawing water out of the cells and into the bloodstream. The excess sugar is excreted in the urine. This is why diabetics present with constant thirst, drinking large amounts of water, and polyuria as the cells try to get rid of the extra glucose, which ultimately leads to glycosuria. As long as prolongs the hyperglycemias, the body cells are devoid of glucose due to the lack of insulin, which ultimately forces the cells to seek alternative mobilizable energy sources. At this point, the cells turn to fatty acids stored in adipose tissue. The fats are not fuel sources for the red blood cells, kidney cortex and the brain. Because red blood cells lack mitochondria in which β-oxidation pathway rests. The fatty acids cannot pass the blood-brain barrier. To avail energy to such cells and tissues, the acetyl-CoA arising from catabolism of fatty acids is diverted to ketogenesis to generate ketone bodies, which can serve as alternative fuel sources for such cells and tissues. These ketone bodies are also passed in the urine, thereby leading to ketonuria, which characterizes diabetes mellitus. Build up of ketone bodies in the blood produces ketosis. Ketone bodies are acidic in nature and therefore, their build up in blood lowers blood pH, leading to acidosis. A combination of ketosis and

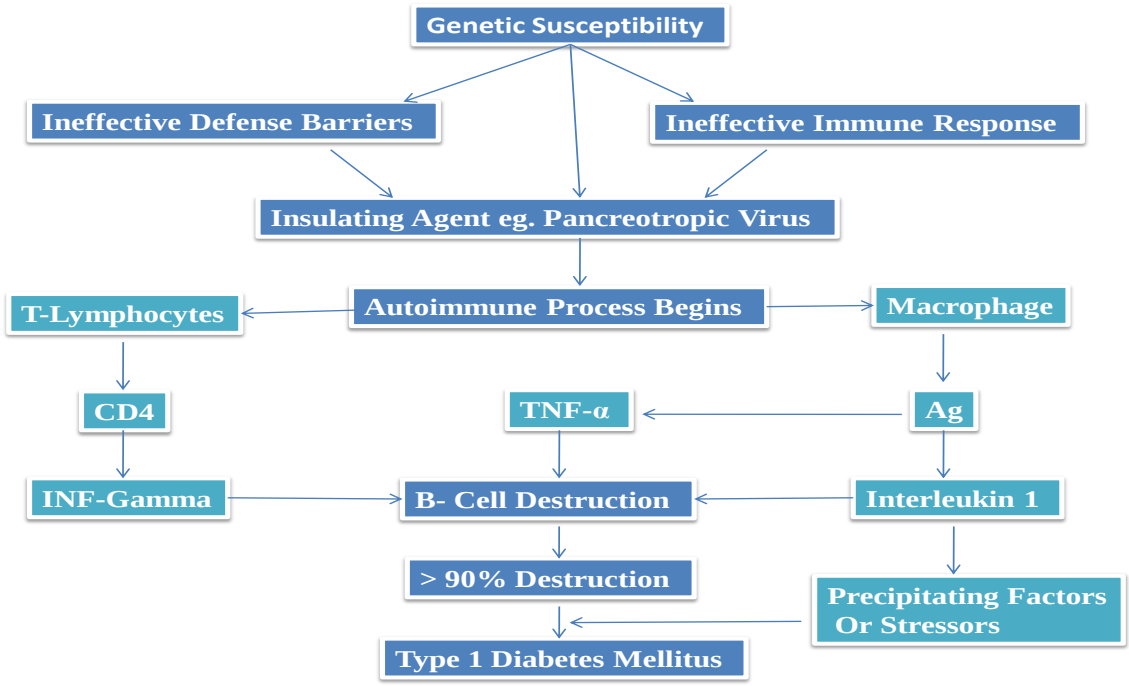
acidosis lead to a condition called ketoacidosis. If left untreated, ketoacidosis leads to coma and death (Piero *et al*., 2014).

Classification of Diabetes

Type 1 diabetes mellitus

The β cells destruction in the pancreas ultimately leads to develop Type 1 DM, which is accounts for approximately 10 percent of all patients with DM. The main cause of Type 1 DM is absolute insulin deficiency. There are two forms of type 1 DM. One is an immune-mediated disease with autoimmune markers such as islet cell antibodies (ICAs), insulin autoantibodies (IAAs), and autoantibodies to glutamic acid decarboxylase (GAD). As many as 85−90% of patients with fasting hyperglycemia are positive for one or more of these markers. Strong human leukocyte antigen (HLA) associations also exist. A second form of type 1 DM, now called idiopathic diabetes, has no known cause. Only a minority of patients fall into this group, which occurs mainly in individuals of African and Asian origin. Idiopathic diabetes is strongly heritable, but it lacks autoimmune markers and is not HLA associated. Although it can occur at any age, type 1 DM is more common in persons less than 30 years of age. The rate of pancreatic destruction is variable and is generally more rapid in infants and children and slower in adults (Harikumar *et al*., 2015).

Pathophysiology of type-1 diabetes



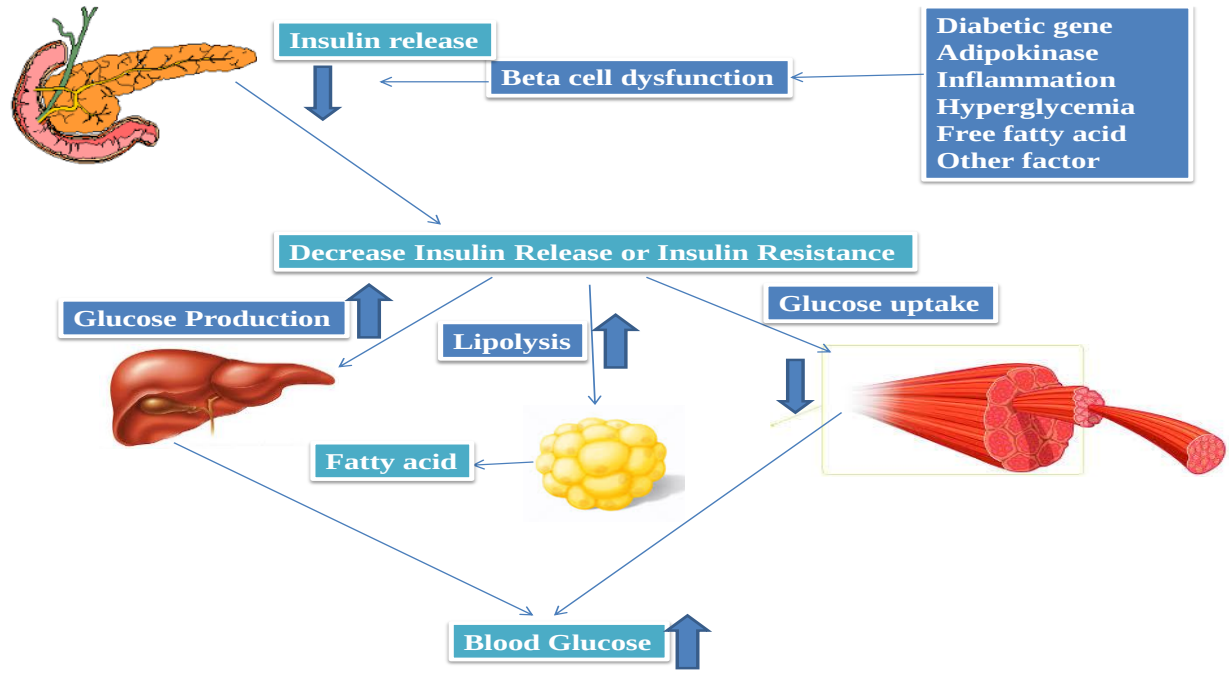
Type 2 diabetes mellitus

Type 2 diabetes mellitus is considered as a serious medical condition which is mainly characterized by an elevation of blood glucose level, this is a metabolic disorder will taken place as a result of either insulin resistance and/or insulin deficiency. This medical condition consider as one of the most predominant type of diabetes since it represent 90% of diabetic cases (Hassan, 2013).

(T2DM) is basically a metabolic disorder and mainly considered due to excess caloric intake and over energy expenditure. Which ultimately leads to defect in progressive insulin secretion or insulin resistance, to retain glucose homeostasis the body demand for insulin. If pancreatic β-cells are unable or fail to secrete enough insulin to compensate for increasing insulin demand, the blood glucose level will be elevated gradually. Chronic hyperglycemia which is strongly

associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels resulting in increasing levels of morbidity and mortality. Poor lifestyle, progressive reduction of physical activity and changes of dietary habits is a primarily factor leading to development the of the disease T2DM. As a consequence of these factor, a greater percentage of the population will become overweight and obese. T2DM is the one of the most prevalent chronic diseases worldwide and one of the major public health challenges of the 21st century. The epidemic of T2DM in the United States and the rest of the world continue to grow rapidly. The vast majority of patients with diabetes suffer from T2DM (Zhao Y *et al*., 2015).

Pathophysiology of type-2 diabetes: (Baynes, 2015).



Other specific types of diabetes (American diabetes association, 2008).

1. Chromosome 12, HNF-1 (MODY3)
2. Chromosome 7, glucokinase (MODY2)
3. Chromosome 20, HNF-4 (MODY1)
4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
5. Chromosome 17, HNF-1 (MODY5)
6. Chromosome 2, NeuroD1 (MODY6)
7. Mitochondrial DNA
8. Others

Genetic defects in insulin action

1. Type A insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipoatrophic diabetes
5. Others

Diseases of the exocrine pancreas

1. Pancreatitis
2. Trauma/pancreatectomy
3. Neoplasia
4. Cystic fibrosis
5. Hemochromatosis
6. Fibrocalculous pancreatopathy
7. Others

Endocrinopathies

1. Acromegaly
2. Cushing's syndrome
3. Glucagonoma
4. Pheochromocytoma
5. Hyperthyroidism
6. Somatostatinoma
7. Aldosteronoma
8. Others

Drug- or chemical-induced

1. Vacor
2. Pentamidine
3. Nicotinic acid
4. Glucocorticoids
5. Thyroid hormone
6. Diazoxide
7. Adrenergic agonists
8. Thiazides
9. Dilantin
10. -Interferon
11. Others

Infections

1. Congenital rubella
2. Cytomegalovirus
3. Others

Uncommon forms of immune-mediated diabetes

1. “Stiff-man” syndrome
2. Anti–insulin receptor antibodies
3. Others

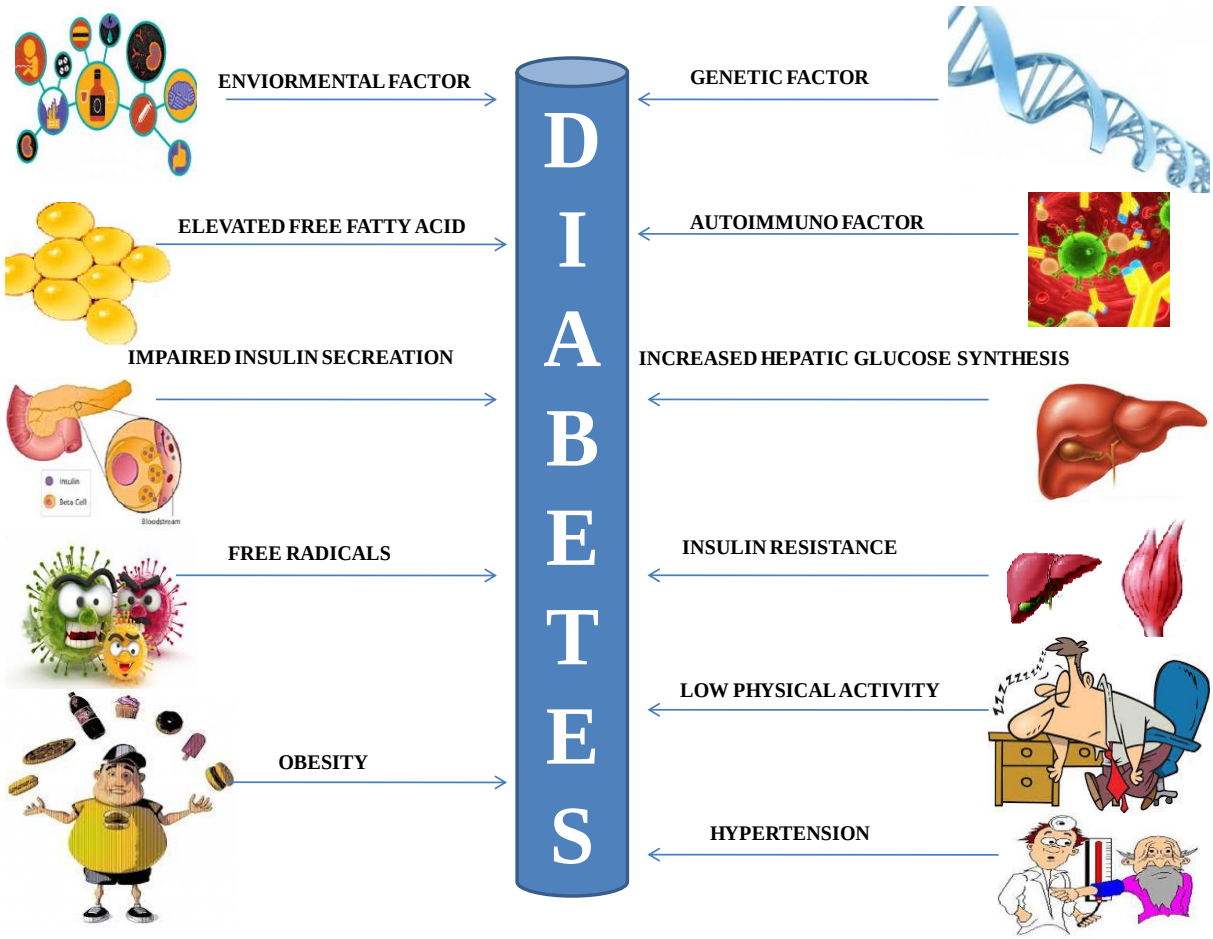
Other genetic syndromes sometimes associated with diabetes

1. Down’s syndrome
2. Klinefelter’s syndrome
3. Turner’s syndrome
4. Wolfram’s syndrome
5. Friedreich’s ataxia
6. Huntington’s chorea
7. Laurence-Moon-Biedl syndrome
8. Myotonic dystrophy
9. Porphyria
10. Prader-Willi syndrome
11. Others

Gestational diabetes mellitus (GDM) P

These types of conditions occurs during pregnancy, and blood glucose returns to normal after delivery. Gestational diabetes mellitus is not an pathophysiologic condition it's an operational classification identifying women who develop diabetes mellitus during gestation. Women who develop Type 1 diabetes mellitus during pregnancy and women with undiagnosed asymptomatic Type 2 diabetes mellitus that is discovered during pregnancy are classified with Gestational Diabetes Mellitus (GDM). In most women who develop GDM; the disorder has its onset in the third trimester of pregnancy (Baynes, 2015).

Etiology of Diabetes



Genetic component

Genetic connection plays an important role in type 2 DM, having relatives (especially first degree) with type 2 DM increases the risks of developing type 2 DM substantially. Concordance among monozygotic twins is close to 100%, and about 25% of those with the disease have a family history of DM. Recently, genes discovered to be significantly associated with developing type 2 DM, include *TCF7L2*, *PPARG*, *FTO*, *KCNJ11*, *NOTCH2*, *WFS1*, *CDKAL1*, *IGF2BP2*, *SLC30A8*, *JAZF1*,and *HHEX*. *KCNJ11* (potassium inwardly rectifying channel, subfamily J, member 11), encodes the islet ATP-sensitive potassium channel Kir 6.2, and *TCF7L2* (transcription factor 7-like 2) regulates proglucagon gene expression and thus the production of glucagon-like peptide-1. (Olokoba *et al*., 2012).

Susceptibility loci

In addition to a considerable number of genetic components associated with T2DM, segregation analysis also suggests the polygenic nature of T2DM. The susceptibility loci of T2DM have been discovered by genome-wide association studies (GWAS) since early 2007. Then, numerous GWAS conducted in different countries and ethnic groups have reported linkage signals at the same or different chromosomes with T2DM, and have successfully identified approximately 75 susceptibility loci related to T2DM(Wu *et al*.,2014).

Gene	Gene Region	SNPs	Population
KCNQ1	11p15.4	rs2237897	Japanese
	11p15.4	rs2237895	Chinese
	11p15.4	rs231362	European
	11p15.4	rs2237892	Japanese
TCF7L2	10q25.2	rs7903146	European
KCNJ11	11p15.1	rs5219	European
	11p15.1	rs5215	UK
IRS1	2q36.3	rs7578326	European
MTNR1B	11q14.3	rs1387153	European
IGF2BP2	3q27.2	rs4402960	European
	3q27.2	rs6769511	European
CDKN2A/B	9p21.3	rs564398	UK
	9p21.3	rs2383208	Japanese
	9p21.3	rs10811661	European
HHEX	10q23.33	rs1111875	European
	10q23.33	rs5015480	European
PPARG2	3p25.2	rs1801282	European
	3p25.2	rs17036101	European

Obesity and Physical Inactivity

A strong correlation was there between Lifestyle factor and diabetes mellitus. Lifestyle factors play an important role to the development of T2DM, such as sedentary lifestyle, physical inactivity, smoking and alcohol consumption. Substantial epidemiological studies have shown that obesity is the most important risk factor for T2DM, which may influence the development of

insulin resistance and disease progression. As per World Health Organization Nearly 90% of diabetic patients develop T2DM related to excess body weight. Furthermore, obesity is considered as strongly inherited .Obstructive sleep apnea (OSA), a treatable sleep disorder that is pervasive among overweight and obese adults, has become a novel, modifiable risk factor relevant to insulin resistance and glucose intolerance, and may influence on the development of prediabetes (20%-67%) and T2DM (15%-30%), independent of shared risk factors.

Development of T2DM diet is also considered as a risk factor. Studies have shown that a low-fibre diet with a high glycaemic index is positively associated with a higher risk of T2DM, and specific dietary fatty acids may affect insulin resistance and the risk of diabetes in varying degrees. Total and saturated fat intake is also associated with an increased risk of T2DM independently of BMI. Frequent consumption of processed meat, but not other meats, may increase the risk of T2DM after adjustment for BMI, prior weight change, and alcohol and energy intake. Soft drinks have also been bounded up with increased risk of T2DM and metabolic syndrome, because they are directly associated with BMI (Ding *et al*., 2014).

Insulin Resistance

An increase in Free Fatty Acid concentration has important physiological consequences of T2DM, during pregnancy, FFA level are increased which induces insulin resistance and so valuable glucose is conserved for the developing fetus. Thus, FFA plays both a physiological and a pathological role in the body. But excess amount of FFA in blood may cause serious metabolic syndrome. More then 80% of people with type 2 diabetes are obese and insulin resistant. Obesity and insulin resistance may be linked with via some mediators including FFA, tumor necrosis factor-α (TNF-α), leptin and adinopectin. Adipocytes are considered to be a site of insulin resistance. Insulin resistance is responsible for development of lipolysis, leading to increased

concentration of circulating FFA and to the development of insulin resistance in skeletal muscles and the liver. Thus, there is a strong association between increased plasma FFA, intramyocellular lipid accumulation and insulin resistance. Lot's of studies are strong link of FFA and insulin resistance, the mechanism is not fully understood and more research is required to find the exact molecular basis (Mukherjee *et al*., 2013).

Oxidative stress in diabetes mellitus

Oxidative stress plays important role in the development of vascular complications in diabetes particularly type 2 diabetes. Reactive oxygen species (ROS) level elevation in diabetes may be due to decrease in destruction or/and increase in the production of enzymatic and non-enzymatic antioxidant defense system (like: catalase (CAT—enzymatic/non-enzymatic, superoxide dismutase SOD and glutathione peroxidase GSH–Px). The alteration in the levels of these enzymes makes the tissues susceptible to oxidative stress leading to the development of diabetic complications. According to epidemiological studies, diabetic mortalities can be explained notably by an increase in vascular diseases other than hyperglycemia.

Pahtophysiology of oxidative stress in diabetes

Scientific evidence support the role of oxidative stress in the pathogenesis of both type 1 and type 2 diabetes. Generation of free radicals in diabetes by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation leads to damage of enzymes, cellular machinery and also increased insulin resistance due to oxidative stress. According to latest research, lipid is not only but also the apolipoprotein component of LDL that forms insoluble aggregates oxidatively due to hydroxyl radical-induced cross-linkage between apo-B monomers that is responsible for oxidative damage in diabetic complications. In diabetes mellitus patient, the main sources of free radicals are oxidative stress in mitochondria. During oxidative

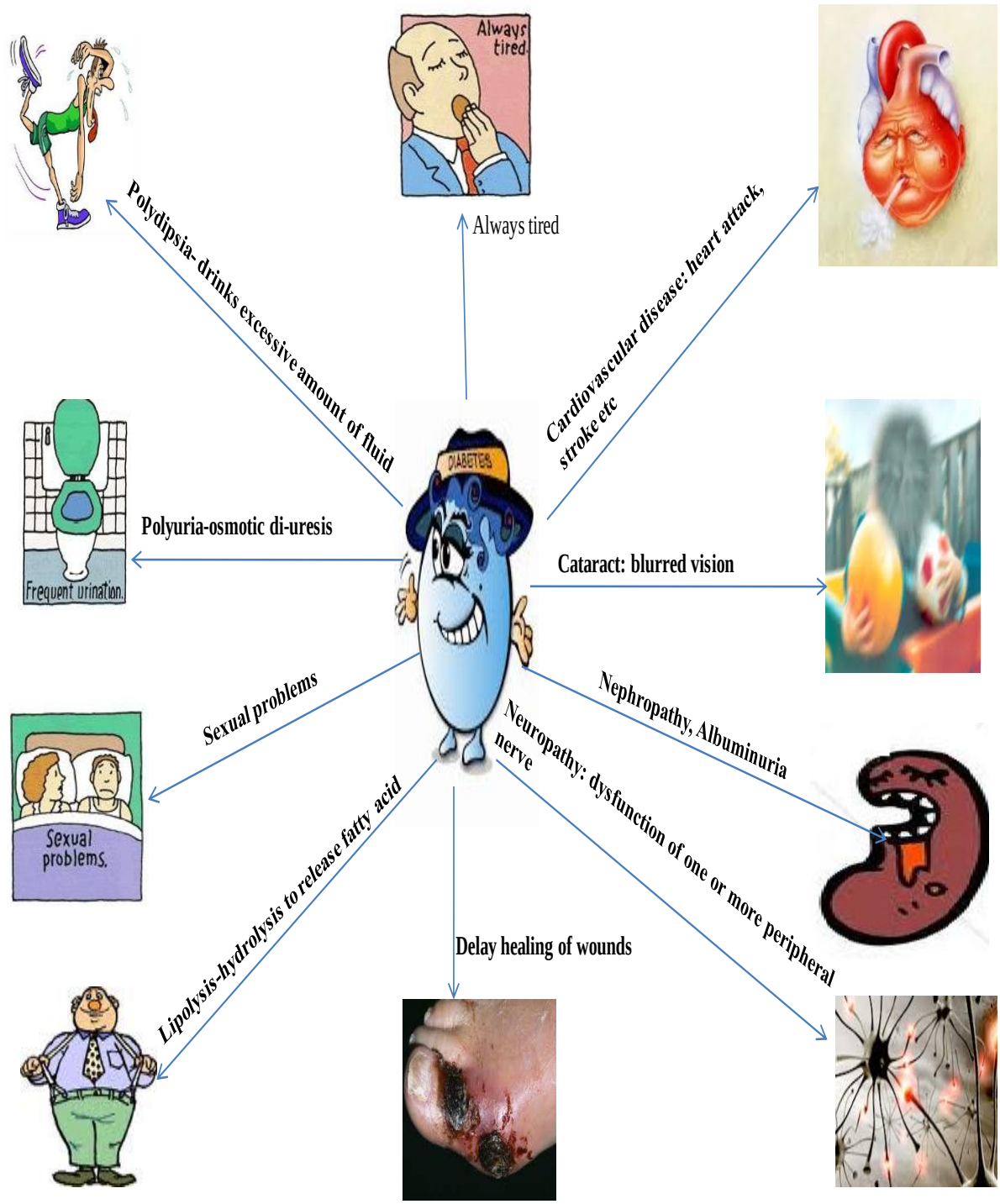
metabolism in mitochondria, a component of the utilized oxygen is reduced to water, and the remaining oxygen is transformed to oxygen free radical ($O^{\bullet}$) which is an important ROS that is converted to other RS such as $ONOO^{-}$, OH and H_2O_2. Insulin signaling is modulated by ROS/RNS by two ways. On one side, in response to insulin, the ROS/RNS are produced to exert its full physiological function and on the other side, the ROS and RNS have got negative regulation on insulin signaling, interpreting them to develop insulin resistance which is a risk factor for diabetes type 2(Ullah *et al*.,2016).

The metabolic syndrome (MetS) is a major and escalating public-health and clinical challenge worldwide in the wake of urbanization, surplus energy intake, increasing obesity, and sedentary life habits. MetS confers a 5-fold increase in the risk of type 2 diabetes mellitus (T2DM) and 2-fold the risk of developing cardiovascular disease (CVD) over the next 5 to 10 years. Further, patients with the MetS are at 2- to 4-fold increased risk of stroke, a 3- to 4-fold increased risk of myocardial infarction (MI), and 2-fold the risk of dying from such an event compared with those without the syndrome regardless of a previous history of cardiovascular events. MetS is considered as a first order risk factor for atherothrombotic complications. Its presence or absence should therefore be considered an indicator of long-term risk (Kaur, 2014).

B cell demise and dysfunction

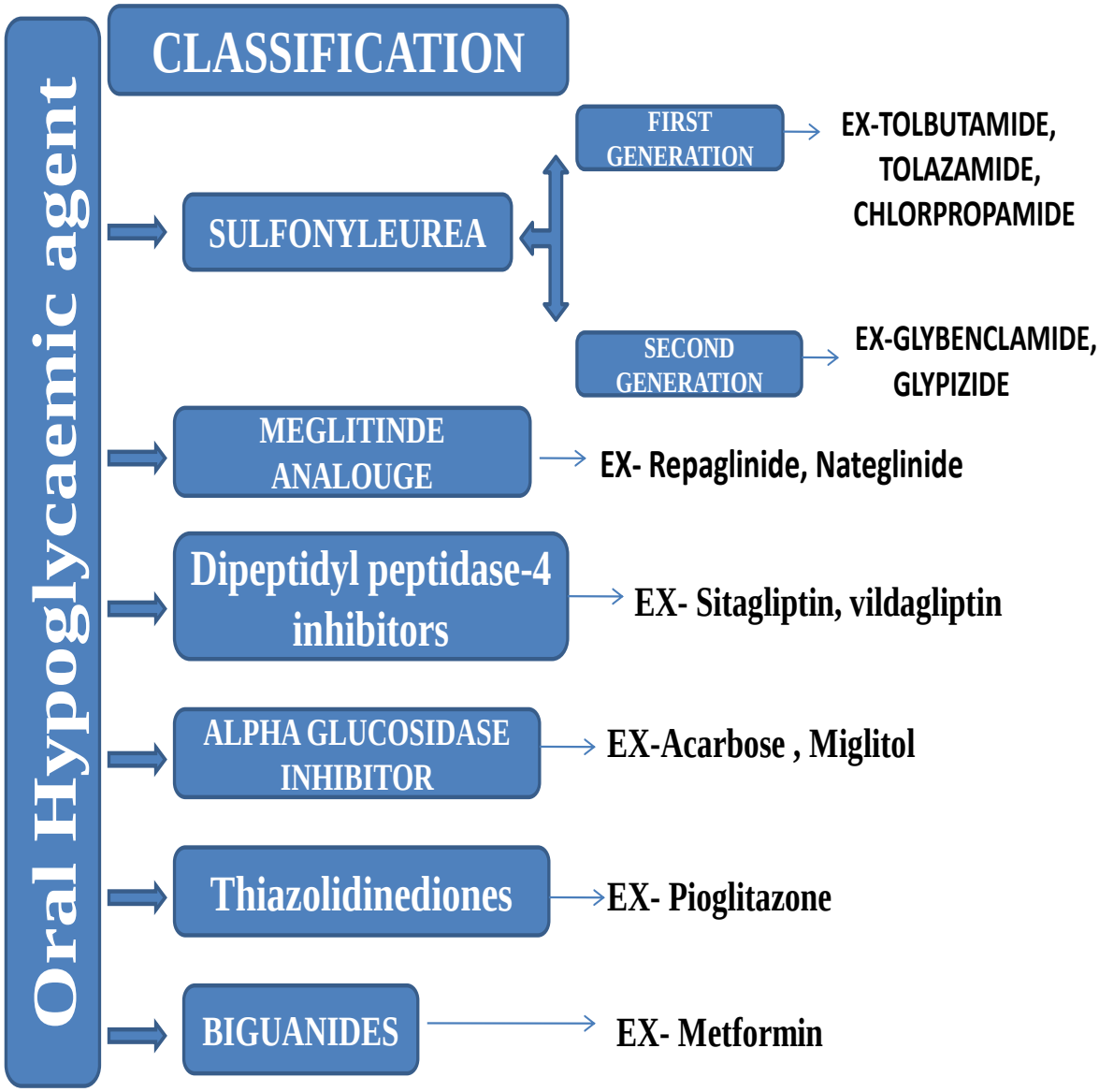
B cell destruction or dysfunction occur may be due to cytokine-induced inflammation, obesity and insulin resistance, and overconsumption of saturated fat and free fatty acids (FFA). A progressive decline or destruction of β cell function leading to β cell exhaustion precedes β cell demise. Loss of β cell mass and function are central to the development of both type 1 and 2 diabetes (Cerf, 2013).

Complication of Diabetes:(Baynes, 2015).



Treatment: Classification of drugs used in the Treatment of type-2 diabetes mellitus

(Brunetti and Kalabalik, 2012).



Natural Products and Diabetes

To the existence of human civilization History of medicine dates back practically. Historically and scientifically, the majority of new drugs have been generated from natural products (secondary metabolites) and from compounds derived from natural products (Lahlou, 2013).

Humans have long history to used naturally occurring substances for medical purposes. Most cultures plants play an important role in medicinal field. Modernization of the science of chemistry at the beginning of the 19th century, plants began to be examined more closely to understand why they were medically useful (Beutler, 2009).

Many of the currently available drugs have been derived directly or indirectly from plants sources. If we look into the relationship plants use as medicine, it can be traced back to the ancient days and they are as old as mankind itself. Natural product are historically been the major source of pharmaceutical agents which also have an important role in the development of modern medicinal system (Kulkarni, 2014).

Traditional medicines (TMs) make use of natural products and are of getting importance day by day. Some common forms of medicine are traditional Chinese medicine (TCM), Ayurveda, Kampo, traditional Korean medicine (TKM), and Unani employ natural products and have been practiced all over the world for hundreds or even thousands of years, and they have blossomed into orderly-regulated systems of medicine. In their various forms, they may have certain defects, but they are still a valuable repository of human knowledge (Yuan *et al*., 2016).

Nature stands as an inexhaustible source of novel chemotypes and pharmacophores, and has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs find their origin in natural products.

In the area of drug discovery Natural products, have played an invaluable role process. Recently, there has been a renewed interest in natural products research due to the failure of alternative drug discovery methods to deliver many lead compounds in key therapeutic areas such as immunosuppressant, anti-infective, and metabolic diseases. However, continuing improvements in natural products research are needed to continue to be competitive with other drug discovery methods, and also to keep pace with the ongoing changes in the drug discovery process

Natural products have provided a important role to the pharmaceutical industry over the past half century, in the areas of infectious diseases and oncology have benefited much from numerous drug classes derived from the natural form and as templates for synthetic modification. About 40 new drugs launched on the market between 2000 and 2010, originating from terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates are reported (Brahmachari).

There is an urge to find out an efficient and economic way to manage diabetes and its complications. The scientists are coming up with new synthetic molecules, but they are having a range of limitations including their side effects and the total treatment cost. On this backdrop, the use of natural products is gaining popularity among the people (Kulkarni, 2014).

List of important phytoconstituents used in the treatment of Diabetes mellitus (Gaikwad *et al*.,2014).

Phytoconstituents	**Plant Name Part used**	**Part used References**
Alkaloids		
Berberine	*Berberis* spp. *Tinospora cordifolia*	Roots, stem-bark
Casuarine 6-o-α-glucoside	*Syzygium malaccense*	Bark
Catharanthine, vindoline and	*Catharanthus roseus*	Leaves, stems

vindolinine		
Calystegine B2	*Nicandra physalodes*	Fruits
Cryptolepine	*Cryptolepis sanguinolenta*	
Harmane , norharmane,	*Tribulus terrestris*	
Jambosine	*Syzygium cumini*	Seeds, fruits, bark
Jatrorrhizine, magnoflorine, palmatine	*Tinospora cordifolia*	
Javaberine A, javaberine A hexaacetate, javaberine B hexaacetate	*Talinum paniculatum*	Roots
Lepidine and semilepidine	*Lepidium sativum*	Seeds
Lupanine	*Lupinus perennis*	
Mahanimbine	*Murraya koenigii*	Leaves
Piperumbellactam A	*Piper umbellatum*	Branches
Radicamines A and B	*Lobelia chinensis*	
Swerchirin	*Swertia chirayita*	
Tecomine	*Tecoma stans*	
Trigonelline	*Trigonella foenum-graecum*	Seeds
1-deoxynojirimycin	*Morus alba*	Leaves, bark
Glycosides		
Kalopanax	*Kalopanax pictus*	Stem bark
Jamboline or antimellin	*Syzygium cumini*	Seeds
Myrciacitrins I and II and myrciaphenones A and B	*Myrcia multiflora*	Leaves
Neomyrtillin	*Vaccinium myrtillus*	Leaves
Perlargonidin 3-o- α - l rhamnoside	*Ficus bengalensis*	Bark
Pseudoprototinosaponin AIII & prototinosaponin AIII	*Anemarrhena asphodeloides*	Rhizome
Vitexin, isovitexin and	*Microcos paniculata*	Leaves

isorhamnetin 3-O- β-D-rutinoside		
Flavonoids		
Bengalenoside	*Ficus benghalensis*	Stem bark
Epigallocatechin gallate	*Camellia sinensis*	Leaves
(-)-3-O-galloylepicatechin, (-)-3- O-galloylcatechin	*Bergenia ciliata*	
Genistein	*Glycine* spp.	Soya beans
Hesperidin, naringin	*Citrus* spp.	
Prunin	*Amygdalus davidiana* var. *davidiana*	Stems
Kaempferitrin	*Bauhinia forficate*	Leaves
Kaempferol	*Jindai soybean*	Leaves
Kolaviron	*Garcinia kola*	
Leucodelphinidin	*Ficus bengalensis*	Bark
Mangiferin	*Anemarrhena asphodeloides*	Rhizomes
Marsupsin, pterostilbene	*Pterocarpus marsupium*	Heartwood
Quercetin	*Chamaecostus cuspidatus*	
Shamimin	*Bombax ceiba*	Leaves
Terpenoids and steroids		
α -amyrin acetate	*Ficus racemosa*	Fruits
Andrographolide	*Andrographis paniculata*	Leaves
3 β -acetoxy-16 β -hydroxybetulinic acid	*Zanthoxylum gilletii*	Stem bark
Bassic acid	*Bumelia sartorum*	Root bark
Charantin	*Momordica charantia*	Seeds, fruits
Christinin A	*Zizyphus spina-christi*	Leaves
Colosolic acid, maslinic acid	*Lagerstroemia speciosa*	Leaves
Corosolic acid	*Vitex* spp.	Leaves
Elatosides E	*Aralia elata*	Root cortex

Escins-IIA and IIB	*Aesculus hippocastanum*	Seeds
Forskolin	*Coleus forskohlii*	
Ginsenosides	*Panax species*	Rhizomes
Gymnemic acid IV	*Gymnema sylvestre*	Leaves
Momordin ic	*Kochia scoparia*	Fruit
β -sitosterol	*Azadirachta indica*	
Senegin derivatives	*Polygala senega*	
Polysaccharides		
Aconitans A-D	*Aconitum carmichaeli*	Roots
Atractans A	*Atractylodes japonica*	Rhizomes
Ganoderans A and B.	*Ganoderma lucidum*	Fruit bodies
Galactomannan gum	*Cyamopsis tetragonolobus* *Amorphophallus konjac*	Seeds Tubers
Miscellaneous		
Allicin	*Allium sativum* *Allium cepa*	Bulbs
Bellidifolin	*Swertia japonica*	
Bakuchiol	*Otholobium pubescens*	
Curcuminoids	*Curcuma longa*	Rhizomes
Ellagitannins	*Terminalia chebula*	Fruits
Ferulic acid	*Curcuma longa*	Leaves seeds
Ginseng polypeptides	*Panax ginseng*	Roots
4-hydroxyisoleucine	*Trigonella foenum-graecum*	Seeds
Kotalanol	*Salacia reticulate*	

The world market for safe and effective phytomedicine is 60-100 billion US $ where India's share is 0.1 billion $. Estimated global imports of raw medicinal and aromatic plants and plant parts in the year 2001 exceeded a billion US dollars. India being the second largest exporter, next to China, of raw medicinal plant materials, accounted for about 13 % of global imports. The

domestic market for medicinal plants or related products is about Rs. 4000 crores. This along with as export level of Rs. 1200 crore makes the commercialization of Indian medicinal plant sector at Rs. 5200 crores (Subramoniam, 2014).

Quality control of traditional medicines is a critical and essential issue to be considered in assuring the therapeutic efficacy, safety and to rationalize their use in the health care. For the quality control of traditional medicine, the traditional methods are procured, studied, documented and then the traditional information about identification and quality assessment is interpreted properly in terms of modern assessment. Quality assurance is an integral part of traditional medicine, which ensures that it delivers the required quantity of quality medicament. Today, quality assurance is the thrust area for traditional formulations like churnas, bhasmas, liquid orals, Lehas, etc. (Mukherjee PK, 2001 and Mukherjee PK, 2002).

Safety Issue of Herbal Medicines

Traditional herbal products are heterogeneous in nature. They impose a number of challenges to qualify control, quality assurance and the regulatory process. Most herbal products on the market today have not been subjected to drug approval process to demonstrate their safety and effectiveness. Some of them contain mercury, lead, arsenic and corticosteroids and poisonous organic substances in harmful amount. Hepatic failure and even death following ingestion of herbal medicine have been reported. A prospective study shows that 25% of the corneal ulcer in Tanzania and 26% of the childhood blindness in Nigeria and Malawi were associated with the use of traditional eye medicine. Side effect of some medicinal plant is currently reviewed.

Need for Clinical Trials

Need for Clinical Trials To gain public trust of today health care system, the researchers, the manufacturers and the regulatory agencies must apply rigorous scientific methodologies and clinical trails to ensure the quality and lot-to-lot consistency of the traditional herbal products. Since the identities of the final products are not well defined and there are essentially no purification steps involved in the productions of herbal products, the quality and lot to lot consistency of the products rely mostly on the quality control of source materials and their manufacturing into the final products. Using modern technologies the quality and consistency of the heterogeneous herbal products can be monitored. A well-designed clinical trail is the method of choice to prove the safety and effectiveness of a therapeutically product. Manufacturers of the herbal products must adhere to the requirements of good manufacturing practices (GMPs) and preclinical testing before these products can be tested on human (Kumar and Yogeshwer, 2002).

Future Prospects of Herbal Medicine

It is estimated that nearly three fourths of the herbal drugs used worldwide were discovered following leads from local medicine. According to WHO about 25% of modern medicines are descended from plants first used traditionally. Many others are synthetic analogues built on prototype compounds isolated from plants. Almost, 70% modern medicines in India are derived from natural products. Proper utilization of these resources and tools in bioprospecting will certainly help in discovering novel lead molecules from plants by employing modern drug discovery techniques and the coordinated efforts of various disciplines. Tribal healers in most of the countries, where ethno medical treatment is frequently used to treat cut wounds, skin infection, swelling, aging, mental illness, cancer, asthma, diabetes, jaundice, scabies, eczema, venereal diseases, snakebite and gastric ulcer, provide instructions to local people as how to

prepare medicine from plants. They keep no records and the information is mainly passed on verbally from generation to generation. World Health Organization (WHO) has shown great interest in documenting the use of medicinal plants used by tribals from different parts of the world. Many developing countries have intensified their efforts in documenting the ethno medical data on medicinal plants. Research to find out scientific evidence for claims by tribal healers on Indian herbs has been intensified. Once these local ethno medical preparations are scientifically evaluated and disseminated properly, people will be better informed regarding efficacious drug treatment and improved health status (Pathak *et al*.,2013).

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CHAPTER-2

COLLECTION, IDENTIFICATION, AUTHENTICATION, EXTRACTION.

Introduction

The southern "Himalaya" is home to more than 8000 species of vascular plants of which 1748 are known for their medicinal properties. The life of tribal peoples living in the Himalaya depends upon these plants which have played a key role by providing food and medicinal support. Numerous wild and cultivated plants have been utilized as curative agents since ancient times, and medicinal plants have gained importance recently, not only as herbal medicines, but also as natural ingredients for the cosmetic industry (Joshi *et al*, 2016).

Given the wide range of ethnomedicinal uses and also the fact that this plant is obtained from high altitude has given us sufficient reasons to select this plant for our study. Moreover, parts of this plant are edible and hence it is likely to pose fewer side effects if used for therapeutic purposes so the plant *Campylandra aurantiaca* was selected for the study.

Literature review of *Campylandra aurantiaca*

Classification

Kingdom- Plantae

Phylum- Tracheophyta

Class- Liliopsida

Order- Asparagales

Family- Asparagaceae

Genus- Rohdea

Synonyms

Rohdea nepalensis (Raf.) N.Tanaka (accepted name), *Campylandra liangshanensis* , *Rohdea aurantiaca* (Baker), *Rohdea liangshanensis* , *Tilcusta nepalensis* Raf, *Tupistra aurantiaca*, *Tupistra liangshanensis*

Local Name: Nakima, ThuloNakim (Species, 2000).

Scientific name: *Campylandra aurantiaca*

Family: Asparagaceae (Uprety *et al*, 2016).

Morphology

Herbs perennial, rhizomatous, monopodial. Rhizome ascending or less often creeping, thick, stout, sometimes slightly woody. Stem very short or sometimes slightly elongate. Leaves basal or on short stem, usually distichous equitant, sometimes spaced, basally distinctly petiolate or not; leaf blade lorate to ovate-elliptic. Scape axillary. Inflorescence a terminal spike, several to many

flowered, sometimes with several sterile bracts apically; fertile bracts lanceolate to ovate, often longer than flowers, sometimes shorter. Fruit a berry, 1–3-seeded.

Geographical Distribution

Bhutan, China, India, Nepal, Sikkim. (Flora of China, 2000).

Ethnomedicinal Uses

The rhizome of the plant *Campylandra aurantiaca* is used for various medicinal purpose like antidiarrhoeic, antidysenteric, analgesic, antimalarial, antiarthritic, vermicidal, antipyretic and stomachic. (Kagyung *et al*, 2010). Bhutia and Lepcha people are used as a vegetable, pickle (Tamang and Thapa, 2014). The flowers of the plant are made into curry and taken with staple food two times per week for 4–6 weeks to treat diabetes (Chhetri *et al*, 2005). Whole parts are grinded and consume to treat indigestion (Gibji *et al*, 2012).

Roots: Stocks given orally in case of food poisoning (Darjeeling).

Inflorescence: Powdered and taken with water to relieve body pain (Sikkim) and given in food poisoning (Darjeeling).

Flowers: Used as appetizer and taken in diabetes (Sikkim). Taken as curry (Sikkim) (Uprety *et al*,2016).

Rhizome: Rhizome decoction is administered as antidiarrhoeic, antidysenteric, analgesic, antimalarial, vermicidal and stomachic (Badwaik and Sakure, 2015). Bitter inflorescence purifies blood and is eaten as curry. Roots used to treat Jaundice (Bhattacharya and Ghosh, 2014).

Flavonoid Fraction used in Diabetic Study

Hypoglycaemic action of the flavonoid fraction of *Artocarpus heterophyllus* leaf (Chandrika *et al*, 2006).

A flavonoid fraction purified from Rutaceae aurantiae (Daflon(R)) inhibiting AGE formation, reduces urinary albumin clearance and corrects hypoalbuminemia in normotensive and hypertensive diabetic rats (Urios *et al*, 2014).

Flavonoid identification and hypoglycaemic Studies of the Butanol Fraction from Gynura procumbens (Akowuah *et al*, 2002).

Collection and Identification

The rhizome of *Campylandra aurantiaca* was collected from the upper hilly region of Eastern Himalayan, Sikkim, India. Authenticated by Botanical Survey of India, Central National Herbarium, Howrah, West Bengal, India. A voucher specimen No-(CNH/Tech.II/2016/38b) was preserved at Phytotherapy and Pharmacology Lab of Department of Pharmacology, Jadavpur University, Kolkata-700032.

Extraction

The air dried whole bark (500 g) was powdered in a mechanical grinder, and the powdered materials were extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus at controlled temperature. The solvents were completely evaporated under reduced pressure. Methanol extract was used for further fractionation.

Qualitative analysis

Preliminary qualitative analysis has been performed to know the type of compounds present in the extracts. Chemical group test were performed for Alkaloids, Flavonoids, Saponins, Tannins, Steroids and others by using the standard procedures.

Phytochemical tests

Test for Steroids

Libermann-Burchard Test (Zhou *et al*, 2004)

- 10mg of extract was dissolved in 1ml of chloroform. 1ml of acetic anhydride was added following the addition of 2ml of concentrated sulphuric acid. Formation of reddish violet colour indicated the presence of steroids.

Salkowski Test (Bosila *et al*, 2005)

- 1ml of concentrated sulphuric acid was added to 10mg of extract dissolved in 1ml of chloroform. A reddish-blue colour exhibited by chloroform layer and green fluorescence by the acid layer suggested the presence of steroids.

Test for Flavonoids (Saija *et al*, 1995)

- 5 ml of extract solution was hydrolyzed with 10% sulphuric acid & cooled. Then it was extracted with diethyl ether and divided with three portions in three separate test tubes. 1 ml of dilute ammonia, 1ml of dilute sodium bicarbonate and 1 ml of 0.1 (N) sodium hydroxide were added to the first, second and third test tube respectively. In each test tube, development of yellow colour indicated the presence of flavonoids.
- The extract was dissolved in alcohol. One piece of magnesium followed by concentrated hydrochloric acid was added drop wise to that and heated. Appearance of magenta colour demonstrated the presence of flavonoids.

Test for Saponins (Sinha *et al*, 1985)

- 1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 min. Development of stable foam suggested the presence of saponins.

- 1 ml extract was treated with 1% lead acetate solution. Formation of white precipitate indicated the presence of saponins.

Test for Alkaloids (Raffauf, 1962)

- 1.2 ml of extract was taken in a test tube. 0.2 ml of dilute hydrochloric acid and 0.1 ml of Mayer's reagent were added. Formation of yellowish buff coloured precipitate gives positive test for alkaloids.
- 0.1 ml of dilute hydrochloric acid and 0.1 ml of Dragendroff's reagent were added in 2 ml solution of extract in a test tube. Development of orange brown coloured precipitate suggested the presence of alkaloids.
- 2 ml of extract solution was treated with dilute hydrochloric acid and 0.1 ml of Wagner's reagent. Formation of reddish brown precipitate indicted the positive response for alkaloids.
- 2 ml of extract was allowed to react with 0.2 ml of dilute hydrochloric acid and 0.1 ml of Hager's reagent. A yellowish precipitate suggested the presence of alkaloids.

Test for Tannins (Segelman, 1969)

- 5 ml of extract solution was allowed to react with 1 ml of 5% ferric chloride solution. Greenish black colouration indicated the presence of tannins.
- 5 ml of extract was treated with 1 ml of 10% aqueous potassium dichromate solution. Formation of yellowish-brown precipitate suggested the presence of tannins.
- 5 ml extract was treated with 1ml of 10% lead acetate solution in water. Yellow coloured precipitation indicated the presence of tannins.

Fractionation and characterization of plant material

Fractionation of the methanol extract was performed by separating funnel using solvent system of petroleum ether, chloroform and ethyl acetate (Das *et al*,2011). Two flavonoid enriched fractions of *Campylandra aurantiaca* (FEFCA) was obtained from the chloroform and ethyl acetate fraction as per the protocol described earlier (Das *et al*, 2011 and Dua *et al*, 2015).

HPLC Characterization:

HPLC Characterization of isolated fractions of *Campylandra aurantiaca* was performed by HPLC system Ultimate 3000 Germany), using a reverse phase C-18 column (250 × 4.6 mm, particle size 5μ) and UV detector. HPLC grade methanol was used to prepare samples and filtered by cellulose nylon membrane filter (0.45 μm). The aliquots of the filtrate were eluted with an isocratic solvent mixture comprising methanol: acetonitrile: acetic acid: o-phosphoric acid: water (20:10:1:1:20) for flavonoids. Methanol: water: acetic acid (75:24:1) for phenolic compounds. Flow rate of 1 ml/min was used in the system and detected at 352 and 254 nm respectively. Standard phytochemical markers i.e. gallic acid, myricetin, quercetin and apigenin were used to characterized the fraction (Dua *et al*, 2015). Flavonoid enriched fraction obtained from ethyl acetate portion was used for further evaluation and consider as FEFCA in the study.

Results

Percentage yield of 2%, 2.7% and 3.4% obtained in Soxhlet extraction apparatus with by petroleum ether, chloroform, and methanol respectively. Preliminary quantitatively analysis confirmed the presence of 24.7 μg /mg of phenolic compounds in the methanol extract.

HPLC characterization of fraction obtained from chloroform portion confirms the presence of myricetin having characterized Rt 4.08 min. However the presence of myricetin and apigenin

was confirmed by the HPLC chromatogram having Rt 4.08 and 8.16 min respectively Representative HPLC chromatograph of standard flavonoids viz. myricetin, quercetin, apigenin and gallic acid with specific Retention time (Rt) 4.08, 5.70, 8.16 and 4.03 min respectively was obtained in the chromatogram using same system.

Phytochemicals analysis

Extract	Alkaloids	Flavonoids	Steroids	Tannins	Saponins
Methanolic Extract	+	+	+	+	+

HPLC chromatograph for flavonoids determination

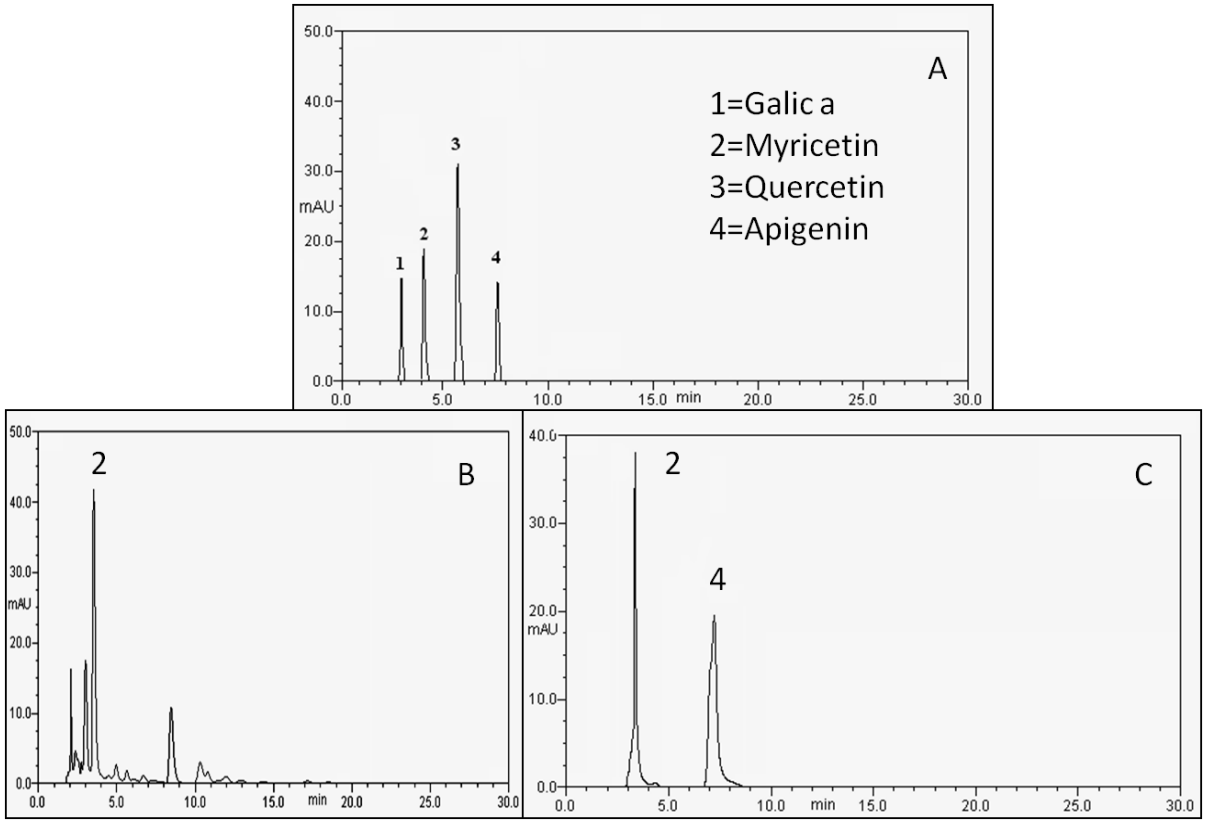

Figure: 1. HPLC characterization of FEFCA. A. Representative HPLC chromatograph of standard flavonoids viz. myricetin, quercetin, apigenin and gallic acid with specific Retention

time (Rt) 4.08, 5.70, 8.16 and 4.03 min respectively. B. HPLC chromatogram of fraction obtained from chloroform portion having characteristic peak of myricetin. C. HPLC chromatogram of fraction obtained from ethyl acetate portion having significant presence of myricetin and apigenin.

Conclusion

Campylandra aurantiaca is utilised as an antidiabetic in Indian folk-medicine; furthermore, its antioxidant properties suggest a potential usefulness in the prevention of diabetes complications associated with oxidative stress. The contribution of a flavonoid-rich fraction, identified through HPLC there is a presences of flavonoid which have good antioxidant property and that is useful in further antidiabetic study.

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CHAPTER-3

ACUTE TOXICITY STUDY

Introduction

Natural resources were the basis of treatment in human diseases since the existence of Human civilization. The modern day medicine or allopathic medicines are the result of scientific and observational efforts of scientists. But the concept and development of modern medicine remains rooted in traditional medicine and therapies. (Patwardhan *et al*., 2004).

When a chemical substance or drug administered to a biological system, different types of interactions can occur and also observed series of dose-related responses. In some cases these responses are desired and useful, but in some condition there are a number of other effects which are undesired or toxic.

In the process of new drug development and Pharmaceutical R&D the types of toxicity tests which are routinely performed acute, sub-acute and chronic toxicity. Acute toxicity is involved to determine the LD_{50} of the drug and new chemicals. The dose which has causing death to 50% of the tested group of animals is termed as LD_{50} of the drug.

To determine the toxic characteristics of any compounds acute oral toxicity is usually an initial screening step (Akhila *et al*., 2007). Acute toxicity is the observation of effect produced after administration of a single dose or multiple doses in a period not exceeding 24 hours, up to a limit of 2000 mg/k g of body weight. The main objective of acute toxicity studies is to identify a dose causing major adverse effects and an estimation of the dose that causing lethality (Sathya *et al*., 2012).

Toxicity is an observation of being poisonous, which is mainly indicating the level of toxic effects due to the interaction between toxicants and cells. This adverse effect may vary depending on the chemical properties of the toxicants and the cell membrane, which is mainly

occur on the cell surface, within the cell body, or in the tissues beneath as well as at the extracellular matrix. Prior to the binding of the toxicants to the vital organs such as liver and kidneys the toxic effects may take place. So it is very difficult to evaluation of toxic properties of a substance when considering for public health protection because exposure to chemicals can be hazardous and results to adverse effects on human being. In practical, the toxicity is typically evaluated by acute, sub-chronic, chronic, carcinogenic and reproductive effects (Zakaria *et al*., 2011).

In developing countries the natural products from medicinal plants have become universally popular in primary healthcare, and some have been mistakenly regarded as safe just because they are a natural source. But, there is a not as such proven scientific study on the toxicity and adverse effect of these remedies. Therefore, the acute oral toxicity study is vitally needed not only to identify the range of doses that could be used subsequently, but also to reveal the possible clinical signs or toxic effect elicited by the substances under investigation. Determination of the therapeutic index of any drugs and xenobiotics, also a useful parameter (Zakaria *et al*, 2011).

Various Methods for LD_{50} Determination

Litchfield And Wilcoxson Graphic Method (Litchfield *et al*, 1949)

Here different doses are given to different groups of animals and mortality recorded. Then graph is prepared with percentage mortality vs. log dose.

Miller Unitary Method (Pulgarin *et al*, 2003)

Here any dose is taken say 1 mg or 1 ml or 1 gm or 1 mg/ml. The unit dose is given to two mice and observed for 24 hrs. If the dose is tolerated by the animals, the dose is modified by multiplication with 3/2; not multiplied by 2/3. This has to be carried out until a dose causes a death of one animal, i.e. 50% death as two animals were used. However, this result is not reliable

until and unless it is verified with more number of animals. But the method is easy, less time consuming and economical.

Trevan's Method

In order to avoid biological response and to get authentic result LD_{50} and LD_{100} are to be measured first and then some doses are chosen in between them and percentage mortality is observed. LD_{50} is determined graphically.

Up and Down Procedure (UDP)-OECD TG425

For determination of acute toxicity (LD50) an up-and down method has been developed and statistically evaluated. This method permits a major reduction in the number of animals used as Compared with the "classical" procedure. In this procedure, animals are dosed one at a time. If an animal survives, the dose for the next animal is increased; if it dies, the dose is decreased (Bruce, 1985).

Each animal is observed for 1 or 2 days before dosing the next animal. Surviving animals monitored for delayed death for a total of 7 days.

Method of toxicity study

The method followed for acute toxicity was Up and Down Method following OECD guidelines TG425. Five healthy Swiss albino mice were used for the experiment. These pre-acclimatized fasted animals were received sequentially with dose of 2000 mg/kg body weight of EFCA with each animal were observed for 48 hrs for safety. The animals were scrutinized for increased motor activity, anesthesia, tremors, arching and rolling, clonic convulsions, tonic extension, lacrimation, Straub reaction, salivation, muscle spasm, writhing, hyperesthesia, loss of righting reflex, depression, ataxia, stimulation, sedation, blanching, hypnosis, cyanosis and analgesia.

Result

Oral administration of methanol extract of FEFCA up to 2000 mg/kg body weight did not produce any significant alteration in the behaviour, breathing, cutaneous effects, sensory nervous response or gastrointestinal effects. During the toxicity study no deaths occurred which inferring that is safe upto dose 2000 mg/kg body weight.

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CHAPTER-4

IN VIVO ANTIOXIDANT STUDY

Introduction

Oxidation gets importance and recognized widely in the body and in food stuffs. It is important and essential for survival of cells. Major drawbacks of these system is the production of free radicals and other reactive species causing oxidative changes (Antolvich *et al*, 2002). Oxidative stress has played a crucial role in various pathological conditions like cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing. These diseases are divided into two parts: (i) the first group has shown its effect by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance—the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) the second group acts by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS implicated in ischemia and reperfusion injury (Valko *et al*, 2007). The ROS and RNS generated further radicals and inactive by antioxidants (Bala *et al*, 2009). The Human body has its own protective system to neutralize or scavenge the radicals by using body's own antioxidant enzymes like glutathione, catalase and superoxide dismutase by donating the hydrogen ions to the respective radicals (Chakraborty *et al*, 2015) though these radicals can be scavenged by the in vivo antioxidant component, but they are not sufficient to completely remove them to maintain a balance. As a result, external sources of antioxidants are required to scavenge excess free radicals (Bala *et al*, 2009). The immune cells are directly linked to ROS and RNS generation and mainly propagated the redox potential of body. So the free radicals and antioxidants balance are important indicators of immune cells activity (Bala *et al*, 2009).The antioxidant plays an important role to protecting them from free radicals induced oxidative stress to maintain the normal cellular activity (Chakraborty *et al*, 2015). It was clearly established that excessive

amounts of free radicals are dangerous for the immune cells, by damaging cellular components, oxidizing the membrane lipids, protein, carbohydrates and nucleic acids. To minimize or neutralize the unwanted effect of free radicals body has developed an own antioxidant environment against the free radicals to protect the cells (Fuente *et al*, 2000). Macrophages are one of the most important immune cells which work by multifunctional way. Extend from clearance of micro-organisms, xenobiotic material and apoptotic cells to regulating both innate and acquired immune responses through antigen presentation to secretion of various cytokines and chemokines. In order to maintain these tasks, they have evolved several strategies; which include the recognition and adherence to particular substrates, migration in response to chemoattractant, activation of inflammatory responses towards removal of a pathogen, and phagocytosis of foreign bodies or apoptotic cells. Phagocytosis of apoptotic cells is an immunologically silent process that plays a major role in the resolution of an inflammatory response (Kirkham, 2007). To mimic immunosuppressed condition in mice, carbon tetra chloride (CCl_4) was used because earlier it was correlated with not only in liver damage but also confers an immunocompromised state particularly concerning macrophage function (Chakraborty B and Sengupta M, 2012). The abnormal functioning of the immune system leads to several chronic illnesses and protection of immune system using plant compounds or plant extracts is in recent times getting renewed interest because of their minimal side effects (Chakraborty B and Sengupta M, 2012). The plant *Campylandra aurantiaca*, belongs to Asperagaceae family, grown in high altitudes (Chhetri *et al*, 2005). The rhizome is traditionally used for it's antidiarrhoeal, antidysentric, analgesic, antimalarial, antiarthritic, vermicidal, antipyretic and stomachic (Kagyung *et al*, 2010). Therefore, the present study was performed to focus on antioxidant

profile and DNA protective effect of *Campylandra aurantiaca* on oxidative stress induced toxicity in murine peritoneal macrophages.

Materials and methods

Chemicals

1,1- diphenyl- 2- picryl- hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, napthyl ethylene diamine dihydrochloride, ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, H_2O_2, butylated hydroxy anisole, deoxyribose, Folin- Ciocalteu's phenol reagent, and carbon tetrachloride (CCl_4) were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were used in high analytical grade.

Extraction, Isolation and Characterization of Plant Material

The rhizome of *Campylandra aurantiaca* was collected from the upper hilly region of Eastern Himalayan, Sikkim, India. Authenticated air dried whole bark (500 g) was powdered in a mechanical grinder, and the powdered materials were extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus. The solvents were completely evaporated under reduced pressure. Methanol extract was used for further fractionation. Fractionation was performed by separating funnel using solvent system of petroleum ether, chloroform and ethyl acetate (Das *et al*, 2012). Two flavonoids enriched fraction of *Campylandra aurantiaca* (FEFCA) were obtained from the chloroform and ethyl acetate fraction as per the protocol described earlier (Das *et al*, 2012 and Dua *et al*, 2015).HPLC Characterization of isolated fractions of *Campylandra aurantiaca* was performed by HPLC system Ultimate 3000 Germany), using a reverse phase C-18 column (250 × 4.6 mm, particle

size 5 μ) and UV detector. HPLC grade methanol was used to prepare samples and filtered by cellulose nylon membrane filter (0.45 μm). The aliquots of the filtrate were eluted with an isocratic solvent mixture comprising methanol: acetonitrile: acetic acid: o-phosphoric acid: water (20:10:1:1:20) for flavonoids. Methanol: water: acetic acid (75:24:1) for phenolic compounds. Flow rate of 1 ml/min was used in the system and detected at 352 nm and 254 nm respectively. Standard phytochemical markers, i.e. gallic acid, myricetin, quercetin and apigenin were used to characterized the fraction (Dua *et al*, 2015). Flavonoid enriched fraction obtained from ethyl acetate portion was used for further evaluation and consider as FEFCA in the study.

Animals

Male Swiss Albino mice (20–25 g) were taken from Chakraborty Enterprise, Kolkata, India. The animals were maintained under standard laboratory conditions and were allowed free access to standard dry pellet diet and water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the procedures described were reviewed and approved (AEC/PHARM/1413/2014) by the University Animal Ethical Committee.

Acute Toxicity

The acute oral toxicity of FEFCA in Swiss albino mice was performed as per OECD guideline 425 (OECD 2008).

***In vivo* Antioxidant Activity in Mouse Peritoneal Macrophages** (Bala *et al*., 2012b).

The animals were divided into four groups (n = 12):

Group I: Normal vehicle control: Received liquid paraffin (1 ml/kg i.p.) as vehicle control for 2 days (Day 1 and 2).

Group II: CCl_4 control: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2).

Group III: N- acetyl cysteine (NAC) control: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2). NAC (150 mg/kg i. p.) treatment was started before 24 h of the CCl_4 treatment and continued for Day 1 and Day 2.

Group IV and V: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2). FEFCA (50 and 100 mg/kg i. p. respectively) treatment was started before 24 h of CCl_4 treatment and continued for Day 1 and 2. At the day third, the six mice were sacrificed under ether anesthesia, and peritoneal macrophages were lavaged aseptically using ice cold PBS (0.02 M, pH- 7.4).

Both Superoxide anion production in macrophages cells and SOD in the cell lysate were estimated by NBT methods. Oxidative damage to the DNA was estimated according to the standard protocol (Bala *et al*,2012a and Bala *et al*, 2012b). The lipid peroxidation was measured by TBA- reactive substances (TBARS) assay. The nonenzymatic antioxidant reduced GSH in the cells lysate was estimated by 5,5'- dithiobis- 2- nitrobenzoic acid method (Bala *et al*, 2012b).

Data Analysis

All the values are given as mean ± SEM by using GraphPad Prism software 5.1. One-way ANOVA followed by Dunnett's post hoc test of significance was performed by GrapPad Instat software (GraphPad Software Inc,San Diego, CA, USA) where ($p<0.01$) reduced the oxidative DNA damage in macrophages (Figure. 1).

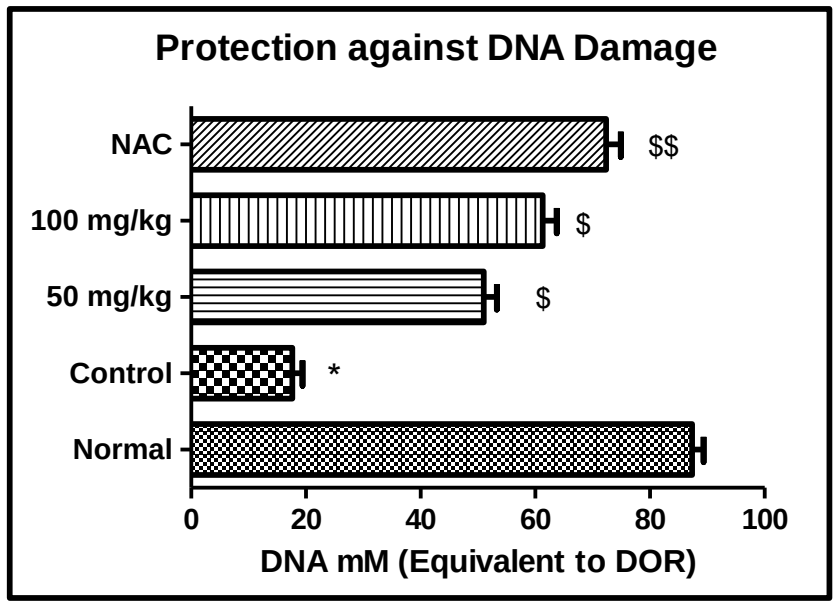

Figure: 1. Protection on DNA damage in macrophages. DNA estimated with respect to deoxy ribose standard (DOR, mM). Values are expressed as mean ± SEM. *p<0.05 (with respect to normal) and $^{\$}p$<0.05, $^{\$\$}p$<0.01, with respect to control.

NAC and FEFCA significantly reduced the superoxide anion level (Figure. 2A). Activity of SOD was measured for further investigation of the effect of CCl4 on the regulation of intracellular superoxide anions level. There was a significant reduction of SOD level in CCl_4 treated mouse cells which was significantly increased by NAC and FEFCA treatment respectively due to free radicals scavenging property (Fig. 2B).

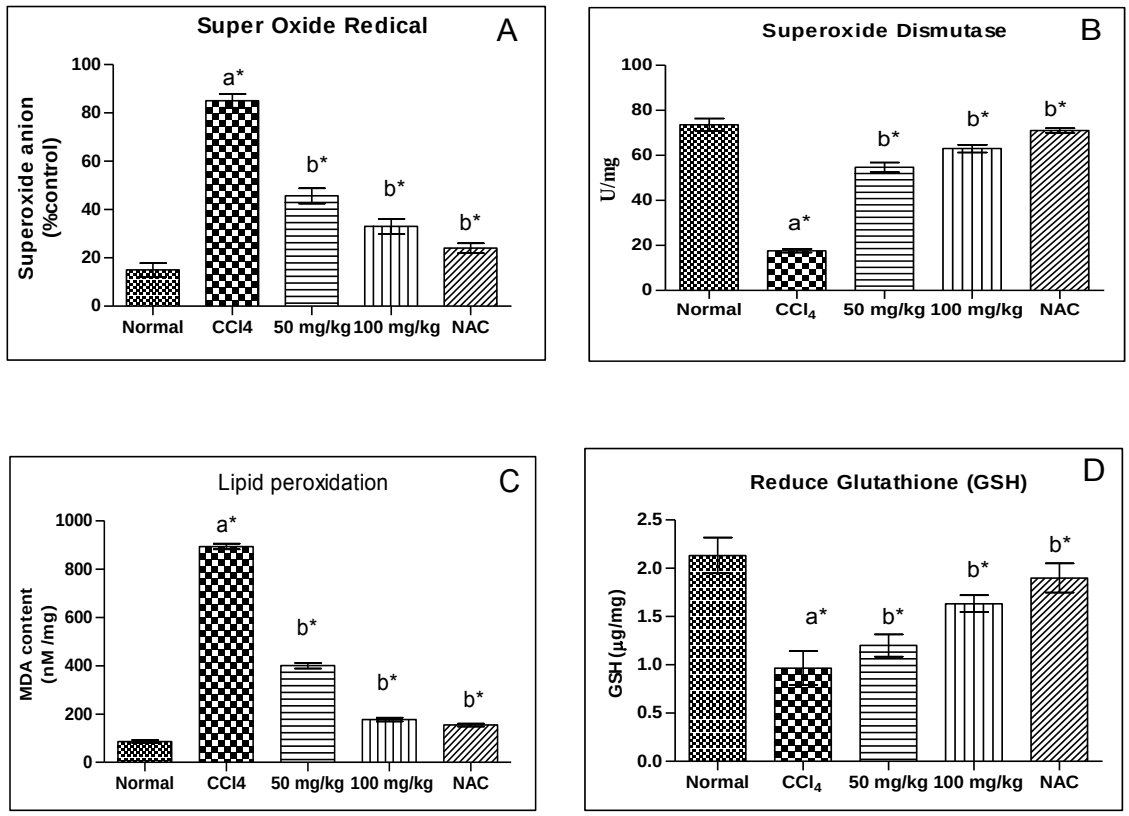

Figure: 2. Effect of FEFCA on O_2^- (A), SOD (B), LPO (C), GSH (D). Each value is expressed as Mean ± SEM where n=6. a* p<0.05 when compared to normal and b* p<0.05when compared all treated group vs. respective control group**.**

Interestingly, treatment with FEFCA and NAC significantly reduced the MDA levels as compared with CCl_4 control group, in the present study the MDA level was significantly increased in CCl_4 control animals when compared with normal control animals (Fig. 2C). Administration of FEFCA and NAC in a dose dependent manner significantly raised the reduced glutathione levels as compared with CCl_4control animals. The levels of reduced GSH were significantly decreased in CCl_4 control group when compared with normal control group (Fig. 2 D).

Discussion

Most of the modern diseases are due to the results of free radicals induced "oxidative stress" which mainly causes due to formation and neutralization imbalance of free radicals. By electron pairing with biological macromolecules such as proteins, lipids, and DNA in healthy human cells free radicals get stability and ultimately cause protein, DNA, and lipid oxidation (Chakraborty *et al*, 2016). Free radicals are continuously generated and causes damage to the body either by damaging antioxidant system or by targeting the signalling routes and expression of molecules involved in the inflammatory cascade (Karmakar *et al*, 2011). In the present study we investigated the antioxidant activity of FEFCA in some in vitro as well as in vivo antioxidant models. In all the models, FEFCA showed its ability to scavenge the free radicals in a concentration dependent manner. We also correlated the in vitro antioxidant models with the in vivo CCl_4 induced generation of free radical directly on macrophage. Different approaches are now introduced to reduces the oxidative stress which mainly focus the neutralizing or scavenge the radicals or inactivation of free radical producing enzymes, influence the antioxidant system or by targeting the signalling routes and expression of molecules involved in the inflammatory cascade (Bala *et al*,2012a and Demopoulos *et al*, 1980). A number of synthetic drugs having the protective effects also cause side effects. To overcome these problems an alternative solution is to consume natural antioxidants from food supplement or in the form of traditional medicines (Gilgun-Sherki *et al*, 2001). Here oxidative DNA damage induced by H_2O_2 and the effect of FEFCA in the mouse macrophages cells (*in vivo*) and isolated DNA from macrophage was estimated by diphenylamine reagent. H_2O_2 (10 mM) significantly ($p < 0.01$) caused DNA damage in mouse macrophages that was attenuated by FEFCA in a concentration dependent manner. Most prevalent ROS that affect profoundly the lipid molecules are mainly hydroxyl

radical and superoxide (Kundu *et al*, 2011). Cellular physiology and pathology also have a great link to lipid peroxidation which can be measured by increase in the level of malondialdehyde (MDA) in the in vivo model of CCl_4 treated mice. Production of free radicals via CCl_4 involved at least two mechanisms. The first is via biotransformation of CCl_4 to a free radical species or formation of Conversion of CCl_4 to the trichloromethyl radical by the hepatic mixed function oxides system which ultimately responsible for lipid peroxidation, protein-lipid cross linkages and altercation of DNA. The second way is formation of less stable free radical intermediates via reduction which are further going to oxidize with the help of molecular oxygen and generate superoxide Bala *et al*, 2012b and Ayala *et al*, 2014). The *in vitro* neutralization of OH– may be correlated with the *in vivo* reduction in MDA content. In the present study the MDA level was significantly increased in CCl_4 control animals when compared with normal control animals. However decreased activity was found when treated with FEFCA. With help of NADPH oxidase, xanthine oxidase, nitric oxide synthase (NOS), lipoxygenase, and mitochondrial enzymes superoxide is generated which is further neutralized by the enzyme superoxide dismutase (SOD) to H_2O_2, which, in turn, is reduced to water by catalase, glutathione peroxidases (GPx), and peroxiredoxins (Prx) (Fukai T and Fukai UM, 2011). The generation of superoxide anion and the reduction of the SOD level in mouse peritoneal macrophages by CCl_4 and their subsequent reduction by FEFCA were observed. Cells are highly rich source of soluble antioxidant GSH which play a major role in antioxidant mediated defence system. GSH functions as an antioxidant in several ways. It neutralizes the hydrogen peroxide and lipid peroxides via action of GSH-Px (Fukai T and Fukai UM, 2011). By donating an electron it converts H_2O_2 to H_2O and O_2. GSSG is again reduced into GSH by GSH reductase that uses NAD(P)H as the electron donor (Birben *et al*, 2012 and Chakraborty SP and Roy S, 2014). GSH-

Pxs has shown their antioxidant effect by protecting the cell membrane from lipid peroxidation. Reduced glutathione has shown its antioxidant effect by donating protons to membrane lipids (Ferrari, 2000 and Noori, 2012). The decreased level of GSH was observed in this study after administration of CCl_4 to the mice is associated with an increased level of lipid peroxidation and DNA damage. However, it has been demonstrated that the level of GSH could be enhanced in mice pre-treated with NAC and FEFCA.

Conclusion

Therefore, FEFCA showed good free radical scavenging activity as well as reduced oxidative DNA damage in mouse peritoneal macrophages in animal model.

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CHAPTER-5

IN VITRO ANTI DIABETIC ACTIVITY

Introduction

Diabetes mellitus is a metabolic disorder, which is mainly characterized by an abnormal postprandial increase of blood glucose level. The control or normalised of postprandial hyperglycemia is believed to be important in the treatment of diabetes mellitus because diabetes mellitus is directly associated with blood glucose level (Yin *et al*; 2014).

Disorders of carbohydrate uptake may cause severe health problems such as diabetes, obesity, and oral diseases, all of which threaten an increasing worldwide population (Michelle de Sales *et al*; 2012).

Carbohydrates are normally converted into simple sugars (monosaccharide), which are absorbed through the intestine. So one of the antidiabetic therapeutic approaches is to reduce gastrointestinal glucose production and absorption. α- amylase and α- glucosidase enzymes digest carbohydrates and increase the post prandial blood glucose after a mixed carbohydrate diet. Therefore blood glucose can be controlled by inhibiting these carbohydrate digesting enzymes such as α- amylase and α-glucosidase (Thilagam *et al*; 2013).

A amylase is an enzyme, found in many tissues but mostly found in pancreatic juice and saliva. The α-amylases are the calcium metallo enzymes which are directly related to calcium and are not able to function in the absence of calcium. Human digestive system there are many digestive enzymes among them the most important one is pancreatic α-amylase, that mainly act as a catalysis in the reaction which involves the hydrolysis of the α-1,4 glycosidic linkages of the starch, amylopectin, amylose, glycogen, and numerous maltodextrins and is responsible for starch digestion (Agarwal and Gupta; 2016).

In the brush border membrane of the small intestine, the enzyme α glucosidase mainly inhibit maltase, sucrase and other disaccharide to hydrolases (i.e., suppress the degradation of disaccharides to monosaccharides. a-Glucosidase inhibitors (aGIs) are widely used for the primary treatment of type 2 diabetes. Therefore, aGIs can improve postprandial hyperglycemia by delaying carbohydrate absorption (Sugihara *et al*; 2014).

α-glucosidase and α-amylase, enzymes are basically involved in the digestion of carbohydrates metabolism, and can significantly decrease the postprandial increase of blood sugar after a mixed carbohydrate diet and therefore it is one of the most important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and borderline patients (Subramanian *et al*; 2008).

Drugs and Chemicals

α-glucosidase (50 µl, 0.5 U/ml) and 0.2 M K3PO4 buffer (pH 6.8, 50 µl), Acarbose, α amylase, PNPG, starch, Na_2CO_3, Iodine solution. All the other reagents used were of analytical reagent grade obtained commercially.

Extraction and Fractionation of plant material:

The rhizome of *Campylandra aurantiaca* was collected from the upper hilly region of Eastern Himalayan, Sikkim, India. Authenticated air dried whole bark (500 gm) was powdered in a mechanical grinder, and the powdered materials were extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus. The solvents were completely evaporated under reduced pressure. Methanol extract was used for further fractionation. Fractionation was performed by separating funnel using solvent system of petroleum ether, chloroform and ethyl acetate (Das *et al.*, 2011). Ethyl acetate fraction was used for the study.

***In vitro* α-amylase inhibitory activity (**Hossan *et al*., 2009)

This study was performed by a modified starch iodine protocol. In short, 1 ml of plant extract or standard (Acarbose) of different concentration (100, 300,500 and 1000 µg/ml) was taken in pre labelled test tubes. A volume of 20 µl of α-amylase was added to each test tube and incubated for 10 min at 37 °C. After the incubation 200 µl of 1% starch solution was added to each test tube and the mixture was re-incubated for 1 h at 37 °C. Then 200 µl of 1% iodine solution was added to each test tube and after that, 10 ml distilled water was added. Absorbance of the mixture was taken at 565 nm. Sample, substrate and α-amylase blank were undertaken under the same conditions. Each experiment was done in triplicate. IC_{50} value was calculated by using regression analysis.

% α-amylase inhibition= [1- {(SA-SBB)-SMB}/ AAB] x 100

where SA=Sample absorbance, SMB=Sample blank, SBB=Substrate blank, AAB=α-Amylase blank.

α-glucosidase inhibition assay (Kim *et al*.,2011**)**

α-glucosidase (50 µl, 0.5 U/ml) and 0.2 M K3PO4 buffer (pH 6.8, 50 µl) were mixed with 50 µl of the test sample. After pre-incubation at 37 °C for 15 min, 3 mM PNPG (100 µl) was added. The enzymatic reaction was allowed to proceed at 37 °C for 10 min, and was stopped by the addition of 750 µl of 0.1 M Na_2CO_3 . The 4-nitrophenol absorption was measured at 405 nm using a spectrophotometer. A solution without sample was used as a control and a solution without substrate was used as a blank. The antidiabetic drug acarbose was also assayed as a standard reference.

% inhibition of α-glucosidase =1 – {(Abs sample – Abs blank)/Abs control} × 100%

where Abs sample represents the absorbance of the sample Abs blank is the absorbance of the blank and Abs control is the absorbance of control.

Result

Inhibition assay for α-amylase and α-glucosidase activity

The results of the study are summarized in Figure 1 and Figure 2. The flavonoid enriched fraction of the plant *Campylandra aurantiaca* significantly inhibit the enzyme α-amylase and α-glucosidase in concentration dependent manner.

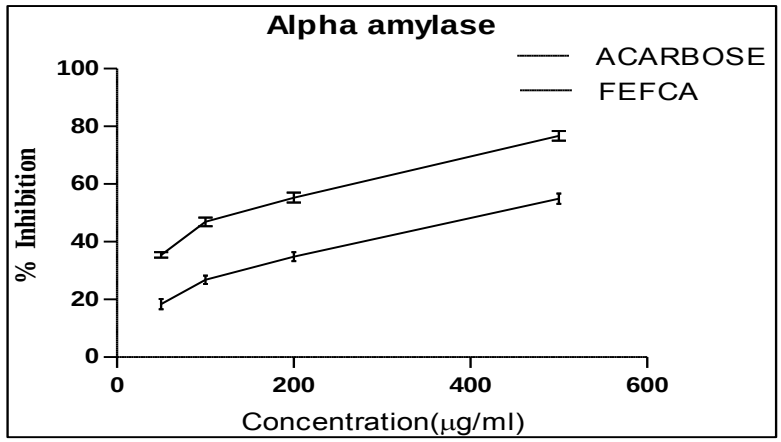

Figure-1: % inhibition of α amylase shown by different concentrations of FEFCA and Acarbose.

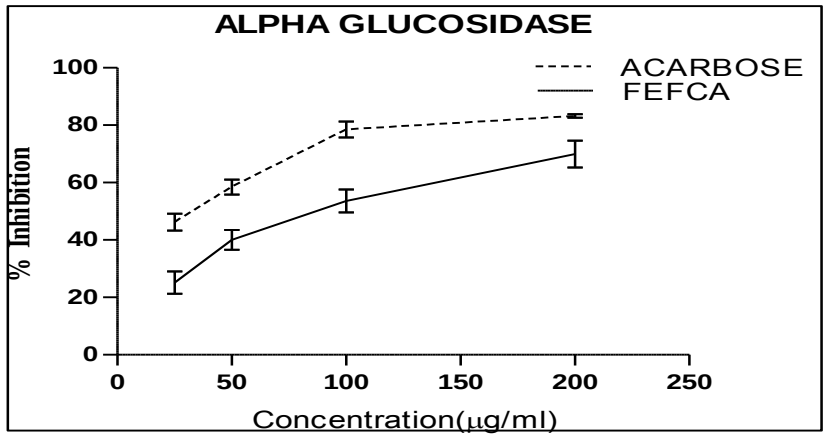

Figure-2: % inhibition of α glucosidase shown by different concentrations of FEFCA and Acarbose.

The IC_{50} value of α-amylase FEFCA was 445±4.21 and standard drug Acarbose was 149±7.37. In case of α-glucosidase case IC_{50} value FEFCA was 86.7±3.41 and Standard drug Acarbose was 36.3±5.21.

Discussion

The present study aimed to evaluate the *in vitro* antihyperglycemic activity of Flavonoid Enriched Fraction of *Campylandra aurantiaca*. The main objective of diabetes is to maintain near normal levels of blood glucose, in both fasting as well as post- prandial conditions.

The key enzymes for carbohydrate metabolism in the small intestine are pancreatic α-amylase and α-glucosidase which convert consumed polysaccharides to monosaccharides. This enzyme action causes postprandial blood glucose level elevation due to absorption of formed glucose from polysaccharides in the small intestine (Ramachandram *et al*; 2013).

Many natural sources have been investigated with respect to suppression of glucose production from the carbohydrates in the gut or glucose absorption from the intestine (Mastsui *et al*., 2001).

The parameters checked were enzyme inhibition capabilities; Activities of enzymes like α-amylase and α-glucosidase in the body are responsible for postprandial hyperglycemia by break down of dietary carbohydrates to glucose. Hence, the inhibitory effect of Flavonoid Enriched Fraction of *Campylandra aurantiaca* (FEFCA) on these enzymes may lead to reduction in post prandial hyperglycemia in diabetes.

Postprandial hyperglycemia has been proposed as an independent risk factor for various diseases. Therefore, control of postprandial hyperglycemia is considered to be important in the treatment of diabetes and prevention of complications related to diabetes.

Drugs which are able to inhibit the action of these enzymes possess an ability to control of postprandial blood glucose level specifically in type 2 diabetic patients or borderline patient. Currently, available drugs in this category which mainly inhibit the action of these two enzymes are acarbose and miglitol. But these drugs have common side effects such as flatulence and abdominal bloating. New drugs or formulations which are devoid of the above side effects will improve the compliance in type 2 diabetic patients. Our present study results clearly demonstrated that FEFCA possesses potent pancreatic α-amylase and α-glucosidase inhibition which confirmed that the FEFCA can control the post prandial hyperglycemia (Ramachandram *et al*; 2013).

Conclusion

The current study clearly demonstrates that daily consumption of Flavonoid Enriched fraction of *Campylandra aurantiaca* (FEFCA) exhibit a pronounced hypoglycemic effect (FBG) and also control the postprandial hyperglycemia. However, investigation of secondary metabolites of this fraction responsible for hypoglycemic effect should be undertaken in order to confirm the compound which is responsible for this activity.

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CHAPTER-6

IN VIVO ANTI-DIABETIC ACTIVITY

Introduction

Diabetes mellitus, a metabolic disorder in which a person has high blood sugar, has become a matter of serious challenge because of its worldwide prevalence. This may be because of the body's inability to produce enough insulin, or because of lesser response of the cells to insulin that is being produced. Diabetes mellitus are of different types based on the causes. The majority of type 1 diabetes (IDDM) is related to immunity, where beta cells are destroyed by T-cell mediated autoimmune attack. Type 2 diabetes (non-insulin-dependent diabetes mellitus or NIDDM) occurs from insulin resistance, and sometimes involves deficiency in insulin production (Diabetes Care, 1997).

The prevalence of diabetes is growing worldwide due to urbanization and improper lifestyle management. In the Western countries, diabetes mostly develops with age but in Asian countries it is attacking mostly young to middle-aged people. This scenario is leaving a huge impact on health and economy of the developing countries. The International Diabetes Federation (IDF) has an estimation of the total number diabetics in India to be around 50.8 million in 2010 and gradually rising to about 87.0 million by 2030. Recent reports collected from various parts of India have detected the invading nature of diabetes in urban areas. Moreover, prevalence of diabetes is also simultaneously increasing in rural areas, as a result of the recent socioeconomic growth (Ramachandran *et al*, 2010).

Obesity is a complex, multi factorial, and largely preventable disease, affecting, along with overweight, over a third of the world's population today. If secular trends continue, by 2030 an estimated 38% of the world's adult population will be overweight and another 20% will be obese.

Obesity greatly increases risk of chronic disease morbidity—namely disability, depression, type 2 diabetes, cardiovascular disease, certain cancers—and mortality. Childhood obesity results in the same conditions, with premature onset, or with greater likelihood in adulthood (Hruby and Hu, 2015).

Recent studies have identified "links" between obesity and type 2 diabetes involving pro inflammatory cytokines (tumor necrosis factor and interleukin-6), insulin resistance, deranged fatty acid metabolism, and cellular processes such as mitochondrial dysfunction and endoplasmic reticulum stress. These interactions are complex (Eckel *et al*, 2011).

Obesity and T2DM frequently occur together, and statistics show that 60–90% of all patients with T2DM are or have been obese. Obesity is generally considered to be a strong risk for the later development of T2DM. Studying this problem over time, the question arises whether obesity is not only a risk factor but also a cause of T2DM (Golay, 2005).

Marketed oral hypoglycemic agents exhibit variety of adverse effects including congestive heart failure with glitazones , gastrointestinal disturbances with glucosidase inhibitors, sulfonylureas and meglitinides. Cardiac problems and weight gain are common adverse effects of sulfonylureas. Therefore it's a urge a develop to less toxic therapeutic agent. Due to the multiple pathophysiological consideration there is a need to develop multi target therapeutic agent which would be helpful for treatment of T2DM and its associated pathogenesis. Plant extract are a multiple compounds mixture and shown there therapeutic value in multiple way. Therefore present investigation has focusing the antidiabetic potential of plant extract considering ethno pharmacological knowledge as reference (Bhattacharjee *et al,* 2016).

Drugs and Chemicals

Trichloroacetic acid (TCA) from Merck Ltd., Mumbai, India;thiobarbituric acid (TBA), streptozotocin (STZ), 5,5´-dithio *bis*-2-nitro benzoic acid (DTNB), phenazonium methosulfate (PMS), nicotinamide adenine din-ucleotide (NADH) and reduced glutathione (GSH) from SISCO Research Laboratory, Mumbai, India;potassium dichromate, glacial acetic acid from Ranbaxy, Mumbai; and glibenclamide from Hoechst, India. All the other reagents used were of analytical reagent grade obtained commercially.

Extraction and Fractanation of plant material

The rhizome of *Campylandra aurantiaca* was collected from the upper hilly region of Eastern Himalayan, Sikkim, India. Authenticated air dried whole bark (500 g) was powdered in a mechanical grinder, and the powdered materials were extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus. The solvents were completely evaporated under reduced pressure. Methanol extract was used for further fractionation. Fractionation was performed by separating funnel using solvent system of petroleum ether, chloroform and ethyl acetate (Das *et al,* 2011). Ethyl acetate fraction which is rich in flavonoid called as flavonoid enriched fraction of *Campylandra aurantiaca* (FEFCA) was used for the study.

Animals

Six- to eight-week-old male Wistar albino rats (180 ± 20 g) were obtained from Chakraborty and Co., Kolkata. They were acclimatized to the laboratory conditions prior to the study for seven days. The animals were kept at 25 ± 2°C and a relative humidity of 40–45% with alternative day and night cycles of 12 hours each. The animals had free access to pellet food (Hindustan Lever, Mumbai, India) and water ad libitum.

Acute toxicity

FEFCA was administered orally to male Swiss Albino mice to evaluate the acute toxicity as per the reported method Chakraborty *et al*, 2015.

Induction of diabetes with high-fat diet and streptozotocin

Male rats were fed with high-fat diet comprising 22% fat, 48% carbohydrate, and 20% protein in blend with standard laboratory chow consisting of 5% fat, 53% carbohydrate, and 23% protein for 4 weeks. After the period of dietary manipulation, rats were injected intraperitoneally (i.p.) with low dose of STZ (35 mg/kg). Then, animals had free access to water and standard food (Ghorbanzadeh *et al*, 2016). One week after streptozotocin injection, the fasting blood glucose levels were appraised and the animals exhibiting fasting blood glucose levels of 170 ± 30 mg/dl were considered to be type 2 diabetic (T2D) rats and included for the further experiments (Bhattacharjee *et al*, 2016).

Oral glucose tolerance test

The oral glucose tolerance test was performed in overnight fasted normal rats. Rats were divided into three groups (n=6). Group I served as normal control and received distilled water (5 ml/kg b.w. p.o), and groups II and III received FEFCA at doses of 50 and 100 mg/kg b.w., respectively. After these treatments, all groups received glucose (2 g/kg b.w.) orally. Blood was withdrawn from the tail vein just prior to and 30, 60, 120 min after oral glucose administration (Haldar *et al*, 2010). Blood glucose levels were measured using single touch glucometer (Accu-check, Roche Diagnostics, USA).

Experimental design was made as per Ghorbanzadeh , *et al* 2016 and Bhattacharjee ,*et al* 2016 with some modification

T2DM was induced by high fat diets *ad libitum* and low-dose of streptozotocin as per

Ghorbanzadeh *et al*, 2016 and Bhattacharjee *et al*, 2017. Briefly, the rats were fed high fat diets ad libitum for 4 weeks and then treated with single dose of streptozotocin (35 mg/kg body weight, intraperitonially) . After 7 days streptozotocin injection, the fasting blood glucose levels were appraised and the animals exhibiting fasting blood glucose levels of 170 ± 30 mg/dl were considered to be type 2 diabetic (T2D) rats and included for the further experiments. The rats were continued with high fat diets throughout the course of the study. The animals were divided into five groups (n = 6) and received the treatment for 28 days:

Group I: Normal control rats were administered normal saline (0.5ml/kg orally by oral gavage) dail.

Group II: Diabetic control rats were administered high fat diets + normal saline (0.5ml/kg daily).

Group III: Diabetic rats were administered high fat diets + FEFCA (100 mg/kg body weight) orally daily.

Group IV: Diabetic rats were administered high fat diets + FEFCA (200 mg/kg body weight) orally daily.

Group V: Diabetic rats were administered high fat diets + glibenclamide (0.5 mg/kg body weight) orally daily

Estimation of FBG level

The rats were divided into five groups (n=6). Except group I, which served as normal control, all other groups were comprised of diabetic rats. Group II served as diabetic (HFD-STZ) control. Groups III and IV received FEFCA(100 and 200 mg/kg b.w., p.o., respectively) and group V received reference drug glibenclamide (0.5mg/kg b.w., p.o.) daily for 28 days (Biswas *et al.*, 2011). Fasting Blood Glucose was measured on day 0, 7, 14, 21 and 28 by using a one touch glucometer (Accu-check®).

Determination of serum biochemical parameters

Twenty-four hours after the last dose, blood was collected from overnight fasted rats of each group by cardiac puncture for estimation of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL), glycosylated hemoglobin (HbA1C), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) and Total protein were estimated using commercially available reagent kits (Erba Diagnostics and Span diagnostics Ltd.) (Panda *et al*, 2016).

Estimation of liver and Kidney biochemical parameters and antioxidant status

Livers and kidneys collected from the sacrificed animals were homogenized separately in 10 ml of phosphate buffer (20mM, pH-7.4) and centrifuged at 12000 rpm for 30 min at 4° C. The supernatants were collected and Lipid peroxidation (LPO), reduced glutathione (GSH), Super oxide dismutase SOD and Catalase were estimated by Chakraborty *et al*,2015 and Ellman, 1959.

Histopathological study

A portion of pancreatic tissue was dissected out and fixed in 10% buffered neutral formal saline and processed. After fixation, tissues were embedded in paraffin. Fixed tissues were cut and stained with hematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken (Gandhi *et al*, 2012).

Statistical Analysis

All the results are shown as mean ± SEM. The results were analyzed for statistically significance by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using Graph Pad Prism 5.0 software (Graph Pad Software, USA). p values of < 0.05 were considered as statistically significant.

Results

While performing the toxicity study no deaths were observed when the animals were given a dose of 2000 mg/kg b.w.

The oral blood glucose tolerance test was done in normal rats, Glucose administration to the normal rats increased blood glucose levels in first 30 mins and gradually decreased in 60 min, 120 mins and returned near to normal at 240 min which is shown in Table 1: Effect of FEFCA on OGTT.

Table 1: Effect of FEFCA on OGTT.

GROUPS	0 min	30 mins	60 mins	120 mins
Normal Control	83.67± 2.33	135.7± 1.76	125 ±1.15	106.3 ±1.76
FEFCA 100mg/kg	88.67 ± 0.88	138.7 ±1.52	132.7 ±1.45*	126.3 ±0.88 *
FEFCA 200 mg/kg	87.33± 1.45	125.7± 1.76 *	119 ±1.15*	115.7 ±1.76*
Glibenclamide	81±2.08	120±1.00 *	115±1.15*	110.3±3.2.40*

Values are represented as mean ± SEM, where n = 6. *p<0.05 when compared to normal control.

There was significantly ($p < 0.05$) elevated FBG level in HFD and STZ-induced diabetic rats as compared to normal control group. Administration of EFCA in diabetic rats at the doses of 100 and 200 mg/kg significant ($p < 0.05$) reduced the FBG level towards normal as compared to the diabetic control group Table2: Effect of FEFCA on Fasting Blood Glucose(mg/dl).

Table:2 Effect of EFCA on Fasting Blood Glucose (mg/dl).

GROUPS	DAY 0	DAY 7	DAY 14	DAY 21	DAY 28
Normal Control (5ml/kg)	75.33±2.72	80.33±1.76	82±1.52	81±1.15	79±3.15
HFD and STZ Control (35mg/kg)	176.3±4.37[a]*	187.3±3.18 [a]*	200±1.73 [a]*	207.7±4.66 [a]*	211.7±3.66 [a]*
STZ+100 mg/kg FEFCA	177.7 ±3.52	172± 2.74 [b*]	163± 2.51 [b*]	153.7 ±1.76 [b*]	139 ±2.30 [b*]
STZ+200 mg/kg FEFCA	172.7±3.28	155 ±3.21 [b*]	143 ±2.02 [b*]	135.7± 2.40 [b*]	127.7± 1.45 [b*]
STZ+0.5mg/kg Glibenclamide	176.3 ±1.76	155 ± 2.64 [b*]	143± 2.30 [b*]	116 ±3.21 [b*]	87.33 ±2.18 [b*]

Each volume expressed as MEAN±SEM, where n=6, a* normal control group vs. diabetic control group (b* all treated group vs. diabetic control group a*) on corresponding day, $p<0.05$.

Glycosylated haemoglobin has been found to be reduced in FEFCA treated animals as compared to HFD and STZ control animals. Table 3: Effect of FEFCA on HbA1c

Table 3: Effect of FEFCA on HbA1c.

Groups	**HbA1c (%)**
Normal Control	6.4 ± 0.20
STZ Control (Diabetic)	8.99 ± 0.24a*
Diabetic + FEFCA (100 mg/kg)	7.33 ± 0.14
Diabetic + FEFCA (200 mg/kg)	6.5 ± 0.14
Diabetic + Glibenclamide (0.5mg/kg)	6.02 ± 0.26

Values are represented as mean ± SEM, where n = 6. a*$p<0.05$ when compared to normal control

Similarly in case of serum biochemical parameters like SALP, SGOT, SGPT, EFCA showed significant (p<0.05) lowering when compared to the HFD and STZ control group. Level of Total protein increased in treated group when compared to the HFD and STZ control.

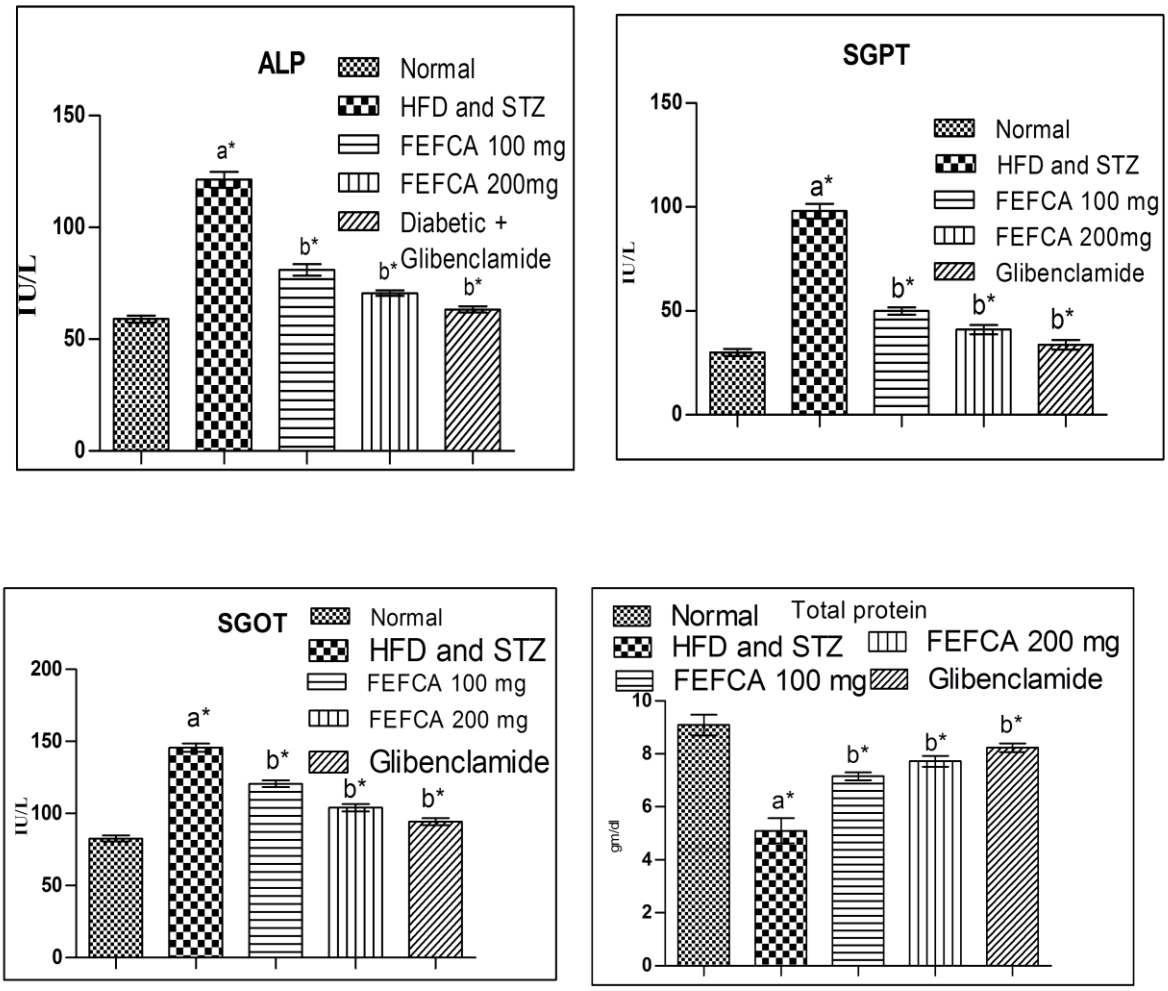

Figure: 1 Serum Biochemical Parameter. Effect of FEFCA on ALP, SGPT, SGOT and Total Protein. Each value is expressed as Mean ± SEM where n=6. a* p<0.05 when compared to normal and b* p<0.05whencompared to diabetic control**.**

Serum lipid profiles like total cholesterol and triglyceride in HFD and STZ-induced diabetic rats HDL level significantly ($p < 0.05$) decreased compared to normal control group. Treatment with FEFCA at the doses of 100 and 200 mg/kg significantly ($p < 0.05$) reduced the total cholesterol, triglyceride level and significantly ($p < 0.05$) increased the HDL level when compared to the diabetic control group

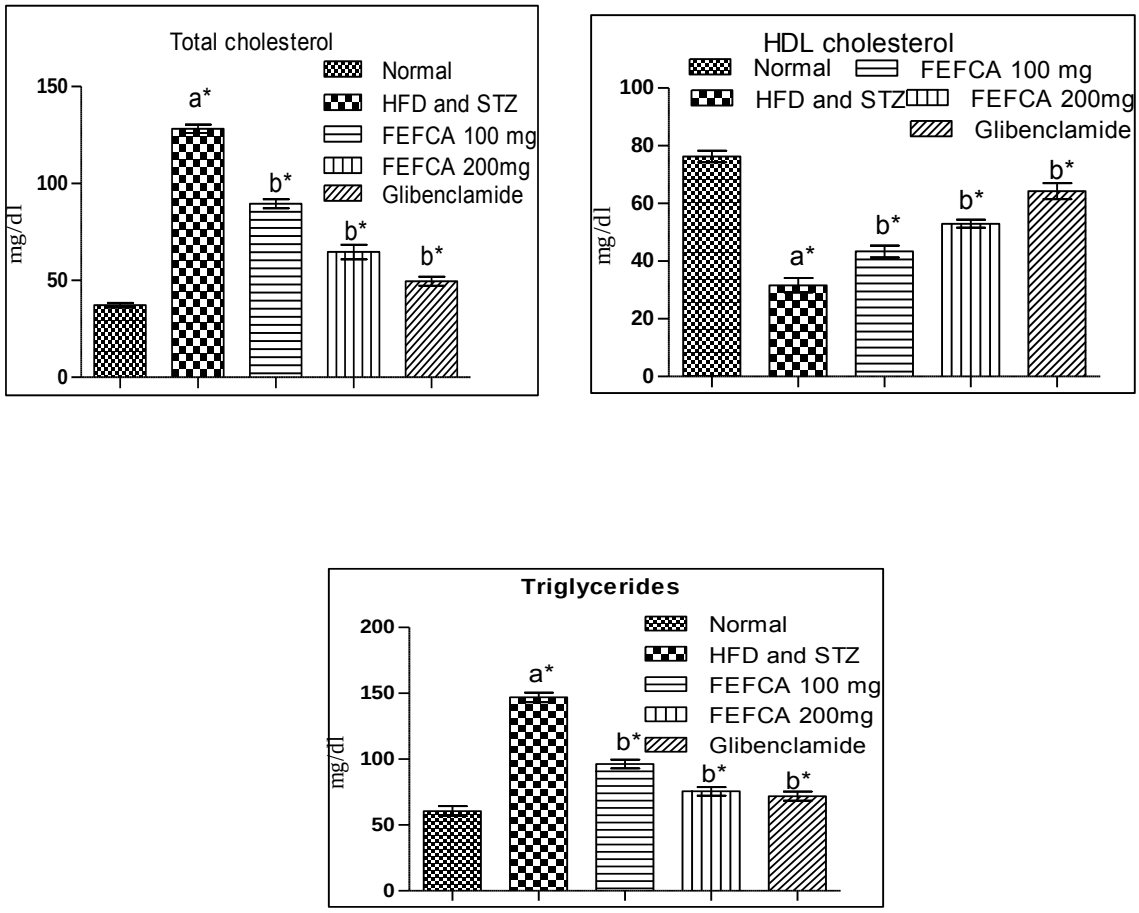

Figure: 2 serum lipid profile. Effect of FEFCA on Total cholesterol, HDL cholesterol and Triglycerides.Each value is expressed as Mean ± SEM where n=6. a* $p<0.05$ when compared to normal and b* $p<0.05$ when compared to diabetic control.

In Liver and Kidney tissue antioxidant studies, MDA level decreased (p<0.05) and SOD, GSH and Catalase level increased (p<0.05) in treated animals.

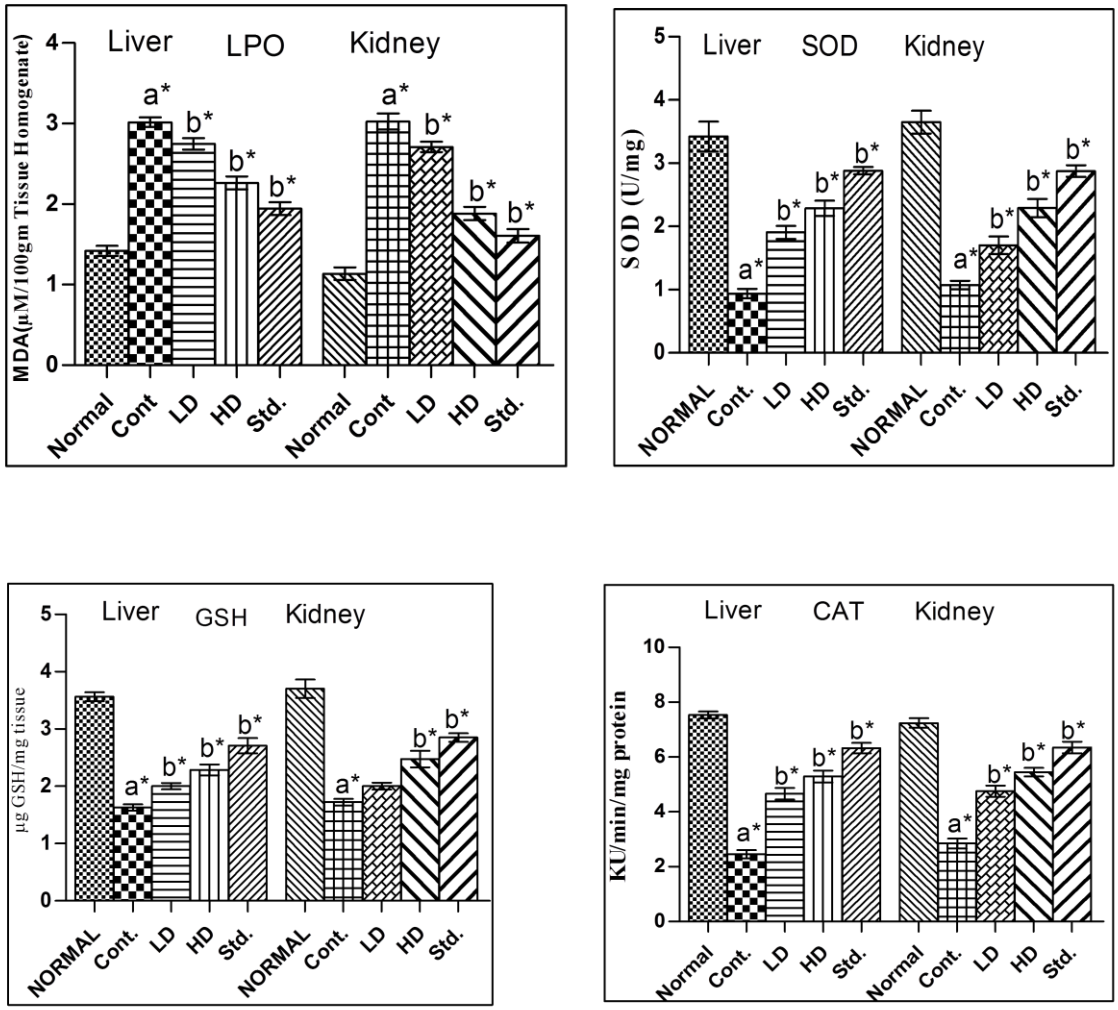

Figure 3: Liver Antioxidant Parameter. Effect of FEFCA on super oxide, lipid peroxidation, GSH and Catalase. Each value is expressed as Mean ± SEM where n=6. a* p<0.05 when compared to normal and b* p<0.05 when compared to diabetic control**.**

The histopathological studies showed gradual improvement in beta cells when treated with FEFCA.

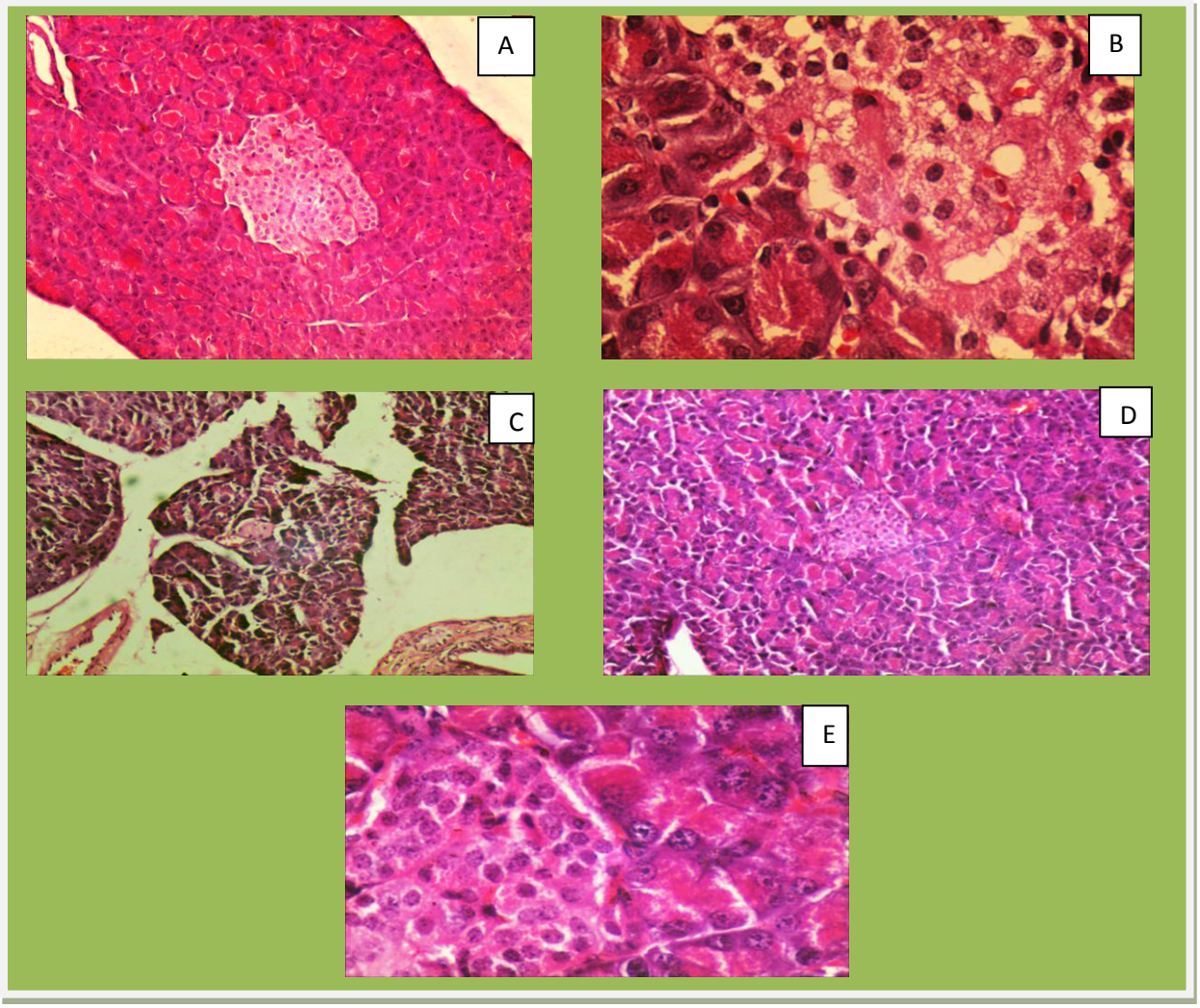

Figure 4: A) Beta cells in normal control rats. B) Total destruction of beta cells in HFD and STZ control rats. C) Remnants of beta cells in FEFCA (100 mg/kg) treated rats. D) Gradual regeneration of beta cells in FEFCA (200 mg/kg) treated rats. E) Functioning secretory granules in the islets of beta cells as seen in Glibenclamide (0.5 mg/kg) treated rats.

Discussion

The present study was aimed to investigate the antihyperglycemic activity of flavonoid enriched fraction of *Campylandra aurantiaca* rhizome **(FEFCA)** in High fat diet and low dose STZ (35mg/kg)-induced diabetic rats. The results of the study revealed that FEFCA at doses of 100 and 200 mg/kg significantly normalized elevated blood glucose level, and restored serum, liver and kidney biochemical parameters towards normal values.

The HFD-STZ-induced diabetic rat is one of the animal models of human non-insulin-dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus. As in human T2DM, diet has a great influence on the development of diabetes as well as hypertension, hyperlipidaemia, and eventually nephropathy in experimental model (Shah *et al*, 2016).

According to many previous studies data, feeding rats a high-fat diet can promote the development of insulin resistance. Injections of high doses of STZ have been shown to critically damage pancreatic β-cell functioning, leading to insulin secretion, which is considered to resemble T1DM. Recently, High fat diet and low-dose injections of STZ have been reported to induce a gradual impairment of insulin secretion, which is similar to the natural progression of T2DM in humans. Therefore, in the current study, a high-fat diet and low-dose injections of STZ (35 mg/kg) were adopted to develop type 2 diabetes in rats (Qian *et al*, 2015).

From the OGTT data, it is clear that administration of flavonoid enriched fraction of *Campylandra aurantiaca* rhizome (FEFCA) at the dose 100 mg/kg and 200 mg/kg effectively prevented the increase in serum glucose level ($P>0.05$) without causing a hypoglycemia as efficiently as the reference drug Glibenclamide ($P>0.05$). This result confirms the reduction of intestinal glucose transporter and is similar to the finding (Panda *et al*, 2016).

Hyperglycemia was observed after 7 days of low dose STZ induction. Treatment with high fat diet and low dose of STZ(35mg/kg) -induced diabetic rats started reducing fasting blood glucose levels in a dose dependent manner after 7, 14 and 21 days and made them normoglycemic after 28 days. The antihyperglycemic effect of FEFCA at a dose of 100 mg/kg and 200 mg/kg was found to be comparable to the effect exerted by the reference drug glibenclamide at a dose of 0.5 mg/kg.

STZ when injected i.p causes beta cell destruction by generating free radicals resulting in gradual depletion of insulin production and elevated blood glucose level. Increased glucose level also hampers the lipid metabolism resulting in hypercholesteremia and hypertriglyceridemia, which are considered to be the primary factors involved in the development of atherosclerosis and coronary heart disease which are the secondary complications of diabetes (Ananthan *et al*, 2003) treatment with FEFCA remarkably restore all the parameter towards normal.

The role of dyslipidemia in the development of diabetes macrovascular complications has long been well established. After treatment with FEFCA fraction at a dose of 100 and 200mg/kg was able to improve the serum lipid profile in diabetic rats. Treatment of diabetic rats with FEFCA fraction at a dose of 100 and 200 mg/kg showed considerable reduction in hepatic lipid accumulation. Lipase functions as a lipolytic enzyme that hydrolyzes Triglycerides and phospholipids in circulating plasma lipoproteins. Reduction of fat absorption by the inhibition of pancreatic lipase is known to be beneficial for the regulation of obesity and related metabolic disorders (Bhandaria *et al*, 2013).

The increase in the activities of plasma AST, ALT, ALP and decreased level of total protein (Table 2) indicated that diabetes may be induced hepatic dysfunction , that liver was necrotized in diabetic patients. Therefore, the increment of the activities of AST, ALT, ALP in plasma may

be mainly due to the leakage of these enzymes from the liver cytosol into the blood stream (El-Demerdash *et al*, 2005).

AST and ALT were used as markers to assess the extent of liver damage in streptozotocin induced diabetic rats (Al-Musa and AL-Hashem 2014).

On the other hand, treatment of the diabetic rats with FEFCA 100 and 200 mg/kg caused reduction in the activity of these enzymes in plasma compared to the mean values of diabetic group. These results are in agreement that the plant having good protective effect on Liver.

Chronic hyperglycemia has shown to play role in the development of diabetic microvascular and macrovascular complications. Four seemingly independent mechanisms are involved in the pathogenesis of diabetic complications: glucose induced activation of protein kinase C (PKC) isoforms, increased formation of glucose-derived advanced glycation endproducts, increased poly ol pathway, and increased production of reactive oxygen species (ROS) (Kumar *et al*, 2012).

Diabetes is a chronic metabolic disease associated with hyperglycemia and oxidative stress which generally causes several tissue damage and subsequently degenerative complications in many organs such as the kidney, Liver etc (Jemai *et al*, 2015).

Lipid peroxide mediated tissue damage has been observed in the development of both Type 1 and Type 2 diabetes mellitus. Insulin secretion is impaired during diabetes and this may evoke lipid peroxidation in biological systems. Enhanced levels of TBARS observed in the liver and kidney of diabetic rats indicate excessive formation of free radicals and activation of lipid peroxidative system. Present study shows that administration of FEFCA 100 and 200 mg/kg and glibenclamide inhibits production of MDA . This indicates the anti-lipid peroxidative potential of EFCA.

SOD and CAT are the two major scavenging enzymes that remove toxic free radicals in vivo and are thought to play important role in protecting the cell against the potentially deleterious effects of reactive oxygen species. Reduced activity of SOD and CAT may result in a number of deleterious effects due to the accumulation of superoxide radicals ($O2^-$) and hydrogen peroxide. Administration of FEFCA 100 and 200 mg/kg and glibenclamide results in the activation of SOD and CAT to near normal levels in diabetic rats. The result of the SOD and CAT activity clearly shows that flavonoid enriched fraction of *Campylandra aurantiaca*(FEFCA 100 and 200 mg/kg) has free radical scavenging activity, which could exert a beneficial action against pathological alterations caused by the presence of O_2^- and OH^- (Kumar *et al*, 2012).

Endogenous nonenzymatic antioxidant system Glutathione plays an important role. Primarily, acting as a reducing agent it detoxifies hydrogen peroxide with the help of enzyme, glutathione peroxidise (Biswas *et al,* 2011). The depleted GSH may be due to reduction in GSH synthesis or degradation of GSH by oxidative stress in HFD and STZ-induced hyperglycaemic animals. But after treatment with FEFCA increase the GSH level in both Liver and kidney tissue.

Conclusion

The current study clearly demonstrates that daily consumption of flavonoid enriched fraction of *Campylandra aurantiaca* (FEFCA) exhibit a pronounced hypoglycemic effect (FBG) and also improve the antioxidant defence system like Lipid peroxidation, SOD,GSH and CAT in the liver and kidney of diabetic rats. These results suggest a promising effect in intestinal glucose transport (OGTT), glycosylated haemoglobin, enzymatic liver biochemical parameters and also improve the histology of pancreas. However, investigation of secondary metabolites of this fraction responsible for hypoglycemic and antioxidant effect should be undertaken in order to confirm the compound which is responsible for this activity.

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CHAPTER-7

SUMMARY AND CONCLUSION

Diabetes is one of the major healthcare problem in today's world. Majority of the population are suffering from the disease called hyperglycaemia. Diabetes mellitus is ranked seventh among the leading causes of death and third when it's fatal complications are taken into account. Many drugs are available in the market to control the high blood glucose but they have lots of side effect. So there is a tendency to develop new drugs which have minimum side effect, and thus the natural product gain the popularity as an alternative source. Traditionally people are used natural products as source of medicines, but there is no as such scientific data available. So our first aim is find out that natural resources and scientifically evaluate them for that activity. There is also another concern of natural product about their safety issue so it is necessary to perform the scientific experiments and validated them.

The thesis entitled Preclinical Evaluation and Molecular Mechanism of Flavonoid Enriched Fraction of *Campylandra aurantiaca* on Type-2 Diabetic Rat.

The plant *Campylandra aurantiaca* rhizome was selected for the entire studies.

The rhizome portion of the plant was cut into small pieces, dried in shade and finally powdered by using a mechanical grinder. Then the dried powdered material was defatted with petroleum ether. The dried mark was then extracted with methanol. After evaporating the solvent, the dark brown concentrated extract was kept in vacuum desiccator for further use. The extract was then investigated phytochemically through several chemical tests for the determination of its constituents and from the result it was found to be containing steroids, flavonoids, tannins and alkaloids. Furthermore methanol extract was used for fractionation and HPLC characterization. Finally the flavonoids rich fraction i.e. ethyl acetate fraction was selected for the *in vivo and in vitro* study. Toxicity studies are essential to carry forward any experiment. As the animal studies

were done only for ethyl acetate fraction, the acute toxicity was therefore determined for it and found to be non-toxic.

Before going to the main targeted experiment some important related studies were done and which supported the further studies. *In vitro* antioxidant studies were already established so we have gone for *in vivo* antioxidant and DNA damage study on specific macrophage cells. An important balance is always needed between oxidants and antioxidants in the cells. *In vivo* free radical and oxidative DNA damage were cheeked on mouse peritoneal macrophage cells by using flavonoid enriched fraction of *Campylandra aurantiaca* (FEFCA). FEFCA gave satisfactory result.

Next, *in vitro* assay methods were carried out to determine the antidiabetic potential of FEFCA. The principles include enzyme (alpha amylase and alpha glucosidase) inhibition which measures intestinal absorption of monosaccharides. The results of both the experiments supported that the FEFCA possess good anti-diabetic potential. So it was promoted further for final *in vivo* studies with molecular mechanism.

Before proceeding to the *in vivo* study, the glycemic control of FEFCA was determined by oral glucose tolerance test (OGTT). FEFCA succeeded in controlling the increasing glucose level with time.

The *in vivo* study was started with induction of diabetes by using high-fat diet and streptozotocin. Male rats were fed with high-fat diet comprising 22% fat, 48% carbohydrate, and 20% protein in blend with standard laboratory chow consisting of 5% fat, 53% carbohydrate, and 23% protein for 4 weeks. After the period of dietary manipulation, rats were injected intraperitoneally (i.p.) with low dose of STZ (35 mg/kg). Then, the animals had free access to water and standard food.

One week after streptozotocin injection, the fasting blood glucose levels were appraised and the animals exhibiting fasting blood glucose levels of 170 ± 30 mg/dl were considered to be type 2 diabetic (T2D) rats and they were treated with FEFCA for further 28days.

On the 29^{th} day the animals were sacrificed. The antioxidant profile of the liver and kidney homogenates were studied. The results showed good free radical scavenging activity of FEFCA. The serum parameters involved levels of SGOT, SGPT, ALP, Total protein whereas lipid parameters included levels of Total cholesterol, HDL cholesterol and triglycerides. The SGOT, SGPT, ALP, Total cholesterol and triglycerides were found to decrease whereas total protein and HDL cholesterol increased in the treated groups. We also measured the fasting blood glucose level on different day interval as well as histopathology of pancreas. Diabetes being a metabolic disease involving lipid metabolism, the control of lipid parameters were also a matter of concern. As the blood glucose level, serum and lipid parameters were come down towards normal level so we can conclude that the FEFCA is quite effective in managing Type 2 diabetes mellitus.

CHAPTER-8

REPRINT OF PUBLICATIONS

Current Drug Discovery Technologies, 2017, *14*, 270-276

RESEARCH ARTICLE

Flavonoid Enriched Fraction of *Campylandra aurantiaca* Attenuates Carbon Tetrachloride Induced Oxidative DNA Damage in Mouse Peritoneal Macrophages in Animal Model

Mainak Chakraborty[1], Asis Bala[2,*] and Pallab Kanti Haldar[1]

[1]*Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India; and*
[2]*Guru Nanak Institute of Pharmaceutical Science and Technology, Sodepur, Kolkata-700114, India*

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Abstract: ***Background*:** Recent studies have sought to draw attention of biological activity of *Campylandra aurantiaca*. The aim of the present study was to evaluate the effect of flavonoid enriched fraction of *Campylandra aurantiaca* (FEFCA) on *in vitro* and *in vivo* antioxidant and DNA protective effect in mouse peritoneal macrophages cells.

***Methods*:** FEFCA was characterized by HPLC analysis. The *in vitro* antioxidant activities of FEFCA was measured by different *in vitro* assays like 1, 1-diphenyl-2-picrylhydrazil radical (DPPH), superoxide anions, nitric oxide and hydroxyl radicals scavenging methods. Isolated mouse peritoneal macrophages were oxidized by carbon tetra chloride (CCl_4) in animal model; subsequently the protective effect of FEFCA was determined in terms of estimation of antioxidant enzyme and the damage to DNA of the cells.

***Results*:** FEFCA exhibited both *in vitro* antioxidant activities in a concentration dependent manner. FEFCA significantly ($^{*}p < 0.05$) attenuated the oxidative DNA damage of mouse peritoneal macrophage cells induced by CCl_4 in an *in vivo* assay.

***Conclusion*:** Therefore FEFCA showed good free radical scavenging activity as well as reduced oxidative DNA damage in mouse peritoneal macrophages in animal model.

Keywords: *Campylandra aurantiaca,* flavonoid, oxidative stress, carbon tetra chloride, DNA protection, macrophages.

1. INTRODUCTION

Oxidation gets importance and recognized widely in the body and in food stuffs. It is important and essential for survival of cells. Major drawbacks of these system is the production of free radicals and other reactive species causing oxidative changes [1].

Oxidative stress has played a crucial role in various pathological conditions like cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing. These diseases are divided into two parts: (i) the first group has shown its effect by pro-oxidants shifting the thiol/disulphide redox state and impairing glucose tolerance-the so-called "mitochondrial oxidative stress" conditions (cancer and diabetes mellitus); (ii) the second group acts by "inflammatory oxidative conditions" and enhanced activity of either NAD(P)H oxidase (leading to atherosclerosis and chronic inflammation) or xanthine oxidase-induced formation of ROS implicated in ischemia and reperfusion injury [2]. The ROS and RNS generated further radicals and inactive by antioxidants [3]. The Human body has its own protective system to neutralize or scavenge the radicals by using body's own antioxidant en-

*Address correspondence to these author at the Guru Nanak Institute of Pharmaceutical Science and Technology, Sodepur, Kolkata-70014, India; Tel: +919903328027; E-mails: asisbala_ju@yahoo.co.in; pallab_haldar@rediffmail.com

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zymes like glutathione, catalase and superoxide dismutase by donating the hydrogen ions to the respective radicals [4] though these radicals can be scavenged by the *in vivo* antioxidant component, but they are not sufficient to completely remove them to maintain a balance. As a result, external sources of antioxidants are required to scavenge excess free radicals [3].

The immune cells are directly linked to ROS and RNS generation and mainly propagated the redox potential of body. So the free radicals and antioxidants balance are important indicators of immune cells activity [3]. The antioxidant plays an important role to protecting them from free radicals induced oxidative stress to maintain the normal cellular activity [4]. It was clearly established that excessive amounts of free radicals are dangerous for the immune cells, by damaging cellular components, oxidizing the membrane lipids, protein, carbohydrates and nucleic acids. To minimize or neutralize the unwanted effect of free radicals body has developed an own antioxidant environment against the free radicals to protect the cells [5].

Macrophages are one of the most important immune cells which work by multifunctional way. Extend from clearance of micro-organisms, xenobiotic material and apoptotic cells to regulating both innate and acquired immune responses through antigen presentation to secretion of various cytokines and chemokines. In order to maintain these tasks, they have evolved several strategies; which include the recognition and adherence to particular substrates, migration in response to chemoattractant, activation of inflammatory responses towards removal of a pathogen, and phagocytosis of foreign bodies or apoptotic cells. Phagocytosis of apoptotic cells is an immunologically silent process that plays a major role in the resolution of an inflammatory response [6].

To mimic immunosuppressed condition in mice, carbon tetra chloride (CCl_4) was used because earlier it was correlated with not only in liver damage but also confers an immunocompromised state particularly concerning macrophage function Chakraborty B *et al*., 2012 [7].

The abnormal functioning of the immune system leads to several chronic illnesses and protection of immune system using plant compounds or plant extracts is in recent times getting renewed interest because of their minimal side effects Chakraborty B *et al*., 2012 [7].

The plant *Campylandra aurantiaca* Family: Asperagaceae grown in high altitudes [8] the rhizome is traditionally used for it's antidiarrhoeal, antidysentric, analgesic, antimalarial, antiarthritic, vermicidal, antipyretic and stomachic [9].

Therefore, the present study was performed to focus on antioxidant profile and DNA protective effect of *Campylandra aurantiaca* on oxidative stress induced toxicity in murine peritoneal macrophages.

2. MATERIALS AND METHODS

2.1. Chemicals

1,1- diphenyl- 2- picryl- hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, napthyl ethylene diamine dihydrochloride, ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, H_2O_2, butylated hydroxy anisole, deoxyribose, Folin- Ciocalteu's phenol reagent, and carbon tetrachloride (CCl_4) were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals were used in high analytical grade.

2.2. Extraction, Isolation and Characterization of Plant Material

The rhizome of *Campylandra aurantiaca* was collected from the upper hilly region of Eastern Himalayan, Sikkim, India. Authenticated air dried whole bark (500 g) was powdered in a mechanical grinder, and the powdered materials were extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus. The solvents were completely evaporated under reduced pressure. Methanol extract was used for further fractionation. Fractionation was performed by separating funnel using solvent system of petroleum ether, chloroform and ethyl acetate [10]. Two flavonoids enriched fraction of *Campylandra aurantiaca* (FEFCA) were obtained from the chloroform and ethyl acetate fraction as per the protocol described earlier [10-11].

HPLC Characterization of isolated fractions of *Campylandra aurantiaca* was performed by HPLC system (Ultimate 3000 Germany), using a reverse phase C-18 column (250 × 4.6 mm, particle size 5μ) and UV detector. HPLC grade methanol was used to prepare samples and filtered by cellulose nylon membrane filter (0.45 μm). The aliquots of the filtrate were eluted with an isocratic solvent mixture comprising methanol: acetonitrile: acetic acid: o-phosphoric acid: water (20:10:1:1:20) for flavonoids. methanol: water: acetic acid (75:24:1) for phenolic compounds. Flow rate of 1 ml/min was used in the system and detected at 352 and 254 nm respectively. Standard phytochemical markers *i.e.* gallic acid, myricetin, quercetin and apigenin were used to characterized the fraction [11]. Flavonoid enriched fraction obtained from ethyl acetate portion was used for further evaluation and consider as FEFCA in the study.

2.3. Animals

Male Swiss Albino mice (20-25 g) were taken from Chakraborty Enterprise, Kolkata, India. The animals were maintained under standard laboratory conditions and were allowed free access to standard dry pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the procedures described were reviewed and approved (AEC/PHARM/1413/2014) by the University Animal Ethical Committee.

2.4. Acute Toxicity

The acute oral toxicity of FEFCA in Swiss albino mice was performed as per OECD guideline 425 (OECD 2008) [12].

2.5. *In Vitro* Scavenging Assay of Toxic Radicals

DPPH radical scavenging activity was measured using the method given by Karmakar *et al.* 2011 [13], with some modifications. The nitric oxide radicals scavenging activity was determined as per previously describe method by Bala *et al.* 2012a [14]. The super oxide radicals scavenging activity was determined as per previously describe method by Bala *et al.* 2012a [14]. The hydroxyl radicals scavenging activity was determined as per previously describe method by Karmakar *et al.* 2011 [13].

The percentage inhibition was calculated from the following formula

$$\% \text{ inhibition} \frac{(\text{Absorbance of control}) - (\text{Absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

2.6. Antioxidant Activity in Mouse Peritoneal Macrophages [15]

The animals were divided into four groups (n = 12):

Group I: Normal vehicle control: Received liquid paraffin (1 ml/kg i.p.) as vehicle control for 2 days (Day 1 and 2).

Group II: CCl_4 control: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2).

Group III: N‑ acetyl cysteine (NAC) control: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2).

NAC (150 mg/kg i. p.) treatment was started before 24 h of the

CCl_4 treatment and continued for Day 1 and Day 2.

Group IV and V: Received CCl_4 (1 ml/kg i.p.) in liquid paraffin (1:1 v/v) for 2 days (Day 1 and 2). FEFCA (50 and 100 mg/kg i. p. respectively) treatment was started before 24 h of CCl_4 treatment and continued for Day 1 and 2.

At the day third, the six mice were sacrificed under ether anesthesia, and peritoneal macrophages were lavaged aseptically using ice cold PBS (0.02 M, pH‑ 7.4). Both Superoxide anion production in macrophages cells and SOD in the cell lysate were estimated by NBT methods. Oxidative damage to the DNA was estimated according to the standard protocol [14-15]. The lipid peroxidation was measured by TBA‑ reactive substances (TBARS) assay. The nonenzymatic antioxidant reduced GSH in the cells lysate was estimated by 5,5'‑ dithiobis‑ 2‑ nitrobenzoic acid method Bala *et al.* 2012b [15].

2.7. Data Analysis

All the values are given as mean ± SEM. The IC_{50} (50% inhibitory concentration) values were calculated from the graphs plotted between concentrations *vs.* percentage inhibition using GraphPad Prism software 5.1. One-way ANOVA followed by Dunnett's post hoc test of significance was performed by GrapPad Instat software

(GraphPad Software Inc,San Diego, CA, USA) where *$p<0.05$ and **$p<0.01$ are considered to be significant and highly significant, respectively, when test group is compared with respective control group.

3. RESULTS

Percentage yield of 2%, 2.7% and 3.4% obtained in Soxhletlation with by petroleum ether, chloroform, and methanol respectively. Preliminary quantitatively analysis confirmed the presence of 24.7 µg /mg of phenolic compounds in the methanol extract.

HPLC characterization of fraction obtained from chloroform portion confirms the presence of myricetin having characterized Rt 4.08 min. However, the presence of myricetin and apigenin was confirmed by the HLC chromatogram having Rt 4.08 and 8.16 min respectively (Fig. **1B, C**). Representative HPLC chromatograph of standard flavonoids viz. myricetin, quercetin, apigenin and gallic acid with specific Retention time (Rt) 4.08, 5.70, 8.16 and 4.03 min respectively was obtained in the chromatogram using same system (Fig. **1A**).

It was found that FEFCA showed good antioxidant activity in all *in vitro* assays whereas the IC_{50} was found in between 17.37- 45.34 µg/ml in all assay condition (Table **1**). The IC_{50} values of standard vitamin C obtained ranges from 7.32-42.34 µg/ml.

CCl_4 in mouse peritoneal macrophages resulted in a significant rise in intracellular superoxide anions with compared to normal mouse macrophages. FEFCA significantly ($p<0.01$) reduced the oxidative DNA damage in macrophages (Fig. **2**). NAC and FEFCA significantly reduced the superoxide

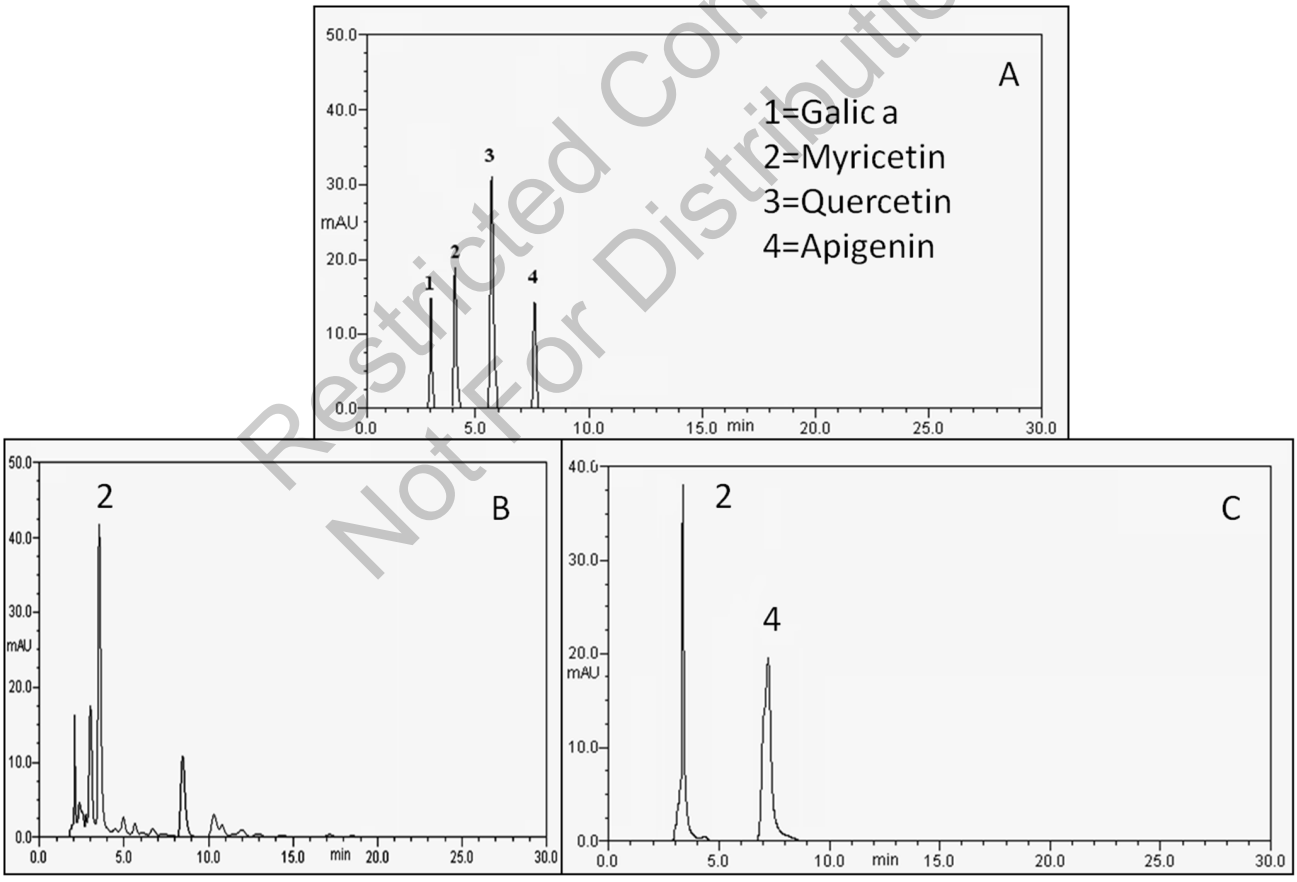

Fig. (1). HPLC characterization of FEFCA. **A**. Representative HPLC chromatograph of standard flavonoids viz. myricetin, quercetin, apigenin and gallic acid with specific Retention time (Rt) 4.08, 5.70, 8.16 and 4.03 min respectively. **B**. HPLC chromatogram of fraction obtained from chloroform portion having characteristic peak of myricetin. **C**. HPLC chromatogram of fraction obtained from ethyl acetate portion having significant presence of myricetin and apigenin.

Table 1. IC_{50} values of all *in vitro* assay models. Vitamin C was used as standard antioxidant. Values are expressed as mean±SEM (n=6).

Scavenging Assays	IC_{50} µg/ml (mean ± SEM)	
	FEFCA	Standard
DPPH	17.37±1.41	7.32±0.52
Superoxide	30.83±1.72	9.21±0.89
Nitric oxide	33.36±1.47	60.36±1.47
Hydroxyl	45.34±1.45	42.34±2.45

anion level (Fig. **3A**). Activity of SOD was measured for further investigation of the effect of CCl_4 on the regulation of intracellular superoxide anions level. There was a significant reduction of SOD level in CCl_4 treated mouse cells which was significantly increased by NAC and FEFCA treatment respectively due to free radicals scavenging property (Fig. **3B**).

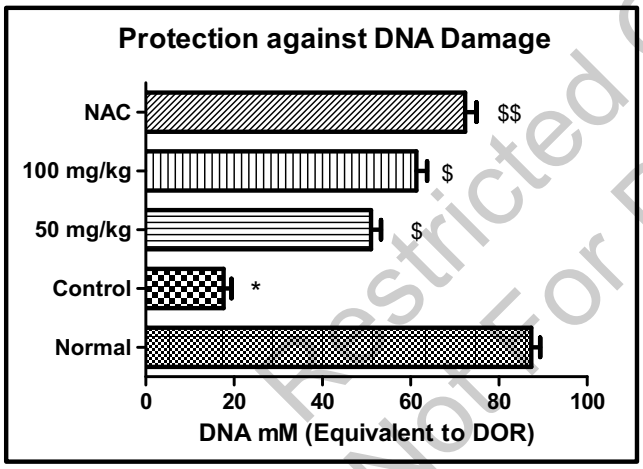

Fig. (2). Protection on DNA damage in macrophages. DNA estimated with respect to deoxy ribose standsrd (DOR, mM). Values are expressed as mean±SEM. *$p<0.05$ (with respect to normal) and $^{\$}p<0.05$, $^{\$\$}p<0.01$, with respect to control.

Interestingly, treatment with FEFCA and NAC significantly reduced the MDA levels as compared with CCl_4 control group, in the present study the MDA level was significantly increased in CCl_4 control animals when compared with normal control animals (Fig. **3C**). Administration of FEFCA and NAC in a dose dependent manner significantly raised the reduced glutathione levels as compared with CCl_4 control animals. The levels of reduced GSH were significantly decreased in CCl_4 control group when compared with normal control group (Fig. **3D**).

4. DISCUSSION

Most of the modern diseases are due to the results of free radicals induced "oxidative stress" which mainly causes due to formation and neutralization imbalance of free radicals. By electron pairing with biological macromolecules such as proteins, lipids, and DNA in healthy human cells free radicals get stability and ultimately cause protein, DNA, and lipid oxidation [16]. Free radicals are continuously generated and causes damage to the body either by damaging antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory cascade [13].

In the present study we investigated the antioxidant activity of FEFCA in some *in vitro* as well as *in vivo* antioxidant models. In all the models, FEFCA showed its ability to scavenge the free radicals in a concentration dependent manner. We also correlated the *in vitro* antioxidant models with the *in vivo* CCl_4 induced generation of free radical directly on macrophage.

Different approaches are now introduced to reduces the oxidative stress which mainly focus the neutralizing or scavenge the radicals or inactivation of free radical producing enzymes, influence the antioxidant system or by targeting the signaling routes and expression of molecules involved in the inflammatory cascade [14, 17]. A number of synthetic drugs having the protective effects also cause side effects. To overcome these problems an alternative solution is to consume natural antioxidants from food supplement or in the form of traditional medicines [18].

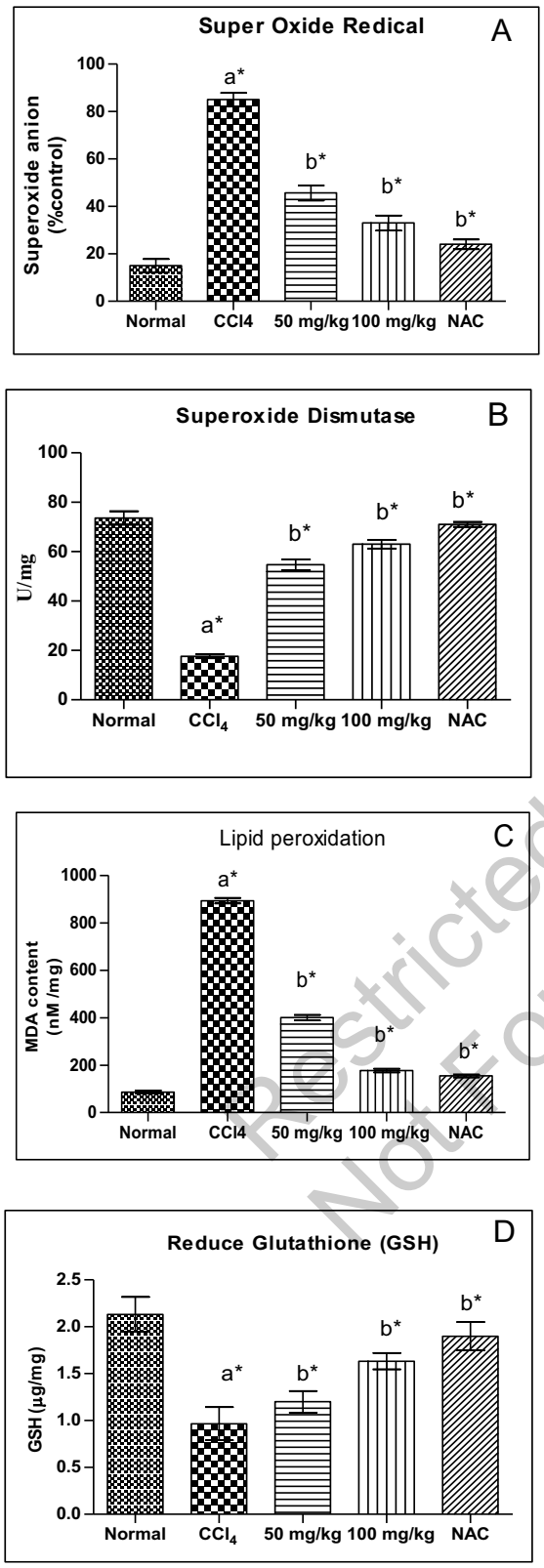

Fig. (3). Effect of FEFCA on O_2^- (**A**), SOD (**B**), LPO (**C**), GSH (**D**). Each value is expressed as Mean ± SEM where n=6. a* p<0.05 when compared to normal and b* p<0.05when compared all treated group *vs.* respective control group.

Here oxidative DNA damage induced by H_2O_2 and the effect of FEFCA in the mouse macrophages cells (*in vivo*) and isolated DNA from macrophage was estimated by diphenylamine reagent. H_2O_2 (10 mM) significantly ($p < 0.01$) caused DNA damage in mouse macrophages that was attenuated by FEFCA in a concentration dependent manner.

Most prevalent ROS that affect profoundly the lipid molecules are mainly hydroxyl radical and superoxide [19]. Cellular physiology and pathology also have a great link to lipid peroxidation which can be measured by increase in the level of malondialdehyde (MDA) in the *in vivo* model of CCl_4 treated mice. Production of free radicals via CCl_4 involvement of atleast two mechanisms. The first is via biotransformation of CCl_4 to a free radical species or formation of Conversion of CCl_4 to the trichloromethyl radical by the hepatic mixed-function oxides system which ultimately responsible for lipid peroxidation, protein-lipid cross linkages and altercation of DNA. The second way is formation of less stable free radical intermediates via reduction which are further going to oxidation with the help of molecular oxygen and generate superoxide [15, 20]. The *in vitro* neutralization of OH^- may be correlated with the *in vivo* reduction in MDA content. In the present study the MDA level was significantly increased in CCl4 control animals when compared with normal control animals. However, decreased when treated with FEFCA.

With help of NADPH oxidase, xanthine oxidase, nitric oxide synthase (NOS), lipoxygenase, and mitochondrial enzymes Superoxide is generated. Which is further neutralize by the enzyme superoxide dismutase (SOD) to H_2O_2, which, in turn, is reduced to water by catalase, glutathione peroxidases (GPx), and peroxiredoxins (Prx) [21]. The generation of superoxide anion and the reduction of the SOD level in mouse peritoneal macrophages by CCl_4 and their subsequent reduction by FEFCA was observed. Cells are highly rich source of soluble antioxidant GSH which play a major role in antioxidant mediated defense system. GSH functioning as an antioxidant in several ways. It neutralized the hydrogen peroxide and lipid peroxides via action of GSH-Px [21]. By donating an electron it converted H_2O_2 to H_2O and O_2. GSSG is again reduced into GSH by GSH reductase that uses NAD(P)H as the electron donor [22, 23]. GSH-Pxs shown their antioxidant effect by pro-

tecting the cell membrane from lipid peroxidation. Reduced glutathione has shown its antioxidant effect by donating protons to membrane lipids [24, 25]. The decreased level of GSH observed in this study after administration of CCl_4 to the mice is associated with an increase in the level of lipid peroxidation and DNA damage. However, it has been demonstrated that the level of GSH could be enhanced in mice pretreated with NAC and FEFCA.

CONCLUSION

Therefore, FEFCA showed good free radical scavenging activity as well as reduced oxidative DNA damage in mouse peritoneal macrophages in animal model.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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ORIGINAL ARTICLE

Hypoglycemic Effect of Ethyl Acetate Fraction of Methanol Extract from *Campylandra aurantiaca* Rhizome on High-fat Diet and Low-dose Streptozotocin-induced Diabetic Rats

Mainak Chakraborty, Asis Bala[1,2], Sanjib Bhattacharya[3], Pallab Kanti Haldar

Department of Pharmaceutical Technology, Jadavpur University, [1]???, Guru Nanak Institute of Pharmaceutical Science and Technology, [3]???, West Bengal Medical Services Corporation Ltd., Kolkata, West Bengal, India, [2]Department of Pharmacology, Faculty of Health Science, University of the Free State, Bloemfontein 9031, South Africa

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ABSTRACT

Background: *Campylandra aurantiaca* (Asparagaceae), commonly known as Nakima in Sikkimese Tibetan, is a plant grown in South-Central China and Northeast India. **Objective:** To evaluate the hypoglycemic activity of ethyl acetate fraction of methanol extract from *C. aurantiaca* rhizome (EFCA) in high-fat diet (HFD) and low-dose streptozotocin (STZ)-induced diabetic Wistar rats. **Materials and Methods:** In rats fed with HFD for 4 weeks, hyperglycemia was induced by single intraperitoneal injection of STZ (35 mg/kg body weight). Seven days after STZ induction, the hyperglycemic rats were treated with EFCA orally at the doses of 100 and 200 mg/kg b.w. daily for 28 days. Glibenclamide (0.5 mg/kg, orally) was used as reference drug. The fasting blood glucose levels were measured on every 7th day during the 28 days of treatment. Serum and hepatorenal biochemical parameters were estimated. Histological study of the pancreas was also performed. **Results:** EFCA at the doses of 100 and 200 mg/kg orally significantly ($P < 0.05$) and dose dependently reduced and normalized blood glucose levels as compared to that of STZ control group. Serum and hepatorenal parameters were significantly ($P < 0.05$) restored toward normal levels in EFCA-treated rats as compared to HFD-STZ control animals. **Conclusion:** The present study concludes that *C. aurantiaca* rhizome demonstrated promising hypoglycemic action in HFD-STZ-induced diabetic rats.

Key words: Diabetes, glibenclamide, hypoglycemic, obesity, streptozotocin

SUMMARY

- The present study evaluated the hypoglycemic activity of ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome (EFCA) in high-fat diet (HFD) and low-dose streptozotocin (STZ)-induced diabetic Wistar rats. In rats fed with HFD for 4 weeks, hyperglycemia was induced by single intraperitoneal injection of STZ (35 mg/kg body weight). Seven days after STZ induction, the hyperglycemic rats were treated with EFCA orally at the doses of 100 and 200 mg/kg b.w daily for 28 days. Glibenclamide (0.5 mg/kg orally) was used as reference drug. The blood glucose levels were measured on every 7th day during the 28 days of treatment. Serum and hepatorenal biochemical parameters were estimated. Histological study of pancreas was also performed. EFCA at the doses of 100 and 200 mg/kg orally significantly and dose dependently reduced and normalized blood glucose levels as compared STZ control group. Serum and hepatorenal parameters were significantly restored toward normal levels in EFCA-treated rats as compared to HFD-STZ control animals. The present study concludes that *C. aurantiaca* rhizome demonstrated promising hypoglycemic action in HFD-STZ-induced diabetic rats.

Abbreviations used: IDDM: Insulin-dependent diabetes mellitus, T2DM: Type 2 diabetes mellitus, NIDDM: Noninsulin-dependent diabetes mellitus, T1DM: Type 1 diabetes mellitus, IDF: International Diabetes Federation, STZ: Streptozotocin, EFCA: Ethyl acetate fraction of methanol extract from Campylandra aurantiaca rhizome, OGTT: Oral glucose tolerance test, HFD: High-fat diet, FBG: Fasting blood glucose, HDL: High-density lipoprotein cholesterol, HbA1c: Glycosylated hemoglobin, AST: Aspartate transaminase, ALT: Alanine transaminase, ALP: Alkaline phosphatase, LPO: Lipid peroxidation, GSH: Reduced glutathione, SOD: Superoxide dismutase, CAT: Catalase, SEM: Standard error of mean, ANOVA: Analysis of variance, HPLC: High-performance liquid chromatography.

Correspondence:

Dr. Pallab Kanti Haldar,
Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700 032, West Bengal, India.
E-mail: pallab_haldar@rediffmail.com

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INTRODUCTION

Diabetes mellitus, a metabolic disorder in which a person has high blood sugar, has become a matter of serious challenge because of its worldwide prevalence. This may be because of the body's inability to produce enough insulin or because of lesser response of the cells to insulin that is being produced. Diabetes mellitus is of different types based on the causes. The majority of type 1 diabetes mellitus (T1DM or insulin-dependent diabetes mellitus [IDDM]) is related to immunity, where beta-cells are destroyed by T-cell-mediated autoimmune attack. Type 2 diabetes mellitus (T2DM or non-IDDM [NIDDM]) occurs

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from insulin resistance and sometimes involves deficiency in insulin production.[1]

The prevalence of diabetes is growing worldwide due to urbanization and improper lifestyle management. In the Western countries, diabetes mostly develops with age; however, in Asian countries, it attacks mostly young to middle-aged people. This scenario leaves a huge impact on health and economy of the developing countries. The International Diabetes Federation has an estimation of the total number diabetics in India to be around 50.8 million in 2010 and gradually rising to about 87.0 million by 2030. Recent reports collected from various parts of India have detected the invading nature of diabetes in urban areas. Moreover, the prevalence of diabetes is also simultaneously increasing in rural areas as a result of the recent socioeconomic growth.[2]

Obesity is a complex, multifactorial, and largely preventable disease, affecting along with overweight, over one-third of the world's population today. Obesity increases risk of chronic diseases namely disability, depression, type 2 diabetes (T2D), cardiovascular diseases, certain cancers, and mortality.[3] Recent studies have identified links between obesity and T2D involving proinflammatory cytokines (tumor necrosis factor and interleukin-6), insulin resistance, deranged fatty acid metabolism, and cellular processes such as mitochondrial dysfunction and endoplasmic reticulum stress.[4] Obesity and T2DM frequently occur together, and statistics show that 60–90% of all patients with T2DM are or have been obese. Obesity is generally considered to be a strong risk for the later development of T2DM.[5]

Marketed oral hypoglycemic agents exhibit a variety of adverse effects, including congestive heart failure with glitazones, gastrointestinal disturbances with glucosidase inhibitors, sulfonylureas, and meglitinides. Cardiac problems and weight gain are common adverse effects of sulfonylureas. Therefore, it is an urge to develop a less toxic therapeutic agent. Due to the multiple pathophysiological considerations, there is a need to develop multitarget therapeutic agent which would be helpful for the treatment of T2DM and its associated pathogenesis. Plant extract is a mixture of multiple compounds and shows their therapeutic value in a multiple way. Therefore, the present investigation has focused the antidiabetic potential of plant extract considering ethnopharmacological knowledge as reference.[6]

Campylandra aurantiaca (Asparagaceae), commonly known as Nakima or Thulo-Nakima in Sikkimese Tibetan, is an herbaceous plant grown in South-Central China including Tibet, Nepal, and Northeast India, including Sikkim. Different parts of *C. aurantiaca* have been used conventionally in India for certain medicinal purposes. Rhizome decoction is administered as antidiarrheic, antidysenteric, analgesic, antimalarial, antiarthritic, vermicidal, antipyretic, and stomachic. Flowers are made into curry and taken with staple food 2 times per week for 4–6 weeks to treat diabetes mellitus.[7,8] However, experimental studies on this plant are scanty. There is no experimental report demonstrating antidiabetic potential of this plant. This study therefore investigated the hypoglycemic effect of the ethyl acetate fraction of methanol extract of *C. aurantiaca* rhizome (EFCA) against streptozotocin (STZ)-induced diabetic Wistar rats to justify the traditional and folkloric attributes.

MATERIALS AND METHODS

Plant material

The mature rhizomes of *C. aurantiaca* were collected during June 2016 from Majhitar region of Sikkim state, India. The plant species were identified and authenticated by Dr. R. Gogoi, Taxonomist, Botanical Survey of India, Central National Herbarium (CNH), Howrah, India, and the voucher specimen (No. CNH/Tech.II/2016/38b) was deposited at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, for future reference. The collected rhizomes were thoroughly washed with running tap water, cut into small pieces, shade-dried (24°C–26°C) for 3–4 weeks, and ground mechanically into a coarse powder.

Drugs and chemicals

Trichloroacetic acid was obtained from Merck Ltd., Mumbai, India; thiobarbituric acid, STZ, 5,5'-dithiobis-2-nitrobenzoic acid, phenazonium methosulfate, nicotinamide adenine dinucleotide, and reduced glutathione (GSH) from Sisco Research Laboratory, Mumbai, India; potassium dichromate and glacial acetic acid from Ranbaxy, India; and glibenclamide from Hoechst, India. All the other reagents used were of analytical reagent grade obtained commercially.

Extraction and fractionation

The powdered material (500 g) was extracted successively by petroleum ether, chloroform, and methanol using Soxhlet extraction apparatus. The solvents were completely evaporated under reduced pressure. The dry methanol extract was used for further fractionation. Fractionation was performed successively using petroleum ether, chloroform, and ethyl acetate.[9] The EFCA was used for the present study. Preliminary phytochemical and high-performance liquid chromatography (HPLC) studies (in our previous work) were performed on EFCA.[10]

Experimental animals

Six to eight-week-old male Wistar albino rats (180 ± 20 g) were obtained from registered breeder, namely Chakraborty and Co., Kolkata, India. They were acclimatized to the laboratory conditions before the study for 7 days. The animals were kept at 25°C ± 2°C and a relative humidity of 40%–45% with alternative day and night cycles of 12 h each. The animals had free access to dry pellet food (Hindustan Unilever, Mumbai, India) and water *ad libitum*. All animal experimental procedures described were reviewed and approved by the University Animal Ethical Committee, Jadavpur University (Reg. no. 367001/C/CPCSEA).

Acute toxicity

EFCA was administered orally to male Swiss albino mice to evaluate the acute toxicity as per the reported method.[11]

Induction of diabetes with high-fat diet and streptozotocin

Male rats were fed with high-fat diet (HFD) comprising of 22% fat, 48% carbohydrate, and 20% protein in blend with standard laboratory chow consisting of 5% fat, 53% carbohydrate, and 23% protein for 4 weeks. After the period of dietary manipulation, rats were injected intraperitoneally (i.p.) with low dose of STZ (35 mg/kg body weight). Then, animals had free access to water and standard food.[12] One week after STZ injection, the fasting blood glucose (FBG) levels of overnight fasted rats were appraised and the animals exhibiting FBG levels of 170 ± 30 mg/dl were considered to be T2D rats and included for the further experiments.[6]

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed in overnight-fasted normal rats. Rats were divided into three groups ($n = 6$). Group I served as normal control and received distilled water (5 ml/kg b.w., p.o), and Groups II and III received EFCA at doses of 100 and 200 mg/kg b.w., respectively. After these treatments, all groups received glucose

(2 g/kg b.w.) orally. Blood was withdrawn from the tail vein just before and 30, 60, and 120 min after oral glucose administration.[13] Blood glucose levels were measured using single touch glucometer (Accu-Chek, Roche Diagnostics, USA).

Experimental design

Hyperglycemia was induced by HFDs *ad libitum* and low-dose of STZ as per recently reported method.[6,12] Briefly, the rats were fed HFDs *ad libitum* for 4 weeks and then treated with single dose of STZ (35 mg/kg b.w., i.p.). After 7 days STZ injection, the FBG levels of overnight-fasted rats were appraised, and the animals exhibiting FBG levels of 170 ± 30 mg/dl were considered to be T2DM rats and included for the further experiments. The rats were continued with HFDs throughout the course of the study. The animals were divided into five groups (n = 6) and received the following treatment for 28 days:

- Group I: Normal control rats were administered normal saline (0.5 ml/kg orally by oral gavages) daily
- Group II: Diabetic control rats were administered HFDs + normal saline (0.5 ml/kg daily)
- Group III: Diabetic rats were administered HFDs + EFCA (100 mg/kg b.w.) orally daily
- Group IV: Diabetic rats were administered HFDs + EFCA (200 mg/kg b.w.) orally daily
- Group V: Diabetic rats were administered HFDs + glibenclamide (0.5 mg/kg b.w.) orally daily.

Estimation of fasting blood glucose level

The rats were divided into five groups (n = 6). Except for Group I, which served as normal control, all other groups were comprised of diabetic rats. Group II served as diabetic (HFD-STZ) control. Groups III and IV received EFCA (100 and 200 mg/kg b.w., p.o., respectively) and Group V received reference drug glibenclamide (0.5 mg/kg b.w., p.o.) daily for 28 days.[14] FBG was measured on day 0, 7, 14, 21 and 28 using a one touch glucometer (Accu-Chek®).

Determination of serum biochemical parameters

Twenty-four hours after the last dose, blood was collected from overnight fasted rats of each group by cardiac puncture for estimation of total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, glycosylated hemoglobin (HbA1c), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), and total protein were estimated using commercially available reagent kits (ERBA Diagnostics, Andheri (E), Mumbai 400072, India, and Span Diagnostics Ltd., Surat, Gujarat 394230, India).[15]

Estimation of liver and kidney biochemical parameters

Livers and kidneys collected from the sacrificed animals were homogenized separately in 10 ml of phosphate buffer (20 mM, pH: 7.4) and centrifuged at 12,000 rpm for 30 min at 4°C. The supernatants were collected and lipid peroxidation (LPO), GSH, superoxide dismutase (SOD), and catalase (CAT) were estimated.[11,16]

Histopathological study

A portion of pancreatic tissue was dissected out and fixed in 10% buffered neutral formal saline and processed. After fixation, tissues were embedded in paraffin. Fixed tissues were cut and stained with hematoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.[17]

Statistical analysis

All the results are shown as mean ± standard error of mean. The results were analyzed for statistically significance by one-way analysis of variance, followed by *post hoc* Dunnett's test using GraphPad Prism 5.0 software (GraphPad Software, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

Preliminary phytochemical studies revealed the presence of flavonoids, alkaloids, and tannins in EFCA. Our previous work on HPLC analysis revealed the presence of two flavonoids, namely myricetin and apigenin, in EFCA.[10] While performing the toxicity, EFCA did not show any toxic effect or death up to the dose of 2000 mg/kg, b.w., p.o. in mice. The oral blood glucose tolerance test was done in normal rats. Glucose administration to the normal rats increased blood glucose levels in first 30 min and gradually decreased in 30 and 60 min and returned near to normal at 120 min [Table 1]. There was significantly ($P < 0.05$) elevated FBG level in HFD and STZ-induced diabetic rats as compared to normal control group. Administration of EFCA in diabetic rats at the doses of 100 and 200 mg/kg significantly ($P < 0.05$) reduced the FBG level toward normal as compared to the diabetic control group [Table 2]. HbA1c has been found to be reduced in EFCA-treated animals as compared to HFD and STZ control animals [Table 3].

Similarly, in case of serum biochemical parameters, EFCA showed a significant ($P < 0.05$) lowering when compared to the HFD and STZ control group. Level of total protein increased in treated group when compared to the HFD and STZ control [Figure 1]. Serum lipid profiles such as total cholesterol and triglyceride in HFD and STZ-induced diabetic rats were significantly ($P < 0.05$) elevated and the HDL cholesterol level significantly ($P < 0.05$) decreased compared to normal control group. Treatment with EFCA at the doses of 100 and 200 mg/kg significantly ($P < 0.05$) reduced the total cholesterol and triglyceride level and significantly ($P < 0.05$) increased the HDL cholesterol level when compared to the diabetic control group [Figure 2]. In liver and kidney tissue antioxidant studies, LPO level decreased ($P < 0.05$) and SOD, GSH, and CAT level increased ($P < 0.05$) in treated animals [Figure 3]. The histopathological studies on pancreas showed gradual improvement in pancreatic beta cells when treated with EFCA [Figure 4].

DISCUSSION

The present study aimed to investigate the hypoglycemic activity of EFCA in HFD and low-dose STZ (35 mg/kg)-induced diabetic rats. The results of the study revealed that EFCA at doses of 100 and 200 mg/kg significantly normalized elevated blood glucose level and restored serum, liver, and kidney biochemical parameters toward normal values.

The HFD-STZ-induced diabetic rat is one of the animal models of human NIDDM or T2DM. As in human T2DM, diet has a great influence on the development of diabetes as well as hypertension, hyperlipidemia,

Table 1: Effect of ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome on oral glucose tolerance test

Groups	0 min	30 min	60 min	120 min
Normal control	83.67±2.33	135.7±1.76	125±1.15	106.3±1.76
EFCA (100 mg/kg)	88.67±0.88	138.7±1.52	132.7±1.45*	126.3±0.88*
EFCA (200 mg/kg)	87.33±1.45	125.7±1.76*	119±1.15*	115.7±1.76*
Glibenclamide (0.5 mg/kg)	81±2.08	120±1.00*	115±1.15*	110.3±3.2.40*

*P<0.05 when compared to normal control. Values are represented as mean±SEM (n=6). EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome; SEM: Standard error of mean

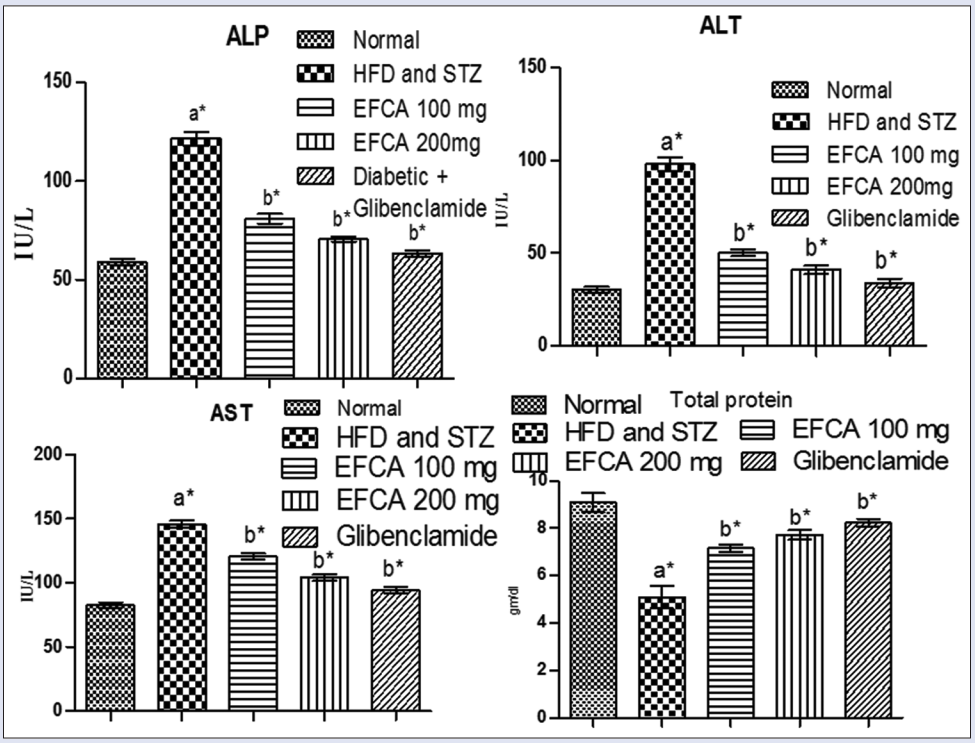

Figure 1: Effect of EFCA on ALP, ALT, AST, and total protein. Each value is expressed as mean ± standard error of mean (n = 6). a*: P <0.05 when compared to normal and b*: P <0.05 when compared to diabetic control. EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome, ALP: Alkaline phosphatase, ALT: Alanine transaminase, AST: Aspartate transaminase, STZ: Streptozotocin, HFD: High-fat diet\

Table 2: Effect of ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome on fasting blood glucose (mg/dl)

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
Normal control (5 ml/kg)	75.33±2.72	80.33±1.76	82±1.52	81±1.15	79±3.15
HFD and STZ control (35 mg/kg)	176.3±4.37*	187.3±3.18*	200±1.73*	207.7±4.66*	211.7±3.66*
STZ + EFCA (100 mg/kg)	177.7±3.52	172±2.74**	163±2.51**	153.7±1.76**	139±2.30**
STZ + EFCA (200 mg/kg)	172.7±3.28	155±3.21**	143±2.02**	135.7±2.40**	127.7±1.45**
STZ + glibenclamide (0.5 mg/kg)	176.3±1.76	155±2.64**	143±2.30**	116±3.21**	87.33±2.18**

*Normal control group versus diabetic control group, **All treated group versus diabetic control group on corresponding day, P<0.05. Each volume expressed as mean±SEM (n=6). HFD: High-fat diet; STZ: Streptozotocin; EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome; SEM: Standard error of mean

Table 3: Effect of ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome on glycosylated hemoglobin

Groups	HbA1c (%)
Normal control	6.4±0.20
STZ control (diabetic)	8.99±0.24*
Diabetic + EFCA (100 mg/kg)	7.33±0.14
Diabetic + EFCA (200 mg/kg)	6.5±0.14
Diabetic + glibenclamide (0.5 mg/kg)	6.02±0.26

Values are represented as mean±SEM (n=6). *P<0.05 when compared to normal control. STZ: Streptozotocin; EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome; SEM: Standard error of mean; HbA1c: Glycosylated hemoglobin

and eventually nephropathy in the experimental model.[18] According to the data from previous studies, feeding rats an HFD can promote the development of insulin resistance. Injections of high doses of STZ have been shown to critically damage pancreatic β-cell functioning, leading to insulin secretion, which is considered to resemble T1DM. Recently, HFD and low-dose injections of STZ have been reported to induce a gradual impairment of insulin secretion, which is similar to the natural progression of T2DM in humans. Therefore, in the current study, an HFD and low-dose of STZ (35 mg/kg) were adopted to develop T2D in rats.[19]

From the OGTT data, it is clear that administration EFCA at the dose 100 mg/kg and 200 mg/kg effectively prevented the increase in serum glucose level without causing a hypoglycemia as efficiently as the reference drug glibenclamide. This result confirms the reduction of intestinal glucose transporter and is similar to the finding.[15] Hyperglycemia was observed after 7 days of low-dose STZ induction. Treatment with HFD and low dose of STZ (35 mg/kg)-induced diabetic rats started reducing FBG levels in a dose-dependent manner after 7, 14, and 21 days and made them normoglycemic after 28 days. The antihyperglycemic effect of EFCA at the dose of 100 mg/kg and 200 mg/kg was found to be comparable to the effect exerted by the reference drug glibenclamide at a dose of 0.5 mg/kg.

STZ when injected i.p. causes pancreatic beta-cell destruction by generating free radicals resulting in gradual depletion of insulin production and elevated blood glucose level. Increased glucose level also hampers the lipid metabolism resulting in hypercholesteremia and hypertriglyceridemia, which are considered to be the primary factors

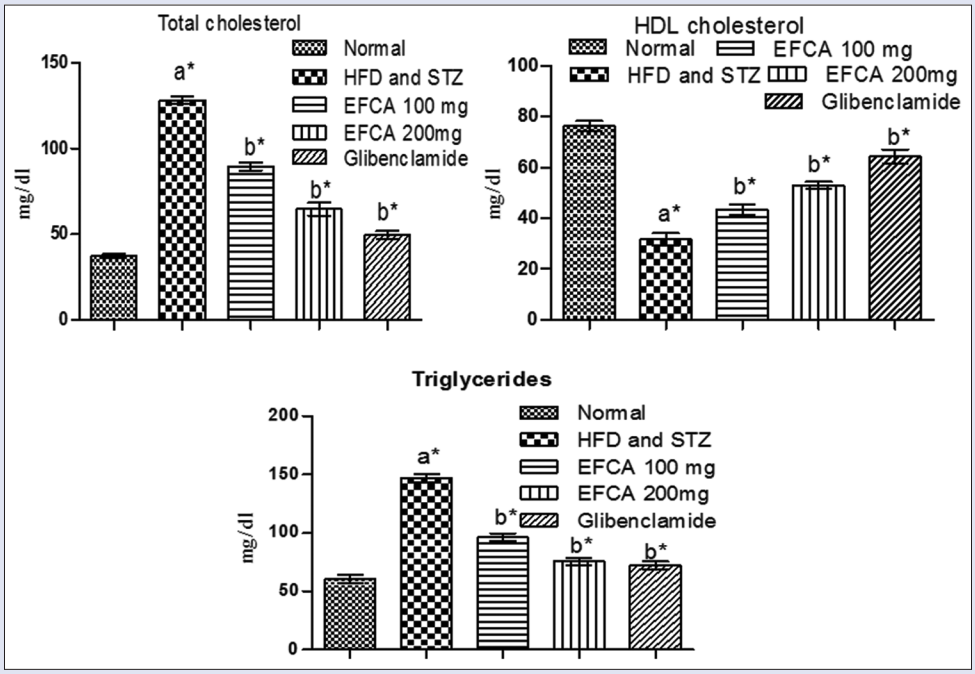

Figure 2: Effect of EFCA on total cholesterol, HDL cholesterol, and triglycerides. Each value is expressed as mean ± standard error of mean ($n = 6$). a*: $P < 0.05$ when compared to normal and b*: $P < 0.05$ when compared to diabetic control. EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome, HDL: High-density lipoprotein, HFD: High-fat diet, STZ: Streptozotocin

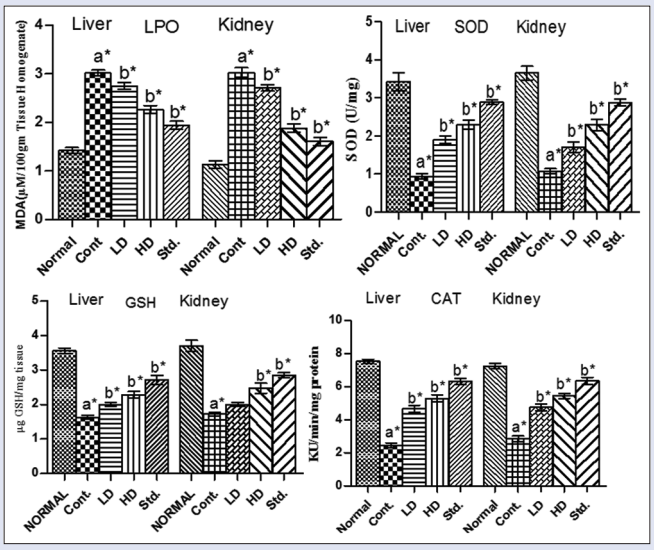

Figure 3: Effect of EFCA on SOD, LPO, GSH, and CAT. Each value is expressed as mean ± standard error of mean ($n = 6$). a*: $P < 0.05$ when compared to normal and b*: $P < 0.05$ when compared to diabetic control. EFCA: Ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome, SOD: Superoxide dismutase, LPO: Lipid peroxidation, GSH: Reduced glutathione, CAT: Catalase

involved in the development of atherosclerosis and coronary heart disease, which are the secondary complications of diabetes.[20] Treatment with EFCA remarkably restores all the parameter toward normal.

The role of dyslipidemia in the development of diabetic macrovascular complications has long been well established. The treatment with EFCA at a dose of 100 and 200 mg/kg was able to improve the serum lipid profile in diabetic rats. Treatment of diabetic rats with EFCA at the doses of 100 and 200 mg/kg shows considerable reduction in hepatic lipid accumulation. Lipase functions as a lipolytic enzyme that hydrolyzes triglycerides and phospholipids in circulating plasma lipoproteins. Reduction of fat absorption by the inhibition of pancreatic lipase is known to be beneficial for the regulation of obesity and related metabolic disorders.[21]

The increase in the activities of plasma AST, ALT, ALP and decreased level of total protein indicated that diabetes may be induced hepatic dysfunction that liver was necrotized in diabetic patients. Therefore, the increment of the activities of AST, ALT, and ALP in plasma may be mainly due to the leakage of these enzymes from the liver cytosol into the bloodstream.[22] AST and ALT were used as markers to assess the extent of liver damage in STZ-induced diabetic rats.[23] On the other hand, treatment of the diabetic rats with EFCA 100 and 200 mg/kg caused reduction in the activity of these enzymes in plasma compared to the mean values of diabetic group. These results are in agreement with that the plant having good protective effect on liver.

Chronic hyperglycemia has shown to play a role in the development of diabetic microvascular and macrovascular complications. Four seemingly independent mechanisms are involved in the pathogenesis of diabetic complications: glucose-induced activation of protein kinase C isoforms, increased formation of glucose-derived advanced glycation endproducts, increased polyol pathway, and increased production of reactive oxygen species (ROS).[24]

Diabetes is a chronic metabolic disease associated with hyperglycemia and oxidative stress which generally causes several tissue damage and subsequently degenerative complications in many organs such as the kidney and liver.[25] Lipid peroxide-mediated tissue damage has been observed in the development of both T1DM and T2DM. Insulin secretion is impaired during diabetes and this may evoke LPO in biological systems. Enhanced levels of LPO observed in the liver and kidney of diabetic rats indicated excessive formation of free radicals and activation of lipid peroxidative system. The present study shows that administration of

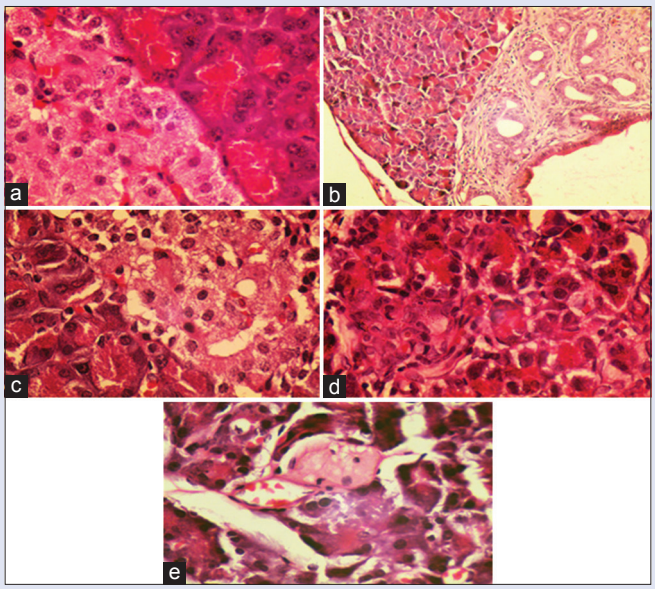

Figure 4: (a) Beta cells in normal control rats. (b) Total destruction of beta cells in high-fat diet and streptozotocin control rats. (c) Remnants of beta cells in ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome (100 mg/kg) treated rats. (d) Gradual regeneration of beta cells in ethyl acetate fraction of methanol extract from *Campylandra aurantiaca* rhizome (200 mg/kg) treated rats. (e) Functioning secretory granules in the islets of beta cells as seen in glibenclamide (0.5 mg/kg) treated rats

EFCA 100 and 200 mg/kg and glibenclamide inhibits production of lipid peroxides. This indicates the anti-lipid peroxidative potential of EFCA.

SOD and CAT are the two major endogenous scavenging enzymes that remove toxic-free radicals *in vivo* and are thought to play an important role in protecting the cell against the potentially deleterious effects of ROS. Reduced activity of SOD and CAT may result in a number of deleterious effects due to the accumulation of superoxide radicals ($O2^-$) and hydrogen peroxide.[26] Administration of EFCA 100 and 200 mg/kg and glibenclamide results in the activation of SOD and CAT to near normal levels in diabetic rats. The result of the SOD and CAT activity clearly shows that EFCA 100 and 200 mg/kg exhibited free radical scavenging activity, which could exert a beneficial action against pathological alterations caused by the presence of $O2^-$ and OH^-.

Endogenous nonenzymatic antioxidant system glutathione plays an important role. Primarily acting as a reducing agent, it detoxifies hydrogen peroxide with the help of enzyme, glutathione peroxidase.[27] The depleted GSH may be due to reduction in GSH synthesis or degradation of GSH by oxidative stress in HFD and STZ-induced hyperglycemic animals but after treatment with EFCA increased the GSH level in both liver and kidney tissues.

Preliminary phytochemical studies revealed the presence of polyphenolic compounds, namely flavonoids and tannins in EFCA. In our previous HPLC study, EFCA revealed the presence of two putative flavonoids, viz., myricetin and apigenin. Polyphenols are believed to be responsible for several important biological activities of plants.[28] The hypoglycemic activity of EFCA may be attributed to its polyphenols, especially flavonoids content.

CONCLUSION

The current study clearly demonstrates that daily administration of EFCA exhibited a pronounced hypoglycemic effect (FBG) and also improved the antioxidant defense system such as LPO, SOD, GSH, and CAT in the liver and kidney of diabetic rats. These results suggest a promising effect in intestinal glucose transport, HbA1c, enzymatic liver, and kidney biochemical parameters and also improve the histology of pancreas. However, investigation of secondary metabolites principally flavonoids of this fraction, responsible for hypoglycemic and antioxidant effect, should be undertaken to confirm the compound which is responsible for these activities.

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Conflicts of interest

There are no conflicts of interest.

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