INDEX

CHAPTER – 1

INTRODUCTION

Diabetes mellitus (DM) is often merely referred as diabetes and it is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because the cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). It is a group of hormonal and metabolic disorder characterized by hyperglycemia and glycosuria, with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The percentage of diabetic patients all over the world is increasing day by day [1-2].

DM is reaching as epidemic proportion across the earth. Today, there are 382 million people affected with DM around the globe. Further, 316 million with impaired glucose tolerance are at high risk of disease and it is an alarming figure that is set to hit 471 million by 2035. DM is on the rise all over the world and countries are struggling to keep up with the progression of this metabolic disorder. Globally, it is believed to be the fifth leading cause of death in 2000 after communicable diseases, cancer and injury[3]. India ranked first with 31.4 million people followed by China (20.8 million) and United State (17.7 million). The statistical data is going to be double by 2030 with expected number of 79.4 million people in India, 42.3 million people in China and 30.3 million people in USA[4]. Prevalence of diabetes in Indian sub-continent countries showed that about one quarter of the population of urban region is affected in Bangladesh, Nepal, Bhutan and Srilanka[5]. Preliminary study conducted by the Indian Council of Medical research (ICMR) revealed the statistical data, that the lower proportion of the population of Chandigarh(0.12 million), Jharkhand (0.96 million) and Uttar Pradesh (4.23 million) was affected in states of Northern India as compared to 9.2 million in Maharashtra and 4.8 million in Tamil Nadu[5].

Glucose is a simple sugar found in food and is an essential nutrient that provides energy for the proper functioning of the body cells. Carbohydrates are broken down in the small intestine and glucose in digested food is absorbed by the intestinal cells into the bloodstream and is carried to all the cells in the body. However, glucose cannot enter the cells alone; it needs insulin for its transport into the cells. Without insulin, the cells starve for energy despite the presence of abundant glucose in the blood stream. In certain types of diabetes the inability of cells to utilize glucose gives rise to an ironic situation of starvation in the midst of abundant quantum of unutilized glucose which is wastefully excreted in the urine.

1.1 Definition and Description of Diabetes Mellitus

Diabetes mellitus is a grouping of metabolic diseases described through hyperglycemia and consequential of deficiencies in insulin secretion or action or sometimes both. Nonetheless, the numerous pathogenic steps are involved in the growth of diabetes. The varieties of the diabetics are starting form autoimmune demolition of the beta cells of the pancreas with subsequent insulin deficit to abnormality and henceforth, causes for resistance to insulin exploit[6]. Nevertheless, due to the abnormalities of protein, carbohydrate and fat metabolism, inadequate action of insulin on target tissues occurs in diabetes. However, lack of secretion of insulin and/or diminished tissue responses to insulin results in deficient insulin action at one or more points in the complex pathways of hormone action.

Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with keto-acidosis or the non-ketotic hyperosmolar syndrome. Long-term complications of diabetes includes retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. Nonetheless, the patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease[7].Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes. The various symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia and blurred vision. However, the

impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia [8].

1.2 Classification of DM

DM is a diverse clinical anarchy with numerous factor turns off normal body condition. The two distinct forms of diabetes mellitus are type I, juvenile or insulin-dependent diabetes mellitus (IDDM) and type II, adult onset or non insulin-dependent diabetes mellitus (NIDDM)[9].

1.2.1 Type - I Insulin Dependent DM (IDDM)

Type- I diabetes mellitus is also called as IDDM or juvenile diabetes and it is characterised by beta-cell obliteration and caused by an autoimmune development, frequently most important to formation of absolute insulin deficit. However, the onset is typically an acute and which is developing from a period of a few days to even few weeks also. Nevertheless, the statistics says that over ninety five percent of persons are suffering from Type-I DM, which develops the diseases prior to attaining of age-twenty to twenty five even with an equivalent occurrence in both male and female sexes; in fact it is an increased prevalence in India and even in the world population history. The family history of Type-I DM, gluten enteropathy certainly called as celiac disease or other form of endocrine disorder is also being found and most of these patients comprise the “immune-mediated form” of type-I DM with islet cell anti-bodies and frequently may have other auto-immune diseases such as a) Addison's disease, b) Hashimoto's thyroiditis, c) Vitiligo and /or d) Pernicious anemia. And usually, few patients especially the patients from Africa or Asia basis they are not suffering from antibodies and in fact they may perhaps have an analogous medical staging; consequently, they are also incorporated in this categorization and these kind of disease is called the “idiopathic form” of type-I DM[10].

1.2.2 Type 2-Non-Insulin Dependent DM (NIDDM)

Similar to Type-1 DM, NIDDM also categorized and distinguished. Generally, Type-2 DM (It is formerly called it as NIDDM, type II and/ or adult-

onset DM) is distinguished by insulin conflict in the peripheral tissues and it is also notable by the imperfection in the insulin secretion by beta cell present in the body[10]. Nevertheless, it is the most important common form of DM and is extremely depended with a family unit narration of diabetes, adult-older age, obesity or fatness and even lack of exercise. However, NIDDDM is highly frequent even in women population, especially women with an account of gestational diabetes (GDM), and in black peoples like hispanics and native of American countries. Moreover, the resistance for the insulin secretion and high insulin secretion eventually leads to impaired tolerance to the level of glucose present in the body and defective beta cells become depleted, further fueling to the cycle of glucose intolerance and increased glucose level of the body. The etiology of type 2 DM is multifactorial and it could be due to genetic sequence in general, but it is also has the brawny reactive components.

1.2.3 Other specific types of DM

The different types of DM of various known causes are grouped together to form a separte classification called “other specific types.” This group includes persons with genetic defects in the function of beta-cell and this type of diabetes is formerly called as MODY-maturity-onset diabetes in youth. Nonetheless, it is caused due to numerous reasons like defects in the action of insulin secretion; persons who have defects in the exocrine pancreas such as pancreatitis or cystic fibrosis; persons with impaired endocrine gland function; and the persons who have pancreatic dysfunction due to the action of some drugs, chemicals or infections[10].

1.2.4 Gestational diabetes mellitus (GDM)

The Gestational diabetes mellitus (GDM) is an operational classification rather than a pathophysiologic condition and it is identifying the populations of women who develop DM during gestation. In general, the women with DM before pregnancy are said to have “pregestational diabetes” and these are not included in this category. On the other hand, the women who develop type 1 DM during pregnancy and women with undiagnosed asymptomatic type 2 DM that is discovered during their pregnancy are classified with gestational diabetes mellitus.

Conversely, the most women classified with gestational diabetes mellitus (GDM) have a normal glucose homeostasis during the first half of their pregnancy and develop a relative insulin scarcity during the last half of the pregnancy and resulting which it leads cause for hyperglycemia. The generated hyperglycemia resolves in most of the women after their delivery but it may tend to increase in the risk of developing type-2 DM during their later age of life.

1.3 Risk Factors for Diabetes Mellitus

A very common lifestyle disease was observed now a days is termed as DM. Although it can be triggered by genetic susceptibilities and viruses, there are many other factors that can increase the risk of diabetes as well. Analysing the various factors is very much helpful in managing and preventing the occurrence of DM.The most important factors that aggregate and more susceptible to DM are detailed in below subtitles.

1.3.1 Types of Risk Factors for Type-1 Diabetes

The identification of risk factors for Type-1 Diabetes is a prime importance for the diagnose of the disease. The crucial threat aspect for type-1 diabetes is the family history of patient, chronic diseases etc.

1.3.1.1 Genetics and History of family members

In persons whose parent or sibling suffering from type 1 diabetes illness has chances for the increased threat of developing this condition.

1.3.1.2 Diseases of pancreas

The deficiency or injury of pancreas may lead to reduce in generation of insulin and results in Type-1 diabetes.

1.3.1.3 Infection

Damage of pancreases due to some disorder or infection lead to Type-1 diabetes. Nevertheless, when expose to viruses like “Epstein-Barr” virus, “coxsackie” virus, “mumps” virus and / or “cytomegalo” virus etc trigger the

autoimmune demolition of the islet cells and / or these viruses may also directly transmit a disease to the islet cells.

1.3.2 The type of Risk Factors for Type - 2 Diabetes

The various types of risk factors are responsible for budding the type 2 diabetes in general and the possible causes are listed below;

- High blood Cholesterol and high blood pressure
- Gestational diabetes
- Unfit diet
- Sedentary life style
- Obesity or over weight
- Ethnic background
- Ageing
- Lack of tolerance in glucose level
- Family History
- Polycystic ovary condition

1.3.2.1 High blood Cholesterol and high blood pressure

Uncontrolled cholesterol level in the blood and increased blood pressure level can make a person more susceptible to DM.The basic reason behind in this is the viscosity of the blood increases due to increase in the blood glucose level results to the high pressure in the vessels and certainly it leads to the damage of blood vessels within.

1.3.2.2 Gestational diabetes

Women who acquire DM during the pregnancy period have high risk of getting the disease even after the delivery. The condition of gestational diabetes

improves when the release of hormones from the placenta attains the cells of the mother to insulin resistant.

1.3.2.3 Unfit diet

It is also considered to be an important factor in generating the DM. Consuming high cotent sugar foods, high carbohydrate diet; fatty foods may aggregate the condition of diabetes and even may worsen the existing disease condition.

1.3.2.4 Sedentary lifestyle

Life style change can also be a major cause for the occurrence of DM.Obesity, lack of exercise and physical activity induce the risk of DM.Generally in the body insulin receptors are more available in the muscle cells when compared with the fat cells and other cells. The muscle cells are much involved in the regulation of amount of glucose in the body hence the exercises involves the muscle cell to utilize more glucose and its regulation.

1.3.2.5 Obesity or being overweight

It is reported in many cases that obesity is the number one factor in contributing to the high risk of diabetes. From the datas of researchers it is observed that the patients who are obese and overweight are 55% more prone to attain DM when compare with the non-obese people.

1.3.2.6 Ethnic background

The reason under the ethnic background for the diabetes occurrence is more often in certain groups, such as

- African-Americans
- Alaska citizens
- Asian-Americans
- Latino Americans

- Native Americans
- Pacific Islanders

1.3.2.7 Aging

Age is one of the common factor in the occurrence of DM. Maximum at the middle age of 45 years is considered to be at high risk to attain DM and it increases thereafter when age increases.Many researchers highlighted in various studies that the function of pancreas reduces when an individual is aged.Hence the ability of the organ to produce and pump insulin is decreased and so automatically the body system become inefficient in their functions. Ageing of cells are more insulin resistant and contribute for the progression of disease.

1.3.2.8 Lack of tolerance in glucose level

The lack of tolerance in glucose level is directly arbitrated as pre-diabetes or impaired fasting glucose level. It is a mild variety of diabetes and is a foremost risk aspect for budding the type-2 diabetes and cardiovascular complications.

1.3.2.9 Family history

DM risk factors are high, if there is a family history of diabetes especially in the case of blood relations with the history of type 2 diabetes.

1.3.2.10 Polycystic ovary condition

The women with polycystic ovary condition are at elevated risk of type 2 diabetes. The factor which is associated with the polycystic ovary condition is insulin resistance and polycystic ovarian condition and which are interrelated.

1.4 Conditions of Diabetes Mellitus

1.4.1 Conditions of Type-1 Diabetes

The type 1 DM is identified in the childhood period or in the early period of adolescence may be adjoined with some illness like urinary tract infection. The conditions of the disease may be unexpected and occur suddenly. Over stress,

ketoacidosis cause DM with nausea and vomiting. Ketoacidosis treatment is essential in time or otherwise it leads to serious conditions like coma and last death.

1.4.2 Conditions of Type 2 diabetes

The conditions of DM associated with the obesity and aging process.An individual can lead a life without any symptoms of DM even if he had the disease devolped before long time. Type 2 DM can aggregate the hyper-glycemic, hyper-osmolar and non-ketotic condition and it shall be impulsive with steroids and as well as stress. These conditions develop multiple complications include blindness, kidney failure, heart disease and damage in the nerves if not properly treated.

1.4.3 Common conditions of both Type 1 and Type 2 diabetes

The following common conditions are observed in both type 1 and type 2 diabetes and the details are explained below.

1.4.3.1 Tiredness or fatigue, constantly tired

In diabetic condition body is incapable of doing its function and occasionally unable to use glucose for functioning of the body and it turns for metabolizing the deposits of fat as moderately or utterly for the energy sources. Henceforth, it may be in need of using extra energy and it results in feeling fatigue or constantly tired.

1.4.3.2 Weight loss

The peoples with DM are sometime inefficient to process many of the calories in the foods what they have consumed. Henceforth, the peoples may loose their weight even though they take apparently appropriate or even an excessive amount than the normal intake of their food. Nonetheless, the loss of sugar component and/or water content through urine and adjoining de-hydration of body also contributes for causes of loss in body weight.

1.4.3.3 Thirstiness (Polydipsia)

The person, who has DM with high level of glucose in blood, shatters the capability of kidney to re-absorb the glucose level when the blood is screened to

make urine. And it may be noticed that large quantity of urine could be discharged as the kidney spills the excess level of glucose in the body. Therefore, the body alarm the brain and send the signals accordingly in order to dilute the blood for coinciding the action and consequently the action becomes converted to feel thirsty and the body becomes encouraged for the level of water uptake and to dilute the high blood glucose until it attains normal level and also recoup for the loss of water level by undue urine discharge.

1.4.3.4 Excessive urination (polyuria)

In the urine, the excessive sugar in the blood may be excreted from the body and it might be the way for reducing the sugar level. In this way loss of water may be adjusted since more water is needed to expel the excess sugar.

1.4.3.5 Excessive eating (polyphagia)

In type 2 DM the body is resistant to insulin action. Insulin plays an important role in hunger stimulation. Hence insulin raises hunger. In spite of this, the person may gain due to high food intake or weight loss.

1.4.3.6 Delay in wound healing

The functions of WBC are retained because of high sugar level like removing dead tissues, cleaning up of cells and fighting against bacteria. Therefore the wound healing gets delayed.

1.4.3.7 Infections

Infections such as genitial yeast infection, skin and urinary tract infections occur frequently due to the suppression of the immune system due to chronic disease condition of DM and also by the bacterial growth in the tissues due to the accumulation of glucose. This conditions show that the person have very poor control over the blood sugar

1.4.3.8 Altered mental status and blurry vision

In case of high blood glucose level, ketoacidosis, hyperosmolar hyperglycemia nonketotic syndrome and hypoglycemia there will be inattention, lethargy or irritability and high emotions. In DM, blurry vision is found to be one of the complications now days**.**

1.5 Complications of Diabetes

In persons with controlled sugar levels have very less common complications. Some problems include smoking, high cholesterol level, obesity and BP may aggregate the problems of DM.

1.5.1 Acute complications

DM is accompanied by many acute complications.

1.5.1.1 Hypoglycemia

It occurs in most of the diabetic patients when they take excess of antidiabetic medication or external insulin, heavy exercises, alcohol intake or by other drugs ingested for other conditions. Its symptoms are headache, dizziness, sweating etc. It should be recognized and treated in time.

1.5.1.2 Diabetic ketoacidosis

Diabetic ketoacidosis defined as a serious condition characterized by unrestricted hyperglycemia in the blood and produce excess of ketones in it. These levels of ketones are harmful to the body and usually found in the people affected by type1 diabetes with uncontrolled blood glucose level. This condition may be elevated by infections, trauma, stress, irregular insulin doses.

1.5.1.3 Hyperosmolar hyperglycemic nonketotic syndrome

It is a condition in which the blood glucose rises abnormally and the excess amount of blood glucose is excreted through urine. So, the urination increases significantly & leads to loss of water. This condition may often lead to seizures, coma and death. This happens with persons who have uncontrolled DM.

1.5.1.4 Infections

Infections are mostly linked with DM and these infections make the diabetic condition worse. The reason behind this is the ability of the body becomes reduced to fight against infections**.**

1.5.2 Chronic complications

The blood vessels get damaged in chronic diabetic condition. Intake of glucose by the endothelial cells is commonly more and they are independent in its action and form glycoproteins on its surface. In case of DM glycoproteins change its pattern of forming in various ailments.

1.5.2.1 Diabetic retinopathy

The most important complications of DM are diabetic retinopathy. It is revealed that the pathogenesis of diabetic retinopathy involved with many genes which includes non-enzymatic glycation, accumulation of glycated end products free radical mediated protein damage, upregulation of matrix metalloproteinases and growth factors.

1.5.2.2 Diabetic nephropathy

Diabetic nephropathy accounts for about 14% of all deaths in diabetic patients. Glomerular hyperfusion is the earliest renal functional change continued by renal hypertrophy. At this stage renal morphology is normal, although there may be microalbuminuria which is reversible by strict control of the blood glucose concentration and high blood pressure, if present. Its importance is that it anticipates the development of irreversible renal damage indicated by the development of proteinuria.

1.5.2.3 Diabetic neuropathy

Diabetic neuropathy may involve every component of the nervous system with the potential exception of the psyche. Distinct syndromes can be spotted and several different types of neuropathy may be present in the same patient. The most common is peripheral poly neuropathy; the symptoms include numbness and severe

pain. Hurt to the nerves in the lower extremities is a major case of foot ulcers and wounds, which often lead to foot amputations.

1.6 Metabolic derangements associated with diabetes mellitus

1.6.1 Metabolism of Glucose

High hepatic glucose output will occur in case of chronic DM. In liver glycogen stores are transported and glucose is synthesized in the liver. In insulin deficiency, non hepatic tissue glucose utilization will be impeded. Also insulin provokes the glucose uptake in adipose tissue and skeletal muscle. The glucose uptake action is mediated through insulin and turns the glucose transporter to the plasma membrane. In case of decreased glucose uptake of peripheral tissues the glucose biotransformation struck off. The conjoint action of elevated hepatic glucose synthesizes & decreased peripheral tissue metabolism pay way to raised glucose level in the plasma. When the capability of the kidneys in the absorption of glucose is impaired, glycosuria occurs. An elevation in the renal loss of glucose is coordinated by loss of H_2o molecules & electrolytes. The loss of H_2o molecules activates the thirst centre. The tissue catabolism also causes an increase in appetite and food intake[11].

1.6.2 Metabolism of Lipids

The energy is created through insulin after meal consumption. The energy will be stored in the form of glycogen in liver cells and skeletal muscle. Hepatocytes are stimulated by insulin to produce and store triglycerides in adipose tissue. Against this action insulin also mediates the inhibition of lipolysis. In case of uncontrolled DM triglycerides are transported more and lead to high free fatty acids in plasma. These free fatty acids will be absorbed by tissues and produce energy and it is absorbed by liver also. In the absence of insulin, fatty acids generate acetyl-CoA and further oxidized in TCA cycle. In liver cells, most of the acetyl-CoA will not be oxidized by TCA cycle but it is converted into acetoacetate, ketone bodies & β-hydroxybutyrate. Due to the excess amount of free fatty acids and ketone bodies the glucose utilization decreases and promotes high glucose level.[11]

1.6.3 Metabolism of proteins

Synthesis of many genes is regulated by insulin and has an impact over metabolism. Insulin plays a major role in the protein metabolism by increasing the charge per unit of protein synthesis and reducing the degradation of protein. Hence, insulin defects can alter protein catabolism. The rise in degradation of proteins results to the high concentrations of amino acids in plasma and these amino acids act as a promolecule in the glucose synthesize and leads to increased glucose level[11].

1.7 Insulin and its Role in Diabetes Mellitus

1.7.1 Structure of Insulin

Insulin is a small peptide consisting of fifty-one amino acids synthesized by beta cells on islets of Langerhans and stored inside the pancreas. The protein contains two chains, named as A and B, connected by disulfide bridges between cysteine residues[12] .

1.7.2 Synthesis of Insulin

Pre-pro-insulin is produced as a random coil on membrane linked ribosomes. After membrane transport the leader sequence is cleaved off by a protease and the resulting pro-insulin folds into a stable conformation. Disulfide bonds form between cysteine side chains. The connecting sequence is cleaved off to form the mature and active insulin molecule [13].

1.7.3 Action of insulin

After the release of insulin in the blood stream it binds to its receptor which is present on the cell membrane.within the cell the glucose transporter proteins are released to the surface of the cell membrane. Glucose transporters carry the sugar molecule from the blood to the tissue where it gets metabolized.Insulin is necessary to absorb glucose. The important role of insulin is to regulate the function of hyperglycemia producing certain hormones and to regulate the blood glucose levels. It also activates lipogenesis, depletes lipolysis and stimulates the transportation of amino acid in to cells.It also alers the transcription, modulates the cellular contents

of large number of mRNAs.It triggers the growth, DNA production and replication of cell with IGF along with relaxin. If insulin fails to produce its action that results in DM. In such cases insulin is administered externally to treat DM.

1.8 Diagnosis of Diabetes Mellitus

To diagnose diabetes and to monitor the blood sugar level Doctors use some specific test.Even number of test are available in the laboratories to verify the condition of the diabetic patients.

1.8.1 Fasting plasma glucose test (FPG)

This test is commonly adviced as a primary test to diagnose the fasting plasma glucose level. It is simple and convenient with low cost. It may not be so sensitive and accurate when compare with the other diagnostic tests.

1.8.2 Oral glucose tolerance test (OGTT)

When compare with the FPG test the Oral Glucose Tolerance Test is considered to be more reliable and sensitive diabetes diagnosis test. In this test the patient should undergo fasting for 8 hours before the test and the plasma glucose is determined many times. Approximately 75g of glucose has to be consumed by the patient and the blood is withdrawn before and after the intake of glucose. The blood glucose level before and after consuming of glucose drink will determine whether the person is diabetic or not. Further the results have to be checked by second round test on other day[8].

1.8.3 Glycosylated hemoglobin or HbA1c

Glycosylated hemoglobin is a form of hemoglobin that is measured primarily to distinguish the mean plasma glucose concentration over prolonged periods of time. It is made in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. Normal levels of glucose represent a normal amount of glycosylated hemoglobin. Glycosylated hemoglobin serves as an indicator for the blood glucose levels over the months. Higher HbA1c value indicates the poor control over the blood glucose levels and may lead to several diseases including

nephropathy, retinopathy and cardiovascular diseases. The result of HbA1c less than 6.0% indicates good control and more than 8% indicates high blood glucose level. To diagnose DM a standard test to be performed.[8,14]

1.9 Management of Diabetes Mellitus

Diabetes mellitus is a chronic disease which cannot be cured except in very specific situations. Management concentrates on keeping blood sugar levels as close to normal as possible, without causing hypoglycemia. Diabetes can be managed with balanced diet, excercises and healthy food habits along with proper medication.

1.9.1 Diet

Dietary measures such as nutritional counseling and motivating the patient's compliance to the prescribed dietary plan are important in the management of diabetes mellitus. An estimate is made on the total number of calories needed per day based on ideal body weight. Protein and carbohydrate calories are supplemented with sufficient fat to bring caloric intake to the desired levels.

1.9.2 Oral hypoglycemic therapy

Oral hypoglycemic drugs are used in the treatment of type 2 diabetes. There are four major classes of hypoglycemic drugs.

1.9.2.1 Sulfonylureas

Sulfonylureas are the most widely used drugs for the treatment of type 2 diabetes and appear to function by stimulating insulin secretion. The net effect is increased responsiveness of β-cells to glucose resulting in more insulin being released at all blood glucose concentrations. Sulfonylureas may also have extra-pancreatic effects, one of which is to increase tissue sensitivity to insulin. Sulfonylureas differ mainly in their potency and their duration of action. Glipizide, glyburide (glibenclamide), and glimepiride are commonly used sulfonylureas[15-16].

1.9.2.2 Biguanides

Biguanides reduce hepatic glucose output and increase uptake of glucose by the periphery, including skeletal muscle. One such biguanide is Metformin which is the commonly used and only in the presence of insulin it is effective.It does not stimulate the secretion of insulin like sulfonylureas[15].

1.9.2.3 Thiazolidinediones

Thiazolidinediones also known as "glitazones," bind to peroxisome proliferator activated receptor (PPARγ), a type of nuclear regulatory protein involved in transcription of genes regulating glucose and fat metabolism. The final result is the better use of glucose by the cells[15-16].

1.9.2.4 Alpha glucosidase inhibitors

The alpha glucosidase inhibitors include acarbose and Miglitol. They inhibit the upper gastrointestinal enzymes that convert dietary starch and other complex carbohydrates into simple sugars which can be absorbed. The result is to slowdown the absorption of glucose after meals[15-16].

1.9.3 Insulin treatment

Insulin is the oldest of the currently available medications and, thereby, the one with the most clinical experience. Although initially developed to treat insulin-deficient type 1 diabetes, it has long been used to treat insulin-resistant type 2 diabetes. It is the most effective drug to decrease glycemia. The total daily dose of insulin, the type of insulin preparation and the number and timing of injections required to achieve satisfactory control without undue risk of hypoglycemia, varies widely and is established by clinical trials. Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and α-glucosidase inhibitors which are used as monotherapy or in combination, to achieve better glycemia regulation[17]. Many of these oral antidiabetic agents have a number of serious adverse effects[18]. Thus, the management of diabetes without any side effects is still a challenge.

1.10 Medicinal plants for the treatment of Diabetes mellitus

Mankind has been continuously using the plants in one or the other way in the handling of various ailments. In India, the sacred Vedas dating back between 3500 BC and 800 BC give much reference to medicinal plants. The Rig Veda, dating between 3500 BC seems to be the earliest record available on medicinal plants[19]. Medicinal plants are frequently used by traditional healers to treat a variety of ailments and symptoms including fever, cold, headache, diabetes and cancer. Plants have been the major source of drugs in Indian system of medicine and other ancient systems in the world. Alternative treatments for DM have become more popular during the last years[20]. Many marketed antidiabetic drugs are obtained from plant sources for example metformin, which is a effective antidiabetic drug synthesized on the bases of phytochemicals found on *Galega officinalis.*[21] It has been reported that many plants are used traditionally in the treatment of DM but only less of them have underwent medical researches to evaluate its potency.[22]

Many plant origin contains phenolic substance for instance it is found in vegetables, legumes, fruits, tea, coffee.[23]The proteins and polyphenols are interrelated to each other and has been popular from earlier times.[24] The one such plant *Gymnema sylvestre* have raised the insulin level may be due to the regeneration of beta cells in the pancreas.[25]

In this study, the collected plant is identified as *Marsdenia brunoniana wight & arn* and it has been evaluated for its anti-diabetic properties. The therapeutic effectiveness of the plant is determined by considering its potential in managing different parameters involved in metabolism. The correlation is made between the *M. brunoniana* extracts and the other widely used hypoglycemic drugs, namely glimepride and acarbose.

CHAPTER – 2

AIM AND OBJECTIVE OF THE STUDY

DM is a most important cause for morbidity and death in human beings. It is described by set of symptoms like polyuria, hyperglycemia and polydipsia and it is problematic to the nerves, eyes and kidneys. Then, it is also linked with a higher prevalence of cardiovascular syndrome. The clinical symptoms of diabetes frequently differ considerably among different countries and also between cultural groups in a country. For instance, diabetes at present influence an probable of fifteen million people community in North- America, eighteen million people in Europe, fifty one million people in Asia, and almost one million people in Oceania. It is predictable that internationally, the numeral of people will increase to about three hundred million people by the year 2025.

According to the survey conducted by WHO, it is reported that plant remedies are important one and presently there is an increasing awareness globally and its impacts are more especially in the developing countries like India. In the industrialized countries, the people are looking for the safest alternative to allopathic medicine because of the increasing realization on the adverse effects of many modern remedies. Nevertheless, the investigation for novel antidiabetic medicinal treatment from natural resources requires the involvement of various departments such as Chemistry, Microbiology, Pharmacology, and Toxicology and Clinical medicine.

The present investigation has been undertaken to perceive the efficacy of the plant as antidiabetic drugs. Such investigation might gain paramount importance and provides a practical solution for combating this appalling disease.

The aim of the existing study is to carry out a systematic investigation for the *in- vitro* glucose metabolizing enzymes inhibitory activities, antioxidant activity and antidiabetic potential of extracts of *Marsdenia brunoniana* against streptozocin-nicotinamide induced type-II DM in male wistar rats. Nonetheless, it is anticipated that this investigation might endow with a methodical basis for the use of *Marsdenia brunoniana* plant in folk plant source medicine to ameliorate the impediment of diabetes mellitus.

CHAPTER – 3

REVIEW OF LITERATURE

3.1 Ethnobotanical Information

The different parts of medicinal plant are used in the traditional way of medicine by Indian tribes. Such medical practitioners experienced to treat much kind of diseases like arthritis, rheumatism, inflammation, liver diseases etc. The leaves of the plants were used for stomach ache and other intestinal disorders also. A decoction of the leaves is given for the treatment of gonorrhea.The leaves are widely used by the tribes from Southern Tamilnadu for the treatment of diabetes[26-28].

R.Kottaimuthu et al stated that more than 100 species of the genus *Marsdenia* belonging to the family Asclepiadaceae have been distributed through out the tropical countries. Around 13 species was found in India. Out of which two species are endemic to Western Ghats. Marsdenia tirunelvelica, a rare species was collected by Henry in 1972 from Agastyamalai hills, Tamil Nadu[28].

Many plant species such as *Marsdenia tinctoria, Marsdenia tenacissima, Marsdenia condurango, Marsdenia roylei* and *Marsdenia brunoniana* were used extensively in Indian and Chinese system of medicine to treat various ailments. Among these plants, *M. tenacissima* was used widely in Chinese system of medicine to treat cancer. An ethnobotanical survey among the tribes from southern Tamilnadu used *M. brunoniana* for the treatment of diabetes mellitus[29].

The polysaccharide called Marsdenia tenacissima – polysaccharide (MTP) which is a water soluble compound and its molecular weight lies about 4.6×10(4)Da and it was secluded from the dried palm of thin stem of *M. tenacissima*. The isolated MTP was able to hinder the growth of tissue in H22 tumor-posture mice medicine dose-contingently, and subsequently it is the sources for increasing in index of spleen, thymus and then serum albumin level also increases in the persuaded mice. Nonetheless, MTP raising the blood serum level of TNF-alpha and IL-II and then increases the pursuit of SOD, CAT and GSH-Px in the liver's tissue, and reduces the

content of vascular endothelial growth factor. These findings proved that the MTP might modulate the resistance of certain infection systems in mice and ultimately subdue the development of tumor tissue in H22 tumor-posture mice. The activity of antitumor shall be based on its antioxidant property and the effects of immunomodulatory[30].

A C_{21} steroidal-glycoside (SG) (17-β-Marsdenia-tenacissima glycoside B(MTG) (17-β-tenacigenin B) was isolated then it was screened for its cytotoxic effect using the methods like Western-blot, MTT and flow cytometry. The obtained results revealed that the 17-β-tenacigenin B had no obvious anti-tumor effects[31]. A new flavone kapitone and three known compounds, that are 3,2'-dihydroxyflavone, 1-methylcyclobutene and dimethyl isatoate were isolated from *M. tinctoria* R. Br. Belonging to the family Apocynaceae[32]. A pure esteric-glycoside condurangogenin A was isolated from Marsdenia condurango and it was studied against lung cancer cells. The results revealed that this compound have potent anticancer activity[33].

The new 16 polyoxypregnane-glycosides, marstenacissides B1-B9 and marstenacissides A1-A7 were secluded from the roots of *M. tenacissima*. The obtained structures of those novel compounds were recognized through different spectroscopic proficiency techniques which include nuclear magnetic resonance spectroscopy and mass spectrometry[34].

Pregnane oligoglycoside namely roylinine and a pregnane derivative like marsgenin has been seculated from $CHCl_3$ soluble extracts of stem in the dried condition from Marsdenia roylei then its structures were identified using FABMS, TOCSY , ^{13}C-NMR, ^{1}H-^{1}H COSY and ^{1}H-NMR, HSQC with spectral techniques as well as chemical degradation and derivatisation. The structure of pregnane oligoglycosides,namely rocinine and marsdinine, which was secluded from the $CHCl_3$ extract of the dried stalk of Marsdenia roylei, were elucidated using MS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC and TOCSY experiments as well as chemical degradation and derivatisation[11]. A new pregnane glycoside roylenine was isolated from the mixture of Chloroform and Ethyl alcohol (ration of 4:1) extract of Marsdenia roylei. Nonetheless, the structure also was established on the basis of

spectroscopic studies. The glycoside and its acetylated derivative were evaluated for their antioxidant and antidyslipidemic activities[35-37].

Shivani saini and Sunil sharma reported that the extraction of Helianthus annuus through alcoholic extract and it was administrated at level of doses of 250 mg/kilogram and 500mg/ kilogram and in which the post orally results revealed as lesser significant alterations in level of glucose in blood of normoglycemic induced rats with the p value of 0.05 and the diabetic rats shown much depletion of glucose level in blood when it was estimated in oral-glucose toleration test and its p value is 0.01when the streptozotocin –nicotinamide induced rats administered with extract it shown significant decreased blood glucose level and the p value was 0.001 when compared with the standard drug Glibenclamide of dose 600µg/kg and the diabetic rats restored the lipid profile, body weight, liver glycogen content, glycosylated haemoglobin, glutathione level, plasma malondialdehyde and serum insulin levels[38].

Sunday Oyedemi, Graeme Bradley, and Anthony Afolayan have stated that aqueous bark extract of S. henningsii was administered at different doses such as 125 mg / kilogram, 250 mg / kilogram and 500 mg / kilogram body mass of by streptozotocin-nicotinamide (SN) persuaded rats which are diabetic for fifteen days and the extract has dropped in the glucose level in blood, water, feed intake and also triacylglycerol. The extract shown significant results at the dose levels of 500 mg/kilogram and the extract treated rats have shown normal weight at all doses. After 90 min of extract administration the diabetic rats have shown significant decrease in glucose tolerance level at the dose level of 250 and 500 mg/kg. The results of S. henningsii revealed that it contains flavonoids, tannins and saponins and proposed that it increase the insulin secretion. The detailed study of those extracts showed the properties of antihyperglcemic and antilipidemic[39].

Rakesh Barik et al. revealed that the aqueous extract derived from the roots of Ichnocarpus-frutescens (IF) administration own notable antidiabetic activity by level of dose in 250 mg/kg and 500 mg/kg, p.o.) in streptozotocin convinced rats of type-2 diabetes. The standard drug used was Glibenclamide against the extract and the serum glucose was tested on 0, 5, 10, and 15th days by using glucose oxidase-peroxidase reactive strips and a glucometer. Oral glucose tolerance test has been

taken by administering of glucose dose as 2g/kg, p.o on non-diabetic control rats along with standard drug and aqueous root extract of Ichnocarpus frutescence. The tolerability of serum glucose and the body weight gaining also were reported .The fasting blood glucose level against the streptozotocin-nicotinamide persuaded diabetic rats showed as reduced blood glucose level[40].

B. Jayaprasad, P.S. Sharavanan, R. Sivaraj have evaluated and presented that the bark extract of Chloroxylon swietenia shown effective activity of antidiabetic hostile the streptozotocin persuaded diabetic rats. The administered methanolic and extracted in aqueous solution of Chloroxylon swietenia and the glibenclamide was used as standard for the study. The study result was shown moderate reduction in the glycosylated haemoglobin and blood glucose level .they also reported that there was an elevated plasma insulin level and haemoglobin levels. The carbohydrate complexed metobolised enzymes and levels of glycogen in liver also improved after the administration of the extracts of Chloroxylon swietenia[41].

S. Ramachandran, A. Rajasekaran and N. Adhirajan have investigated and reported that aqueous extract of *Terminalia paniculata* bark (AETPB) have significant antidiabetic property in both experiments of in-vivo and in-vitro. The administration of *Terminalia paniculata* bark (AETPB) through oral to the streptozotocin-nicotinamide (65 mg/kg–110 mg/kilogram; *i.p.*) persuaded type-II rats which is diabetic at the doses level of 100 mg/kg and 200 mg/kg shown that reduction in glucose level of blood and levels of hemoglobin (gycosylated) when comparing with the rats which are diabetic untreated.The extract treated rats increases its body weight with the elevated total insulin, protein and level of haemoglobin. Nevertheless, in the profile of lipid there was a reduction in total cholesterol and increases in HD lipoprotein levels was observed after the administration of extracts. The biomarkers gallic acid, catechin, ellagic acid and epicatechin presence in AETPB was established by HPLC analysis technique. It has been stated that the intensification of uptake in glucose action in presence of insulin available in muscle cells comparatively higher than vehicle control could be due to the presence of trihydroxy benzoic acid and Terminalia paniculata Bark. The

hindrance of pancreatic alpha-amylase and alpha-glucosidase was also observed in this study[42].

W. Pierre et al. studied the both anti-diabetic and anti-hyperlipidemic properties of the methanolic extracts and aqueous extracts of Bersama engleriana leave. The extract has been administered at the dose levels of 300 mg/kilogram or 600 mg/kilogram to the Nicotinamide- streptozotocin (SN) persuaded type two diabetic- rats. The glucose measurement was made in the blood at the interval of 0 day, 1day, 14 days, 28 days after the diabetes induction and sacrificed on 29th day for the estimation of LD lipoproteins, HD lipoproteins, triglycerides and total cholesterol. In this study, Glibenclamide was considered as a standard drug. The leaves of Bersama engleriana in methanolic extracts at the dosage level of 600 mg/kilograms had shown the notable results when compared with the other dose levels. The methanolic extract induced diabetic-rats suffered by decreasing in blood level of glucose, LD lipoproteins, total cholesterol and triglycerides. At the same time, it was observed that remarkable increases in HD lipoprotein levels. Nonetheless, the methanolic extract 600 mg/kilogram of the plant was showed that good therapeutic activity than the usual drug being used glibenclamide (0.25 mg/ kilogram)[43].

U. Sharma et al. investigated the activity of anti-diabetic and ant-oxidant of the grinded bulb of Stephania-hernandifolia (SH). The aqueous extract and the ethanol extract of the corm were tested against the diabetic rats which were persuaded with streptozotocin. The administered extracts in different level of dosage of 400 mg/ kilogram shown remarkable reduction in the level of glucose in blood while examined for specific time intervals when compared with the standard drug glibenclamide.The data of the experiment shown that the aqueous extract and ethanol extracts have potent hypo diabetic and antioxidant activity. The in vitro antioxidant activity was also investigated upon various models like DPPH and Superoxide-free radical scavenging assay. IC_{50} values of the extracts of ethanol and aqueous are 265.33 and 217.90 µg/ml shown that they have strong scavenging property[44].

M. Madhavi, S. Kiran and D. Ramesh were studied the Phytochemical screening, antioxidant activities, hypolipidemic and hypoglycemic of Acacia leucophloea in streptozotocin-nicotinamide (SN) persuaded type two diabetes. The ethanolic extracts of Acacia leucophloea stem bark at two dosage levels of 200 mg/kilogram, 400 mg/k kilogram were administered to the diabetic rats for 14 days. The treatment significantly mitigated the changes in the glucose, lipid profile, oxidative stress, liver enzymes and body weight parameters. The antioxidant activity altered by the extracts within the diabetic rat also shown to be improved[45].

Rekha Bisht and S. Bhattacharya studied on streptozotocin-nicotinamide persuaded diabetic wistar albino-rats with different extracts of Desmodium gangeticum and they have revealed that the extracts have potent antidiabetic effect. The different solvent extracts includes pet. ether, benzene, chloroform, acetone, ethanol and water of Desmodium gangeticum 100 mg/kilogram was administrated for the diabetic -rats to twenty one days. The consequence of the extract was studied on different parameters such as low density L-C, High density L-C, blood glucose, triglycerides and total cholesterol. The diabetic rat's body weight also found to be elevated in extract treated rats. Among all the extract treated groups, they have identified that aqueous treated group has antidiabetic, antihyperlipidemic activity and positive effect on weight of diabetic rats also[46].

Asmawati abd rahim, Jamaludin Mohamad & zazali alias has investigated the antidiabetic activity of leaves and stems of Leptospermum flavescent. In the study the aqueous extract of leaves and stems were taken and estimated for the total phenol and flavonoid content and correlated with antidiabetic activity. LCMS / MS indicated the presence of flavonoids aromadendrin glucoside, kaempferol rhamnoside, quercetin rhamnoside and vindoline.About 85% of glycogen phosphorylase with IC_{50} = 0.18 mg/mL was inhibited in this study. The results showed that the aqueous extract 500 mg/kilogram were remarkable deterioration in fasting plasma level of glucose by 61.9% with the p value of 0.001. The total cholesterol level, triglycerides, low density lipoprotein levels also been found to be in decreased level and the High density lipoprotein was restored on treatment with the leaves and stems extract of Leptospermum flavescent. The high antidiabetic

activity was correlated with high total phenol at 1.57±0.01 GAE/g and total flavonoids at 1.41±0.01 mg QE/g. The higher in activity of the antidiabetic extract was attributed because of availability of aromadendron glucoside, kaempferol rhamnoside, quercetin rhamnoside and vindoline in aqueous extract of Leptospermum flavescens[47].

A.Verma et al. reported that scopoletin, a derivative of coumarin and it posses the hypoglycemic and hypolipidemic activity when studying on streptozotocin persuaded wistar albino rats. In this study, Glimepiride is used used as reference standard for the experiment study and the quantity usage was 0.11 mg/kilogram). The rats were ingested by scopoletin with the dosage level of 1mg /kilogram by post orally and the frequencies were once in a day and two times per day. At the end of the experiment the fasting level of glucose in blood and lipid profiles were measured in both categories of rats like diabetic as well as non-diabetic. The study reveals that the subsequent reduction in glucose level in blood and lipid were observed. The same study found that the scopoletin administered as two times a day results proved the minimisation in glucose level of blood during fasting and lipid profile[48].

PK. Bhateja and R. Singh revealed that the hypoglycemic property of aqueous extract of Acacia tortilis polysaccharide in STZ-nicotinamide (SN) persuaded diabetic-rats. The diabetic rats were administrated with aqueous extract of different in different dosage levels such as 250, 500 and 1000 mg/ kg and glimepiride as the standard drug. The results showed that streptozotocin remarkably increasing in level of glucose in blood, glycated HB, triglyceride, total cholesterol, Low DL, liver function-makers namely SGPT and SGOT and decreased the High DL level during fasting. Finally, during the seventh day study, the blood glucose level was diminished in fasting along with the triglyceride, cholesterol, Low DL, VLDL, liver function levels (SGOT and SGPT) and High DL level was significantly improved when compared to diabetic control group. Thus, the study has been concluded that AEATP may have potential for the treatment of T2DM and its comorbidities[49].

Ranjana, Yamini and Tripati were revealed that the hexane extract of Annona squamosa have a significant activity in increasing the secretion of insulin by the dosage levels of 100 mg/ kilogram and 400 mg/ kilogram when compared with the drug being used as standard glimepiride 1mg/kg. The extract also has potent inhibitory action over alpha glucosidase than the acarbose10 mg/kilogram. The studied extract also minimised the level of glucose and the AUC levels in oral glucose tolerance test and oral sucrose tolerance test and overall it has a good control in reducing blood glucose level and thus confirmed that the extract has a good insulin secretagogue action and also have inhibitory action on the intestinal enzymes responsible for glucose metabolism[50].

Ramachandran et al. evaluated that aqueous extract of Anogeissus latifolia bark has a potent antidiabetic, antihyperlipidemic and antioxidant activity in STZ-nicotinamide persuaded diabetic-rats. The rats were administered with the extract at different dosage levels like 100 mg/kilogram and 200 mg/kilogram for four weeks. The body weight and the blood glucose were estimated at the week ends. The haemoglobin, glycosylated haemoglobin and lipid profiles were studied along with various bio-logical parameters. The study observed that the glycosylated haemoglobin, serum lipid profiles have significantly reduced with the extract treated diabetic groups and the antioxidant level was reversed to near normal than diabetic control rats[51].

Sabitha et al. investigated the anti-oxidant property through in-vivo study of extract Abelmoschus esculentus in STZ persuaded diabetic- rats. The diabetic rats were studied for different dosages level of AEPP and then AESP through orally up to 28 days. The dosage used in this study is 100 mg/kg and 200 mg/kg. After, 28th day, the tested rats were sacrificed and analysed by collecting their organs in order to study the levels of lipid peroxidation, superoxide dismutase, glutathione peroxidase , catalase and reduced glutathione. In this study, only thiobarbituric acid active components were increases and remaining lipid peroxidation, superoxide dismutase, glutathione peroxidase, catalase and reduced glutathione were observed as reduction when compared to the control rats[52].

C.Liu et al. evaluated the antinephritic activity along with anti-diabetic activity of cordyceps militaris.The aqueous extract of the cordyceps militaris was studied at the different dosage levels as 0.5 g/kilogram, 1.0 g/kilogram and 2.0 g/kilogram for four weeks orally and in this study the standards drug is used as metformin by the dosage of 100 mg/kilogram. The results reveled that the reduction in the food, water intake, urine output and plasma glucose level; this reduction indicates the hypoglycemic activity of cordyceps militaris.The normalization of total cholesterol, triglycerides and LDL, HDL with the CM administration denoted the hypolipidemic activity. The renal protective activity of CM was proved by its inhibitory action over albuminuria, creatinine, urea nitrogen, and n-acetyl-β-d-glucosaminidase. When compared with the untreated diabetic rats CM exerted beneficial modulation of inflammatory factors and oxidative enzymes and also it decreased the expression of phosphor-AKT and phosphor-GSK-3β in the kidneys. Therefore it is concluded that the CM has antidiabetic and antinephritic action via attenuating oxidative stress[53].

Ramachandran, S.A, Rajasekaran, A.B, Kumar, K.T.M. stated that the methanolic extract of Tectona grandis flowers (TGF) have potent antidiabetic, hyperlipidemic and antioxidant activity. Toxicity studies have done to determine the the dose for the antidiabetic study by acute toxicity study. The extracts of TGF observed no toxicity up to 2000 mg/ kg. The impact of TGF on elevated blood glucose level was analysed through oral glucose tolerance test. The extract administered study was carried out for twenty eight days and the then level of glucose in blood was monitored in each week of the study. At the last day of study, blood was analysed to assess the various biochemical variances and the level of anti-oxidant was estimated by collecting the kidney & liver. While using the above dosage level, TGF clearly proved that remarkable loss in level of glucose in blood. Nonetheless, in the above dosage level TGF showed significant increases in weight of body, haemoglobin (Hb), serum insulin and then total protein levels of persuaded diabetic rats. Lipid profiles are normalized from the elevated state in diabetic rats[54].

OM Ahmed et al. evaluated the therapeutic efficacy of rutin present in Ruta graveolens in nicotinamide-streptozotocin induced type II diabetes. R. graveolens infusion and rutin were orally induced to diabetic-rats as a dosage of 125 mg/

kilogram and 50 mg/kilogram of rat's body weight per day for totally thirty days. At the end of the study both the R.graveolens infusion and rutin had a good impact on reduction in level of glucose in blood, ameliorates and hyperlipidemia, serum-insulin. It was also stated that the treatment remarkably increases the insulin releases, binding capacity of insulin and peripheral glucose uptake in rat diaphragm whereas intestinal glucose and cholesterol absorption was also found to be reduced[55].

Pari L and Saravanan R. reported the effect of drug in poly-herbal, on level of glucose in blood, plasma-insulin and the various activities. The hepatic glucose metabolic enzymes were used for alloxan admnistered diabetic-rats. The diasulin treated groups of rats showed the results that remarkable decrease in level of glucose in blood and glycosylated haemoglobin.The level of HB was found to be elevated along with good glucose tolerance. The study also defines that, the reduction in glucose, fructose and bi-phosphatase in the liver and the same time the quantum of plasma-insulin activity and hepatic-hexokinase activity gets increased[56].

Raushanara Akter et al., revealed the antioxidant property of methanol extract of the leaves of Opuntia dillenii by DPPH, nitric oxide scavenging activity, reducing power, total phenol and flavonoid content determination assays. In this study 80% of methanol was used in study. The Phytochemical investigation of the extract denotes the presence of flavonoids, steroids, alkaloids and tannins. The antioxidant study was compared with the reference antioxidant and found to have dose dependent activity. In DPPH assay the IC 50 values of the extract and the standard was 15.71µg/mL and 10.84 µg/mL respectively. The presence of high amount of flavonoids and phenols ameliorates the antioxidant activity[57].

Subramanian R, Asmawi MZ and Sadikun investigated the in-vitro evidence for study of inhibition property of different enzymes like α-glucosidase enzyme and α-amylase enzyme then later *in-vivo* studies also was carried out on the same kind of study in order to analyse the efficacy of the EE of Andrographis paniculata and andrographolide. The ethanolic extract (EE) was found to have significant alpha glucosidase inhibitory action and week alpha amylase inhibitory action in concentration dependent manner with the IC 50 values of 17.2+/-0.15 mg/ml and

50.9+/-0.17 mg/ml respectively. The in-vivo studies demonstrated that A. paniculata significant is capable of reducing in the level of glucose in blood. Hence, the study was concluded that due to α-glucosidase reserving capacity is the responsible for the antidiabetic activity exerted by A. paniculata[58].

John WB studied in detail that the glycoxidation products such as carboxymethyl -lysine and hydroxylysine. These products are formed by the stage of reductions reaction in sugars and also proteins during the glycation and oxidation reactions .These formed products are getting gathered in the tissue-collagen and being responsible for increasing of diabetes. These products are found to be biomarkers proteins in the development of diabetes but in small traces. Nonetheless, the other causes for the production of free radicals are through auto-oxidation formed sugars and the same time auto-oxidation of available un-saturated lipids and proteins. The underlying mechanism of these processes is clearly understood in this study and it may provide a way for the limitation of glycation and oxidation reactions which is considered as a key factor in the aggregation of diabetes[59].

3.2 Profile of *Marsdenia brunoniana wight & arn.*

The detailed profile of *Marsdenia brunoniana Wight & arn* is given below and the photograph image of the plant used for the research work is cited the Figure.1

- **Botanical Name** **:***Marsdenia brunoniana wight & arn* .
- **Family** **:** Asclepiadaceae
- **Tamil Name** **:**Perunkurinjan
- **Distribution** **:** Throughout Peninsular India.
- **Parts Used** **:**Leaves

Fig.:1 Photograph of *Marsdenia brunoniana Wight & arn* .

3.2.1 Description *Marsdenia brunoniana*

Marsdenia brunoniana wight & arn is a twining shrub in nature and seldom straight growing plant. The leaves of plant are generally oval outline shaped which are having almost 100x 70 mm in size, some leaves are falt ring or normally quadrilateral appearance also, The leaves top is tapering to the point of plant and the bottom is shorten or somewhat sagittate at the base,

The glands of the plant starts at a pedestal and it is made up of thin layer of tissue, pliable membrane covered below and the nerves are made with five to six pairs; the plants of the stalk joins with leaf to a stem by means of soft layer of tissues about 2.5 cm. The plants has seasonable few flowered cluster in a middle stem and having a single terminal blossom that develops first, the other flowers in the cluster developing as terminal buds of lateral stems; and the calyx part of a

flower is usually formed as a spiral that enclosing the segment of the flower and forms an protective layer around a flower in buds and the shape of the buds is roundish and flattish in nature , which is projecting or hanging part of the plant, normally one or more such parts are divided by a fissure about 0.4 x 0.2 cm in size with unequal adjacent size and projecting to top of the plant . The buds are fleshy in nature and the glands are present within; the petals of a flower turn around hose having about 0.5 cm in length and roundish, flattish then projecting about 0.4 x 0.3 cm flat structure, and it is having a notched margin or tip, as some leaves or wings are at top portion of the plant and it is also a fleshy scenery.

The plant's base is supported with two buttresses and having the shape like a lance head with the size of tip about 0.5 cm; The plants are rational gathering of each another run with a long bounding stride of some flowers [60-62].

3.2.2 Uses

The plant leaf is used for various ailments as listed below.

- Diabetes mellitus
- Stomach ache
- Arthritis

CHAPTER – 4

SCOPE AND PLAN OF WORK

The extensively collected literature review clearly revealed that, the plant selected for the study has not been exposed to any pharmacognostical or pharmacological studies so far, even though the plant *Marsdenia brunoniana Wight & Arn was* used for various ailments. Henceforth, the Research work has been proposed to study on the therapeutic potentials of *Marsdenia brunoniana Wight & Arn*.

The schematic diagram for the research work is given in Figure.2 and the various stages of work and systematic study is detailed below.

- First stage the plant shall be collected and identified based on literature review then the same will be authenticated along with its various parts.
- After authentication, the leaves of the plant shall be extracted with various solvents. The solvents chosen for the study are,
    - Hexane,
    - Chloroform,
    - Ethyl acetate,
    - Ethanol,
    - Water.

The above solvents were chosen and used based on its polarity increasing order.

- The extract of above solvents undergoes for qualitative analysis for phytochemical, *in-vitro* and *in-vivo* and the detailed studies was carried out.
- The *in-vitro* analysis for the study of antioxidant property of different extracts by using the below assays.
    - ABTS assay
    - DPPH assay

- Hydroxyl radical Scavenging
- Superoxide radical Scavenging
- Hydrogen peroxide Scavenging
- Reductive ability measurement

➢ The following method is used for the study of *in-vitro* antidiabetic activity.

- α-amylase enzyme inhibitory assay
- α-glucosidase enzyme inhibitory assay

➢ The extracts undergone for the quantification of below bio-active compounds namely,

- Ascorbic acid
- Flavonoids
- Glycosides
- Phenol
- Saponins
- Tannin

➢ The selected plant extract was studied with the following chromatographic analysis.

- GC-MS Analysis
- HPLC Analysis
- HPTLC Analysis

➢ A toxicological study for the selected plant extract was carried out in order to study,

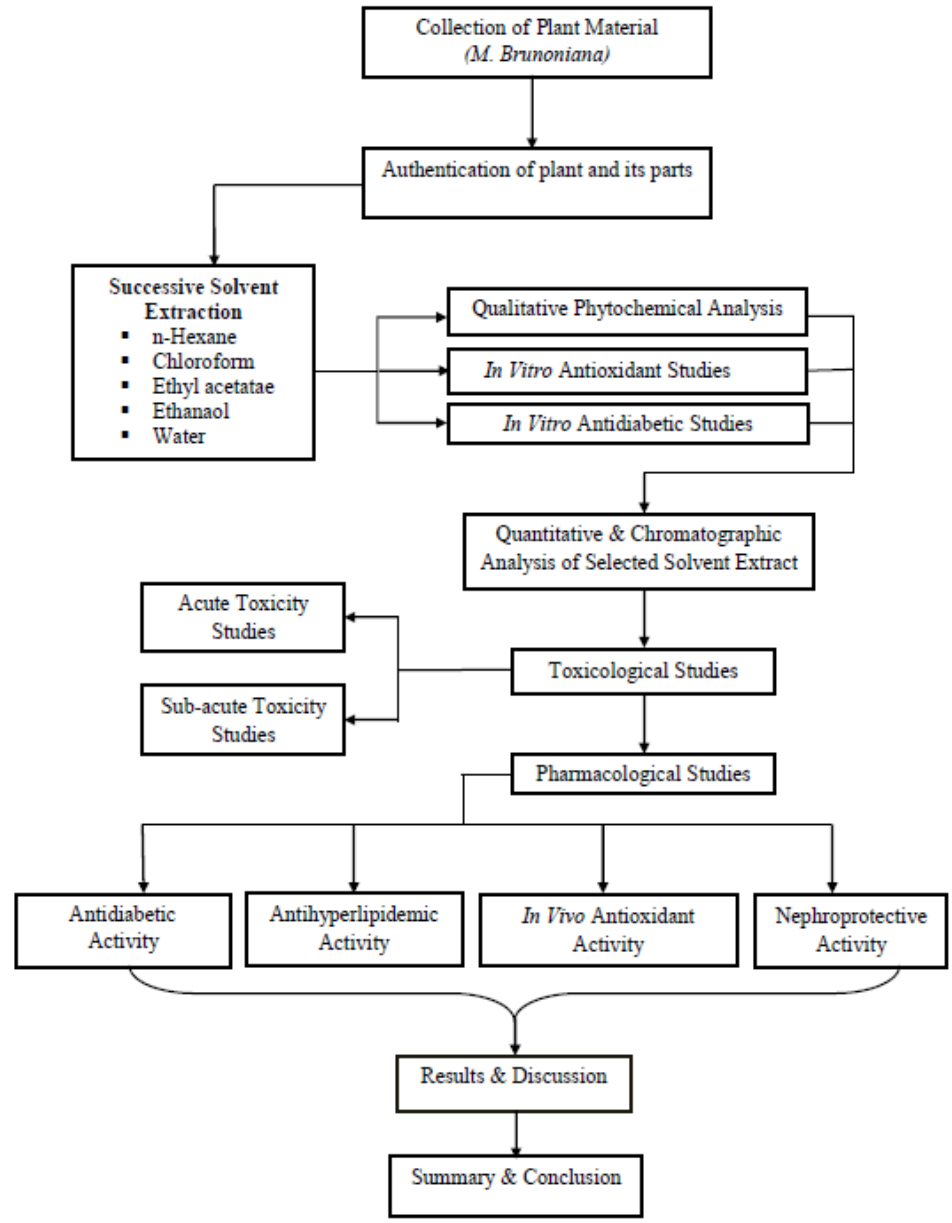
- Acute Toxicity study
- Sub-acute Toxicity study

➢ The below screening of *in-vivo* studies for the selected plant extracts was carried out with STZ and NAA induced Type-II diabetes rodents.

- Screening of anti-hyperlipidemic,
- Screening of anti-oxidant
- Screening of anti-diabetic Activities.

➢ The screening of Nephroprotective activity for the selected plant extract was conducted with STZ and NA persuaded Type-II diabetes rats.

Fig.2 Plan of Research Work



CHAPTER - 5

MATERIALS AND METHODS

5.1 Collection of Plant and its Authentication

The plant *Marsdenia brunoniana* has been collected from Sirumalai Hills, located near Dindigul, Tamil Nadu, India with the help of Valaiyans, a tribal community. The collected plant and its various parts were authenticated through the Botanical Survey of India (BSI)-Coimbatore, Ministry of Environment, Forest and Climate Change, Government of India where the receipt is conserved for further indications.

(Certificate bearing No. BSI / SRC / 5 / 23 / 2013-14/Tech/1002).

5.2 Extraction with Solvents

The shade dried coarse powders of leaves of *M. brunoniana* (1.5 kg) was extracted using soxhlet extractor with below listed solvents.

- Hexane
- Chloroform
- Ethyl acetate
- Ethanol
- Water

The above solvents were chosen and used based on its polarity increasing order in nature. The extracted extract is filtered to remove solid particles and by using rota-vapor equipment and the extracts concentrated to dryness at low temperature & pressure. The temperature used here is 40-50°C. The obtained residues are conditioned in vacuum entrusted desiccators. The percentage (%) of total yield in different solvent extracts is being calculated as per the equation given below:

$$\% \text{ yield} = [\text{Weight of crude extract} / \text{Weight of raw material}] \times 100$$

The extractive yield, colour, odour and nature of the various solvent extracts of *M. brunoniana* were tabulated.

5.3 Qualitative Phytochemical Analysis

The various active constituents of *M. brunoniana* are identified by subjecting to the extracts to chemical tests [63-65].

5.3.1 Estimation of Carbohydrates compound and Glycosides compound

The collected extract is dissolved in four milliliter of water (Only distilled water shall be used) and after dissolution, the aqueous is filtered off. The collected filtrate sample is again subjected to the below mentioned tests in order to the qualitative determination of carbohydrates. The determination of glycosides also found out in the same method.

5.3.1.1 Carbohydrate identification by Molisch's method

The presence of compound-carbohydrate is identified by putting two to three drops of one percentage alcoholic-alpha-naphthol along with conc. H_2SO_4. The sulphuric acid shall be added in the side of chemical test tube carefully. The notification of brown colour band at the intersection of those chemicals prove the the existence of carbohydrate compound.

5.3.1.2 Glycosides identification by Fehling's method

The collected filtrate is first treated with one milliliter of both A and B solutions of Fehling's solution and the mixed Fehling's solution is heated indirectly by using electric heated water bath. The formation of precipitates in red colour proves the presence of carbohydrate in the filtration.

In the same way, another part of extract or filtered off is treated with diluted HCl solution for 1 to 3 Hrs for Hydrolysation process using water bath equipment. The prepared hydrolytes is again subjected to the following identification method in order to identify glycosides compound.

i) Identification of glycosides by Legal's method

The glycoside compound is identified by mixing the hydrolysed solution with one milliliter of pyridine solution along with two to three-drops in nitroprusside-Na compound solution then it is made in to pH-9 to 10 by adding the diluted solution of NaOH. The change in colour into Redish pink shows that the glycoside compound is present in the filtrate solution.

ii) Identification of glycosides by Borntrager's method

The glycosides compound is identified in this method by mixing the hydrolysed solution with $CHCl_3$ solution. After mixing, the layer of $CHCl_3$ is formed then this formed layer is separated out and finally separated layer is mixed with few drops of diluted liquid ammonia solution. The appearance of pinkish colour layer proves the presence of glycoside compound.

5.3.2 Identification of Oil or Fat

i) Identification of Oil or Fat by simple filtration method

The collected extract is passed in to the filter paper in forty two pore size model. If any pre-set oil or fat is present, the filter paper becomes stained due to the presence of oil or fat and it will not allowed into the filter paper immediately. Henceforth, the oil or fat is identified by simple filtration method.

ii) Identification of Oil or Fat by Saponification method

The extract is to be treated with 2 to 4 drops of 0.5 normal solution of alcoholic KOH solution. After mixing the homogeneous solution, heated in water bath heater indirectly for about one to two hours. After, adding 2 to 3 drops of phenolphthalein indicator formation of soap shows the presence of oil or fat.

5.3.3 Identification of Amino acids

The collected sample of extracts is dissolved in few milliliter of distilled water then the following identification test is carried out to identify the protein or amino acids present in the extract.

i) Identification of Amino acids by Millon's method

The above collected extract is mixed with Millon's solution. The formation of Reddish shade indicates the availability of amino acids in the extract.

ii) Identification of Amino acids by Biuret method

The above collected extract is mixed with equal quantity of 5 percent NaOH solution then after thorough mixing add one percent of $CuSO_4$ solution and mixed well. The formation of violet shade indicates the availability of amino acids.

iii) Identification of Amino acids by Ninhydrin method

The above collected extract is mixed with few milliliter of reagent of Ninhydrin solution. The formation of purple shade indicates the availability of amino acids in the prepared extract solution.

5.3.4 Identification of Saponins compound

The above collected extract is diluted with twenty liter of water in measuring cylinder then it is kept in agitator for fifteen minutes. The water used shall be distilled water to identify the formed foams upon agitating for fifteen minutes. Usually, one centimeter of foam layer indicates the availability of Saponins compound in the prepared extract.

5.3.5 Identification of Tannins and compound and Phenolic Compound

The above collected extract is taken in small quantity and dissolved in distilled water and later it is identified while mixing with the below said solutions separately then Tannins and Phenolic Compound is identified based on the colour of the solution / formed precipitates are noticed

- 5.0% solution of $FeCl_3$ turns in to Violet shade.
- 1.0% solution of gelatin containing 10% of NaCl solution forms precipitates in white colour
- 10% of $Pb(C_2H_3O_2)_2$ solution forms precipitates in white colour
-

5.3.6 Identification of Phytosterols compound

The above collected extract is diluted with five milliliter of $CHCl_3$ solution. After mixing thoroughly, the following analysis test is carried out to identify the presence of Phytosterols compound.

i) Identification of Phytosterols compound by Salkowski method

From the above said $CHCl_3$ solution, one milliliter solution is mixed with 2 to 3 drops of prepared Conc.H_2SO_4 solution and the mixed soution's colour turns in to brownish colour indicates the availability of Phytosterols compound

ii) Identification of Phytosterols compound by Libermann Burchard method

From the above said $CHCl_3$ solution, one milliliter solution is mixed with 2 to 3 drops of prepared Conc.H_2SO_4 solution and again two to three drops of dil.CH_3COOH and $C_4H_6O_3$ solution is added. The prepared solution turns into greenish blue indicates the availability of Phytosterols compound.

5.3.7 Identification of Alkaloids compound

The above collected extract sample is mixed with two to three drops of dil. HCl solution then filtered off. The obtained filtrate is used to identify the alkaloids compound by treating with below mentioned reagents and the resultant precipitate is used to identify easily for the alkaloids compound.

- Treating with reagent of Mayer's solution turns in to creamy colour precipitate
- Treating with reagent of Dragendroff's solution turns in to precipitates of brownish orange
- Treating with reagent of Hager's solution turns in to precipitates of yellow in colour
- Treating with reagent of Wagner's solution turns in to brownish red precipitates

5.3.8 Identification of Gums or Mucilages

The above collected extract sample is added with twenty five milliliter of alcohol solution and during mixing, the constant stirring is needed then filtered off. The formed precipitates are dried by air drying technique and assessed for the swelling behavior. If there is no swelling is identified, the result shows that there is no any gum or mucilages present in the prepared extract solution.

5.3.9 Identification of Flavonoids compound

i) Using aqueous NaOH solution

Few milliliter of prepared extract is mixed in dissolved NaOH solution. Then the change in colour is noticed if, it turns into yellowish colour, the result shows that flavonoids compound is available in the prepared extract.

ii) Using conc.H_2S0_4 solution

Few milliliter of prepared extract is mixed in dissolved in prepared conc.H_2SO_4 solution. Then the change in colour is noticed if, it turns into yellowish orange colour, the result shows that flavonoids compound is available in the prepared extract.

iii) Using Shinoda's method

Few milliliter of prepared extract is mixed with alcohol then small quantity of magnesium is added and finally conc.H_2SO_4 solution is dropped gradually and heated in water bath equipment. Then, the change in colour is noticed if, it turns into yellowish orange colour, the result shows that flavonoids compound is available in the prepared extract.

5.4 Analysis of Activity for *in -Vitro* Antioxidant Property

The *in- vitro* assessment is based on the concept of inhibition technique. The prepared sample is added in to the free radical system, the depth of inhibition of free radical process is noted and the found inhibition level is depends on antioxidant property of the tested sample.

The assessment method is differing based on the following conditions:

- Based on generated free radical,
- Capacity of reproducibility in the generation stage,
- Type of endpoint used for assessment

The obtained data through *in-vitro* assessment is tricky to use in system for biological activity at the same time it does not produce same activity. Due to presence of constituents of phytochemical in the different extracts solution of *M. brunoniana*, the possibility of antioxidant property of the different extracts are assessed using various methods available in research technique. The various concentrations of the solution of extract and standard (1000, 500, 250, 125, 62.5, 31.25 and 15.6256 microgram per milliliter) utilized in this study is being measured and analysed.

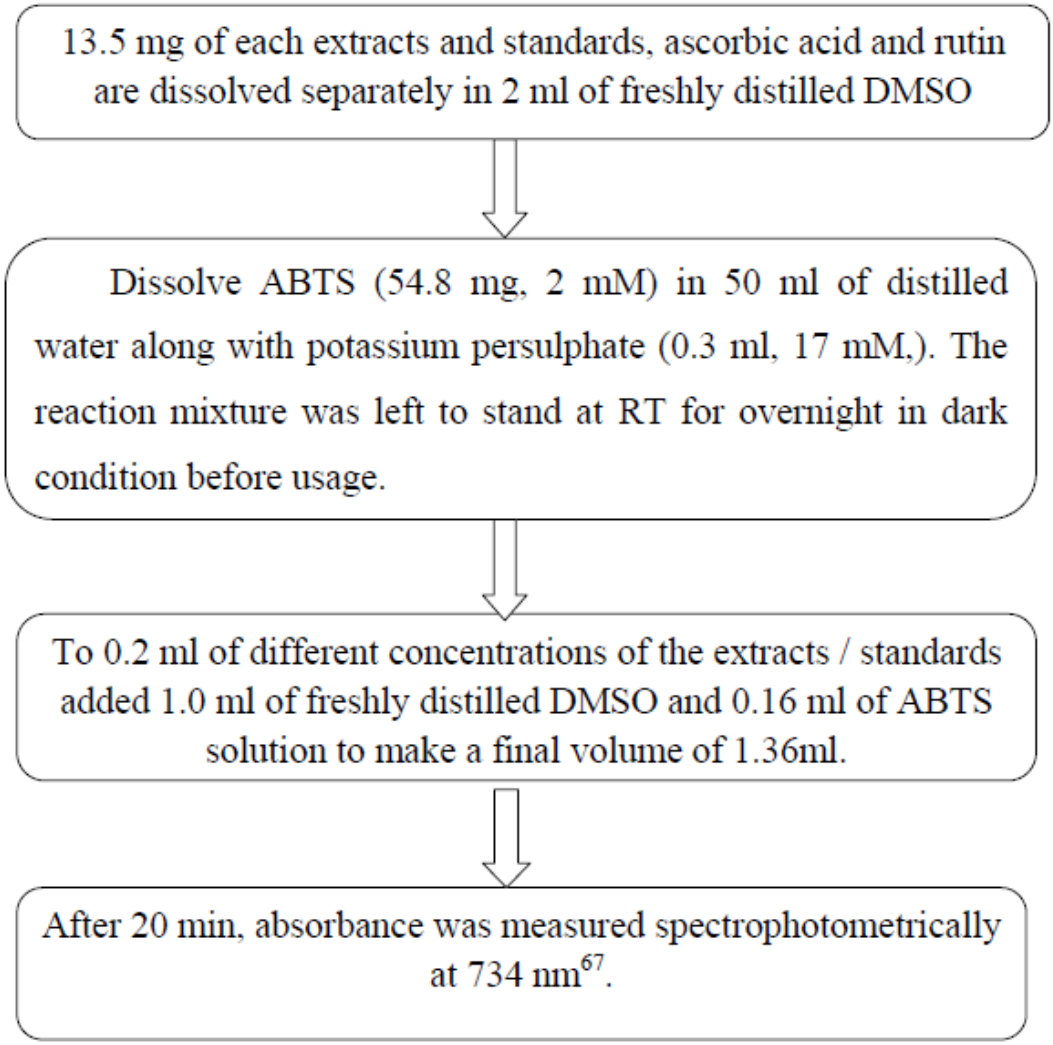
The observed absorbance through spectrophotometric technique is interpreted with the corresponding standard and blank solution.

The total percentage (%) of inhibition is calculated based on the following equation.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100$$

5.4.1 Scavenging of 2-2'-azino bis (3-ethylbenzothiazoline sulphonic acid) ABTS

This 2-2'-azino-bis (3-ethylbenzothiazoline sulphonic acid) assay is a novel method and it involves sudden radicals and used for assessment of complexed anti-oxidant solutions like extracts of plant. Which can be used in almost many ranges of pH, henceforth its application in research of pharmacy discipline in order to study the activity of antioxidant property is dominant area[66].



5.4.2 Evaluation of 2-2-diphenyl picrylhydrazyl (DPPH) compound

The 2-2-diphenyl picrylhydrazyl (DPPH) compound reacts with H donors and it undergoes reduction stage resulting which it produces respected hydrazine molecule. The changes to hydrazine causes for colour changes from purple to yellowish colour since originally radical of DPPH is purple shade and upon reduction it turns in to yellow colour. This assay also called as discoloration evaluation. It is estimated through adding of anti-oxidant compound into 2, 2-diphenyl-1-picrylhydrazyl solution in either C_2H_5OH or CH_3OH and the resultant reduction in light absorbent are measured in the wavelength of 490 nano meter.

The sequence of process is given below,

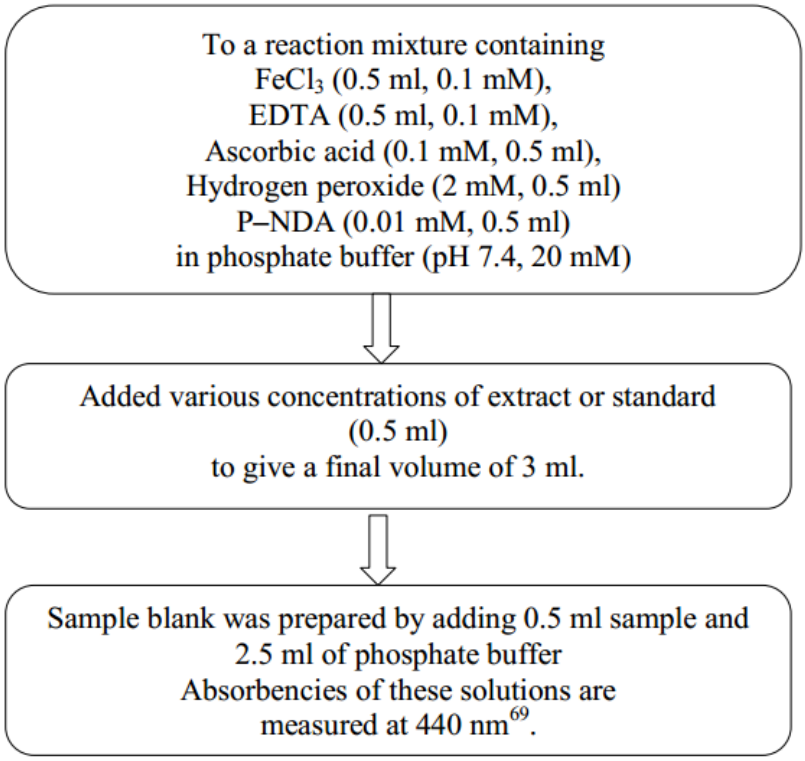
To 200 µl of DPPH solution, 10 µl of each of the extract or standard solution was added separately in wells of the microtitre plate.

The plates were incubated at 37°C for 30 min

Absorbance of each solution was measured at 490 nm using ELISA reader[68]

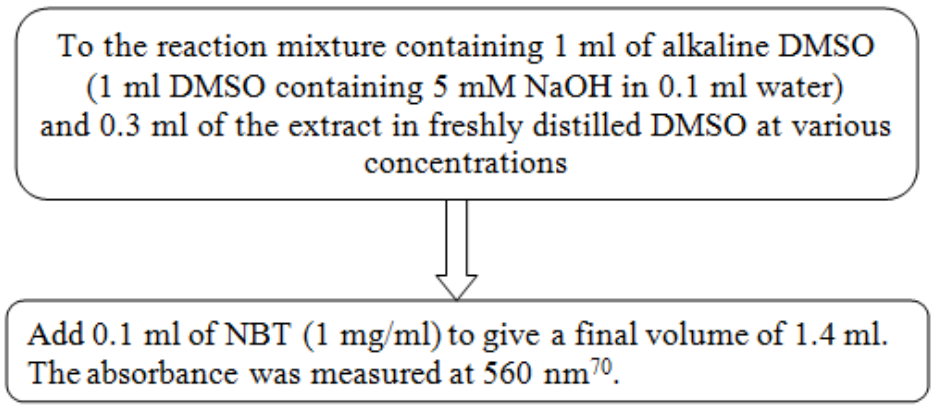
5.4.3 Hydroxyl radical scavenging of by p-NDA technique

The inhibition of p-Nitroso Diamine bleaching[69] technique is used to measure the hydroxyl radical. The fenton treatment is to evolve the generation of hydroxyl radicals through reaction between H_20_2 and Fe-EDTA compounds in the presence of acid like ascorbic acid and resulting which, it can bleach the compound generated through the reaction called as fenton reaction. In this reaction, ferrous iron-Ethylene di-amine tetra adipic acid complex reacts with the hydrogen peroxide in presence of ascorbic acid in order to generate hydroxyl compound, the formed hydroxyl compound shall decolourise the p-Nitroso Diamine purposely. In this reaction, the OH radical scavenger determines the scavenging functions through inhibition of bleaching and nonetheless, the percentage of scavenging is determined by absorbing the wavelength at 440 nano meter. The sequence of procedure is given below,



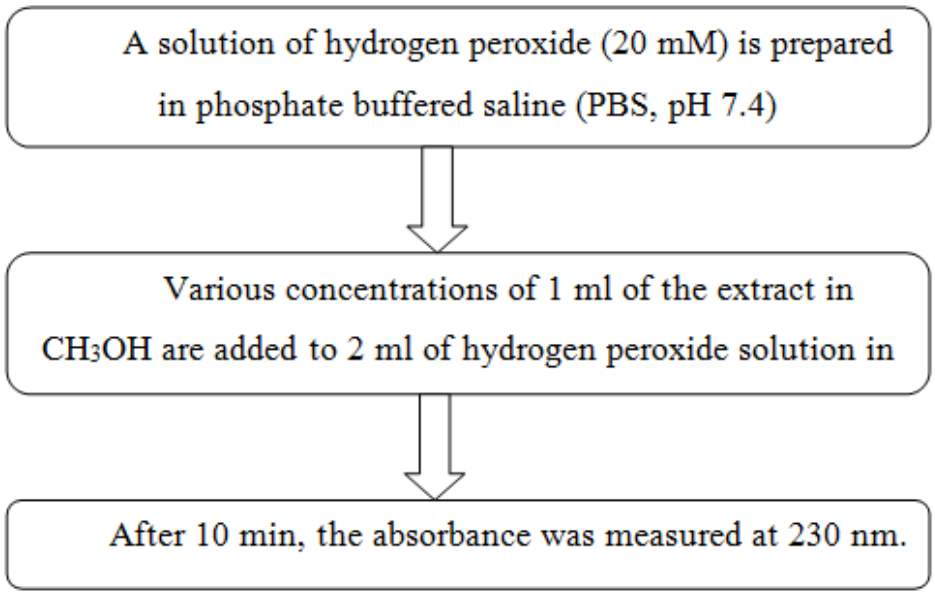
5.4.4 Superoxide radical Scavenging through alkaline dimethyl sulphoxide technic (DMSO)

In this technique, superoxide radical is produced by the adding up of NaOH and dimethyl sulphoxide. The produced superoxide will not reduced in the aqueous solution and at RT reduction of nitro-blue tetra-zolium is takes place resulting which formazan is produced and the absorbance is measured at 550 to 560 nanometer[69].



5.4.5. H_2O_2 Scavenging

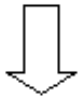
H_2O_2 is produced *in-vivo* through several enzymes which are oxidase based. and it is found to be that H_2O_2 generation of hydroxyl ions is takes place either in alkaline medium or acidic medium and the formed hydroxyl ions causes for damage in biological systems. In this process, the scavenging is done with H_2O_2 and the loss of H_2O_2 is evaluated spectrophotometrically in the range of 230 nanometre[70].The sequence of process is given below,



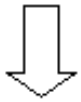
5.4.6. Evaluation of activity for Reductive capability

The evaluation of activity for Reductive capability is carried out by the transforming of metal ions (Ferrous) from ferrous to ferric when the extract is there in the mixture of solution[71].The procedure is given in sequences as below,

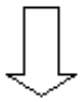
1 ml of extract, 2.5 ml of phosphate buffer, 2.5 ml of $K_3Fe(CN)_6$ are incubated at 50 °C for 20 min



Add 2.5 ml of TCA to the mixture and centrifuged for 10 min at 3000 rpm.



From the upper part, 2.5 ml is diluted with 2.5 ml of distilled water & shaken with 0.5 ml fresh $FeCl_3$.



The absorbance was measured at 700 nm after 20 min[72].

5.5 Evaluation of activity for antidiabetic by *in-vitro*

5.5.1 Inhibitory activity for alpha-glucosidase

The evaluation of inhibitory activity for alpha-glucosidase in *in-vitro* antidiabetic of the extract is carried out by the following procedure,

Alpha-glucosidase is pre-mixed with the prepared plant extracts at various concentration (1000 –15.625 µg/ml)

⇩

Add 3 mM p-nitrophenyl α-D -glucopyranoside in potassium phosphate to start the reaction.

⇩

The reaction was incubated at 37°C for 30 min and stopped by addition of 2 ml of 0.1 M sodium carbonate.

⇩

Activity is determined by measuring release of p-nitrophenyl α-D-glucopyranoside at 420 nm[73].

The percentage-inhibition rate is evaluated using the following equation:

$$\% \text{ inhibition} = \frac{[(A420_{Control} - A420_{Test})}{A420_{Control}]} \times 100$$

The value of IC50 is measured from the curve obtained for dose responses by the inhibition percentage against the concentration of extract then it shall to compare with the value of IC_{50} in response to acarbose. It is also measured under the same condition.

5.5.2 Evaluation of activity for inhibitory of alpha-amylase

The inhibitory action of alpha-amylase is performed as per the procedure given below.

Add Alpha-amylase with plant extracts by various level of concentrations (1000 – 15.625 µg/ml)

⇩

Add 0.5% starch solution in phosphate buffer to start the reaction and the reaction is carried for 20 minutes and terminated by adding 2 ml of DNS reagent (1% 3,5- dinitrosalysalic acid and 12% sodium potassium tartrate in 0.4 M NaOH)

⇩

The reaction mixture is heated for 15 min at 100°C and α amylase activity is determined by measuring the absorbance at 540 nm[74].

The rate of inhibition is measured in percentage while comparing with controls using the below formula:

$$\% \text{ inhibition} = \frac{(A540_{Control} - A540_{Test})}{(A540_{Control})} \times 100$$

The value of IC_{50} is calculated from the dosage response curve in percentage (%) inhibition against the concentration of extract and then compared with IC_{50} of acarbose as measured in the same procedure.

5.5.3 Extract selection for supplementary study

Based on the extractive yield obtained, the preliminary phytochemical analysis and *in-vitro* antioxidant and enzyme inhibitory enzyme assay data of various extracts of *M. brunoniana*, it has been concluded that among all the solvent extracts, ethanol extract of *M. brunoniana* (EEMB) possess noticeably higher activity. Henceforth, the ethanol extract is chosen for further supplementary study.

5.5.4 Assessment of DNA protection

The assessment of DNA protection is carried out by the following procedure.

The supercoiled pUC18 (2686 bp) plasmid DNA is used. Add plasmid DNA (100 ng) in 1 µl TE buffer (10 mM Tris–HCl and 1 mM EDTA), pH 7.2

Incubated with Fenton's reagent (30 mM H_2O_2, 100 µM ascorbic acid and 160 µM FeCl3) in the absence (control) or presence of plant extract then the total volume was makeup to 20 µl by TE buffer.

The mixture is incubated for 30 min at 37°C followed by addition of loading dye.

Electrophoresis is carried out in TAE buffer (40 mM Tris base, 16 mM acetic acid 1 mM EDTA, pH 8.0) and DNA is visualized in UV light under GelDoc (Biorad). The mannitol is used as the standard reference compound[75].

5.5.5 Estimation assay for Cell viability

Actually, preadipocytes (3T3-L1) cell lines mimic as *in-vivo* cells which is having direct impact on sugar homeostasis. These preadipocytes (3T3-L1) cell lines are received from National Centre of Cell sciences - Pune, India. The received cell lines are subjected to regular culturation process and the temperature condition used for culture is 37˚C in a conditioned atmosphere of 5% carbon-di-oxide along with 95% of oxygen in atmosphere. The culture is being supplemented with ten percentages of FBS along with 8 mM L of glutamine and antimycotic-2 % in a DMEM medium.

The typical colorimetric assay is measured on the basis of reducing nature of MTT through dehydrogenase of mitochondrial and resulting which the purple colour formazan product is generated and the same is used to evaluate the assess of cytotoxic activity for the extract of ethanol solvent produced for the plant *M.*

brunoniana. Briefly, monolayer cell culture of 3T3-L1 is dissociated by trypsin and the count of cell is adjusted to 104 x 1 cells/milliliter in the medium of DMEM which consisting of ten percentage of FCS. Step wise culture assay is described further as below,

To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added.

⇩

After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations were added on to the partial monolayer in microtitre plates.

⇩

The plates were then incubated at 37°C for 3 days in 5 % CO_2 atmosphere, and microscopic examination was carried out and observations were noted for every 24 hrs interval.

⇩

After 72 hrs, the extract solutions in the wells are discarded and 50 µl of MTT in PBS is added to each well. The plates are gently shaken and incubated for 3 hrs at 37°C in 5 % CO_2 atmosphere. Later, the supernatant is removed and 100 µl of propanol also added and the plates agitated in the shaker to solubilize the formed formazan. The absorbance is measured using a micro plate reader at a wavelength of 540 nm[76].

The percentage inhibition of growth is evaluated by using the below formula and the drug concentration or test extract required in order to inhibit the growth of cell by fifty percentage is produced from the response of dosage curves against each cell line.

$$\% \text{ Growth Inhibition} = 100 - \frac{[\text{Mean OD of test group}}{\text{Mean OD of control group}]} \times 100$$

5.5.6 Evaluation of assay for Glucose level uptake

The various stages of the evaluation of assay for the Glucose uptake is explained below,

Cells are cultured and plated at a density of 12,000 cells / well in 24-well plate and incubated for 24 hours in the DMEM growth media containing 5mM glucose.

⇩

On first day, the growth medium is replaced by supplemented medium and which is consisted of DMEM; supplemented with 10 % of FCS, insulin (10 µg/ml), DEX (10-8 M) and IBMX (0.1mM).

⇩

After 48 Hrs later, the cells are re-fed for 48 hours with the same supplemented medium and after another 24 hours (day 4) this medium is removed and replaced with growth medium including the treatment protocol of Yang *et al*[77] as followed in Table 1.

⇩

After a further 48 hour of incubation (day 6), the cells are assayed in their appropriate experiments. Ten microliter of the media is removed and placed in the 96 well plates to which 200 µl of GOD/POD reagent is added and incubated for 15 min at 37°C. The change in the color is recorded at 495 nm.

In each well, the content of glucose level is calculated using the below mentioned equation and it is expressed by in mg/dl unit.

$$\text{Conc. of unknown sample} = \frac{(\text{Concentration of sample})}{\text{Abs. of standard - Abs of reagent blank}} \times (\text{Ab. of unknown sample - Abs of reagent blank}).$$

Lastly, the uptake of glucose over control sample is calculated by estimating the difference among the starting and finally attained glucose level content under the medium of incubation.

Table1. Incubation medium for glucose uptake via 3T3-L1

S.No.	Incubation medium
1	1000 µl DMEM containing 5mM glucose
2	900 µl DMEM+100 µl Insulin (1IU/ml)
3	900 µl DMEM+100 µl Metformin(1 mg/1ml)
4	900 µl DMEM+100 µl Plant extract (1 mg/ml)
5	800 µl DMEM+100 µl Insulin (1 IU/ml)+100 µl Plant extract (1 mg/ml)
6	800 µl DMEM+100 µl Insulin (1 IU/ml)+100 µl Metformin (1 mg/1ml)
7	700µl DMEM+100µl Insulin (1IU/ml)+100µl Metformin (1mg/1ml)+100 µl Plant extract(1mg/ml)

5.6 Quantitation of Bio-Active Compounds

5.6.1 Assay of Glycosides

In order to estimate the assay of Glycoside the following steps are carried out

2.5 g of the plant extracts are weighed separately and 15 ml of hot distilled water is added. The flask is kept on a water bath till extract is dissolved.

Add 25 ml of 80 %v/v alcohol is added, shaken well and 50 ml of 95 %v/v alcohol is added. The resultant solution is allowed to settle down and filtered on a filter paper and washed with 80 %v/v alcohol until washings becomes colourless.

Filtrate and washings is transferred to dish and the alcohol is evaporated to get a syrupy mass. Then it is transferred into a 50 ml cylinder with stopper and volume is made up with distilled water up to the level of 30 ml.

3 ml of 10 %v/v sulphuric acid is added slowly, with constant shaking and allowed to stand overnight at room temperature. Supernatant liquid is decanted through a filter paper, washed the pot 2 or 3 times with cold water and passing the washings each time through filter paper.

The residue in the cylinder and on the filter paper is dissolved with a little dilute alcohol (45 %), 2 or 3 drops of ammonia (10 %) is added to neutralize the acid. The contents were evaporated to dryness in a tarred beaker and dried to a constant weight at 105 °C. Increase in weight of beaker represents total glycosides[78].

5.6.2 Evaluation of Saponins

The evaluation of Saponins compound is assessed by the following steps.

5 gm of the plant extract is dissolved separately in 50 ml of 90 % v/v alcohol by refluxing on a water bath for half an hour.

⇩

Filtered and the residue is washed thoroughly to take maximum quantity of soluble matter (refluxation is repeated until dissolution is not completed).

⇩

The alcoholic extract is concentrated to a thick paste.

⇩

50 ml of petroleum ether (40 -60 °C) is added & refluxed for half an hour. The petroleum ether soluble portion is discarded by filtering

⇩

The thimble is transferred to a soxhlet extractor and refluxed with chloroform for half an hour and soluble portion is discarded. The same treatment was done with CCl_4 and ethyl acetate and respective soluble portions are discarded.

⇩

The residue is dissolved in 10 ml methanol and poured drop-wise into 50 ml of acetone with constant stirring.

⇩

The precipitate is collected and dried at 105°C to obtain constant weight[78].

5.6.3 Evaluation of Phenol Content

The evaluation of total phenol content is carried out in crude drug powder, prepared extract and added beverages using Folin Ciocalteu technique. This technique is based on the addition of oxygen compound to the phenolic groups. After this oxidation process, the obtained blue-green compound is measured at 730 to 750 nanometer. The obtained result of a tested extract is correlated with the activity of antioxidant property.

The step wise procedure is given below,

About 0.1 ml of ethanol extract (0.1 mg/ml) is mixed with 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml of sodium carbonate. The mixture is shaken thoroughly and made up to 10 ml with distilled water.

⇩

The mixture is allowed to stand for 2 h. The absorbance is measured at 750 nm using PerkinElmer Lambda 25 UV-VIS Spectrophotometer. The standard curve is prepared and linearity is obtained in the range of 2-10 µg/ml.

⇩

Using the standard curve the total phenol content is obtained. The total phenol content is expressed as gallic acid equivalent in mg/g or % w/w of extract[79].

5.6.4 Evaluation of tannin content

The Tannin content is evaluated by the Schenderl (1970)[80] technique. The presence of Tannins compound is found in almost all the plants. The evaluation of tannin is depends on the stoichiometric potential of oxidation of hydroxyl group present in phenol. The presence of Tannin compound reduces phosphomolybdic acid in the pH range of above 8 to a coloured compound. Then the absorbance is measured in the range of 680 to 700 nanometer. The stepwise procedure followed is given below.

0.2 - 1.0 ml of standard tannic acid solution is pipetted out into a series of test tubes. To another test tube 0.5 ml of extract solution is taken. The volumes of all the tubes were made up to 3 ml with distilled water.

⇩

3 ml of distilled water is taken as blank. To all the tubes 5 ml of 35% Na2 CO_3 is added followed by the addition of 2.5 ml of Folin-Denis reagent and incubated at RT for 30 minutes.

⇩

The absorbance was read against reagent blank at 700 nm. From the standard graph obtained, the amount of tannin compound present is calculated.

5.6.5 Evaluation Flavonoids compound

The presence of flavonoids in the extract is evaluated by means of characterization of its absorption in the ultra-violet region through specific complexation $AlCl_3$ and CH_3COOK. The detailed procedure is given below,

500 µl of each extract is mixed with 1.5 ml of methanol, 100 µl of 10% aluminium chloride, 100 µl of 1M potassium acetate and 2.8 ml of distilled water.

After incubation at RT for 30 min, the absorbance is measured at 415 nm.

Using rutin, standard curve is prepared and from the standard curve the total flavonoids content of extracts are obtained. The total flavonoids content is expressed as rutin equivalent in mg/g or %w/w of the extract[81].

5.6.6 Evaluation of Ascorbic Acid

The principle behind for the evaluation of Ascorbic acid compound is, it will reduce the dye called dichlorophenol dye to the colorless state as leuco-base. During the reaction, the ascorbic acid is oxidized to dehydroascorbic acid compound. Finally, the dye colour is changed from blue colour to pink in the pH range of 3 to 4 and in which oxalic acid is used for the titration purpose.

The titration process is explained below,

5 ml of the working standard solution is pipette out into a 100 ml conical flask. Add 10 ml of 4% oxalic acid and titrated against 2,6-diclorophenol indophenol (V_1 ml).

⇩

End point was the appearance of pink color which persists for few minutes. The amount of dye consumed is equivalent to the amount of ascorbic acid.

⇩

0.5 g of each extract is dissolved separately in 4 % oxalic acid, filtered and made up to 100 ml and centrifuged. 5 ml of the supernatant is pipetted out and treated with 10 ml of 4 % oxalic acid and titrated against the dye (V_2 ml)[82].

⇩

Amount of ascorbic acid present is calculated by the equation
(0.5 mg/V_1 ml) X (V_2/5 ml) X (100 ml/wt. of the sample)

5.7 Investigation of EEMB using GC-MS

The investigation of EEMB compound is performed by using GS-MS instrument. The details of instrument is given below,

Model	: Clarus 500 PE system
Sampling	: Auto sampler
Interface	: chromatograph interfaced to MS instrument
Column size	: 0.25 x 30 mm, 100% dimethyl poly- siloxane,
Electron impact	: 70 eV;
Carrier gas	: Helium 1ml/min flow rate with volume of 0.5 EI
Temperature	: 250°C (injector); 280°C (ion-source)

In this technique, the prepared extract is dissolved in C_2H_5OH, exposed with N gas for about 10 minutes. After that, inject 2 µl of extract to be analysed into the column which was preheated.

The detection of compound is performed through association with relative retention time(s) and MS data attained were compared with data base available for the study.

5.8 Analysis of EEMB through HPLC Technique

The analysis of EEMB is carried out by HPLC Technique and the details of analyser used is given below,

Model : Shimadzu Class; VP-V6.14-SP2

Sampling : Auto sampler.

Column used : C^{18} reverse phase model /size - (0.46 cm X 25 cm)

Column temperature : 40°C.

The steps involved in analysis of EEMB is mentioned below,

Standard stock solutions of the five phenolic compounds are prepared in CH_3OH as 0.4 mg/ml. and all the standard solutions are filtered through 0.45 µm membrane filter (Millipore) and injected by autosampler.

1 g of extract is dissolved in 50 ml of CH_3OH separately under 80 KHz, 45°C in ultrasonic extraction device for 30 min. repeated twice.The extract iscollected, filtered and the filtrate is dried at 50°C under reduced pressure.

The dried extract is dissolved in 100 ml of mobile phase. After filtering through a normal filter paper and 0.45 µm membrane filter (Millipore), the extract is subjected to HPLC analysis

The plant extract is eluted in HPLC using a binary gradient at a flow rate of 1.0 ml/min with water: acetic acid (25:1 v/v) as solvent A and methanol as solvent B

5.9 Analysis of EEMB Using HPTLC Technique

The analysis of EEMB is carried out with HPTLC technique with the following instrument particulars are used.

Chromatography on : 100X30 mm HPTLC Silica gel (60-F_{254}) Plate coated.

Prior Treatment : The plates are washed in CH_3OH & made it in to activation at 50 to 60^0 C for 3 to 5 minutes. The samples are applied on plate and the band is used as 6 mm in CAM-AG Linomat applicator. The slit dimension is kept at 0.45 x 0.5 mm and the scanning takes place as 20 mm/second. Then trial and error method is used for fixing the mobile phase.

The collected extract is weighed about 100 mg accurately by using an electronic weighing balance then dissolved in one milliliter of CH3OH and centrifuged for 3000 rpm rotation speed and continued for 3 minutes. Then, this solution is measured about 10 µl and loaded as 0.5 cm band length in thin layer chromatographic plate.

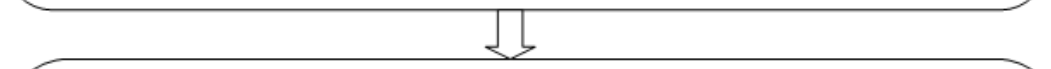
After completion of saturation with solvent vapor, the coated plate is kept in twin trough chamber for developing with concern to typical mobile phase. (CCL_4: $CH_3COOC_2H_5OH$: HCOOH of 2.5:4.5:3) then the TLC plate is developed up to 9 cm. And developed plate is dried through hot air in order to make evaporation of solvents form TLC plate. Finally, the TLC plate is set in the scanner and scanning is performed at UV 254 nanometer. Then, the peaks are recorded and images are captured in ultra violet region at 254 nanometer and 356 nanometer.

5.9.1 EEMB study for Flavonoid Profile using HPTLC Technique

The analysis of Flavonoid compound is carried out as per the procedure given below,

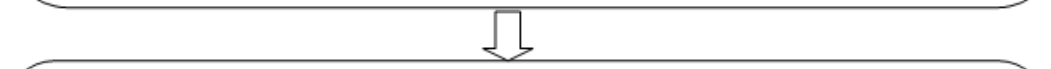
Preparation and application of samples

100 mg of the extract is weighed accurately in an electronic balance, dissolved in 1 ml methanol and centrifuged at 3000 rpm for 3 minutes. 2 µl of the prepared solution and 2 µl of standard (Quercetin) are loaded as 5mm band length in the 3 x 10 Silica gel $60F_{254}$ TLC plate.



Chromatographic development & photo documentation.

The samples loaded plate is kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (Chloroform-Ethyl acetate-Glacial acetic acid, 60:35:5) and the chromatogram is developed up to 90 mm. The developed plate is dried by hot air to evaporate solvents from the plate. The plate is kept in Photo-documentation chamber and captured the images at day light, UV 254 nm and UV366 nm.



Derivatization

The developed plate is sprayed with respective spray reagent (1% Ethanolic Aluminium chloride reagent) and dried at 100 °C in hot air oven. The plate is photo-documented in Day light and UV 366 nm mode using Photo-documentation (CAMAG REPROSTAR 3) chamber.

5.10 Pharmacological Studies

5.10.1 Animals

In order to proceed the pharmacological studies, good healthy swiss albino mice, wistar rats are chosen and procured from M/s. Venkateshwara Enterprises - Bangalore, India. These animals are maintained by standard environmental condition at the temperature of 25 ± 2 ºC for 12 hours dark and light cycle(s). Those animals are fed by using normal animal pellet food, water *ad libitum*. The

institutional level clearance certificate is received from Animal Ethical Committee vide reference 887/AC/05/CPCSEA dated 04/03/2013 at JKK Nattraja college of Pharmacy, Komarapalayam.

5.10.2 Toxicological studies

5.10.2.1 Acute Toxicity Studies

The evaluation of ED_{50} (that is, the dose needed to produce desirable reaction in fifty percentage of the tested animal group) will reveal the effectiveness of the drug with respect to standard reference drug. The computation of ED_{50} assessment is completed while the drug shows adequate reaction in with respect to dose.

LD_{50} is estimated by using OECD guideline[83]. The Acute toxicity study 423 method is explained below,

Healthy, young and non-pregnant female mice are randomly selected and kept in their cages for at least 5 days prior to the study.

⇩

The overnight fasted animals are weighed and administered the plant extract (in 0.3 % sodium CMC) as a single dose (2000 mg/kg) by oral route using oral intubation canula.

⇩

After administration of the extracts, the animals are observed for toxic symptoms and mortality continuously for first 4 hours.

⇩

Finally the number of survivors aree noted after 24 hours and the observation made daily for a period of 14 days.

5.10.2.2 Sub-acute Toxicity investigation

The investigation of sub-acute Toxicity-407 is carried out using OECD guideline[84] as per the procedure explained below.

i) Selection of animals

The rats used for analysis is belong to swiss albino rats of both male and female category. These rats are weighing between 135 to 155 grammes. The animals

are allowed for taking the standard pellets of food and normal drinking water. The rats are grouped in to from four to six rats per group. The groups are categorized as below,

- **Group : 1** Control group and totally, six rats (three male rats & three female rats)
- **Group : 2** Low dose group (Ethanolic extract of *M. brunoniana,* 200 mg/kilogram) , totally six rats (three male rats & three female rats)
- **Group : 3** Intermediate dose group (Ethanolic extract of *M. brunoniana,* 400 mg/ kilogram) , totally 6 rats (three male rats & three female rats)
- **Group : 4** High dose group (Ethanolic extract of. *brunoniana, 1000* mg/ kilogram) totally 6 rats (three male rats & three female rats). The extract is given orally by gavage regularly (one time per day) for twenty eight days.

ii) Collection of blood samples and organs

After completion of the investigational phase, the rats are sacrificed and blood is gathered. The rat's body is cut, opened and the gross pathological changes are observed. Immediately, the liver, heart, stomach, kidney, spleen and lung are excised and preserved using 10 % of formalin solution in order to study histopathological studies. By means of centrifugation, the Serum is removed from the blood through centrifugation at 2000 revolution per minute and it is continued for twenty minutes. The separated serum sample is transferred into dry, clean serum tubes; stoppered and stored in the refrigerator at 2-4°C till it is used for testing**.** The observations and examinations are performed during the study is listed below.

Physical observations: The animals are closely observed daily twice (morning & evening) for overt signs of toxicity, morbidity and mortality. In addition, each animal is removed from its cage and a physical examination of each animal is conducted twice a week for any changes in skin, fur, eyes, respiratory function, autonomic and central nervous system and general behavior etc

Body weight: The body weights of all the animals are measured once per week throughout the study period by using calibrated balance.

Food and water intake: The food and water intake of each animal of both control and test groups are measured once per week throughout the study by using digital Feeding and Drinking Analyser (Ugo basile, 41700, Italy).

Haematological parameters: The blood samples were analyzed for hematological parameters like total red blood cell (RBC), white blood cells (WBC), lymphocytes (LYM), hemoglobin (Hb), MID cells (less frequently occurring and rate cells correlating to monocytes, eosinophils, basophils etc.,) using a blood automatic analyzer (Celldyn, Abbot Inc. USA).

Biochemical parameters: In addition, blood glucose, total cholesterol, serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), Alkaline phosphatase (ALP), total bilirubin, total cholesterol, urea, BUN, total protein, albumin and creatinine were also estimated by using commercially available kits and autochemical analyzer (Landwind – Diatek LW C100, UK).

Weight of isolated organs: After blood collection the animals are sacrificed by cervical decapitation and the vital organs such as liver, kidney, heart, lungs and spleen were subjected to gross examination and later weighed.

Urinalysis: The urine analysis is performed to investigate any abnormalities in excretion pattern after the exposure with test drug for 28 days. The urine is collected from each animal at the end of the study early in the morning and it is analvzed for the detection of abnormal constitutions.

Histopathology: The portions of liver and kidney are fixed in buffered 10 % formalin and 5 µm thick paraffin sections are made and stained with haemotoxylin and eosin for histopathological examination.

5.10.3 Studying of *in- Vivo* Antidiabetic and Antihyperlipidemic Activity

5.10.3.1 Experimentation for Induction of Type-II Diabetes

The dissolution of streptozotocin (STZ) is performed in buffer solution with the help of citrate (0.1M, pH 4.5) and made Nicotinamide (NAA) dissolution in typical physiological saline solution and the temperature is maintained on ice bars

before proceeding for application. Nonetheless, non-insulin dependent DM is persuaded during the night for fasted rats by means of single time IP injection of STZ (45 mg/kilogram b.w), After 15 minutes, the IP injection of NAA is performed using the dose level of 110 milligram per kilogram b.w) [85]. Then, the level of glucose is evaluated after forty eight hours of STZ and NAA administration and rats those are with glucose levels in fasting is higher than 250 mg/dL and becomes diabetic-rats and the same are used for the research work. The treatment using EEMB is commenced from third day upon STZ induction[86]. The experimentation of the study is detailed below,

The rats are grouped into total five groups and each group has 6 rats (Hence, n=six), a total of thirty rats (twenty four diabetic surviving rats, six normal control rats) are used.

Group-1 - Normal control (0.3 %w/v CMC in water) (1 ml/100gm)

Group-2 – Diabetic control (0.3 %w/v CMC in water) (1 ml/100gm)

Group-3 – Disease + Glimepride (5 mg/kg)

Group-4 - Disease+ EEMB (200mg/kg/ day in 0.3 % CMC in water)

Group-5 - Disease+ EEMB (400mg/kg/ day in 0.3 % CMC in water)

The plant extract is dissolved in vehicle solution of 0.3 %w/v CMC in water (1 ml/100gm) and it was administered orally using an intragastric tube for a period of 28 days[87].The body weights of the rats in different groups are recorded. After the investigational stage, the animals are fasted for overnight, blood are collected through cardiac punching. The same blood is used for the assessment of total cholesterol, triglycerides, serum glucose, serum insulin levels and glycosylated haemoglobin. Finally, the rats are sacrificed through decapitation and the collection of liver takes place at the same time and dissected, and washed with ice-cold saline solution in order to expel the blood. In this method, the liver is separated since it is an important tissue for presentation of higher rate of free- radical production. The preparation of 10 percentage weight per volume level of liver homogenate is prepared in ice cold using 10 percentage of potassium chloride solution and it is

centrifuged for fifteen minutes at four degree celcius. The obtained tissues are used for the evaluation of following profiles.

- Catalase
- Glutathione peroxidase
- Glutathione S-transferase
- Lipid peroxidation
- Superoxide dismutase

5.10.3.2 Evaluation of Tissue Lipid Peroxidation

The evaluation of Tissue Lipid Peroxidation is carried as per the steps provided below,

The basal lipid peroxidation system consisted of 1.2 ml of 0.3 M. Tris HCl buffer, 0.2 ml of sodium pyrophosphate and 0.2 ml of diluted tissue homogenate. The inducing system contained 0.2 ml ferrous sulphate, 0.2 ml ascorbate (as an inducer), 0.2 ml sodium pyrophosphate and 0.2 ml of diluted tissue homogenate.

The volume is made up to 2.0 ml with water. The tubes are incubated at 37°C with constant shaking for 20 minutes. The reaction is stopped by the addition of 1.0 ml of 10% TCA. The tubes are shaken well with 1.5 ml TBA reagents and heated at 90°C for 20 min. The tubes are centrifuged and the colour developed in the supernatant is read at 532 nm.

Standards (1-5 nmoles) are taken in 2.0 ml volume and are processed as above along with blank containing 2.0 ml water. The basal and inducers added lipid peroxidation in the experimental setupis compared with respective controls. A level of lipid peroxidation is expressed as amount of MDA formed /mg protein[89].

5.10.3.3 Evaluation of Catalase

The evaluation of catalase is carried as per the steps provided below,

0.1ml of the tissue homogenate is taken to which 1.0 ml of phosphate buffer and 0.5 ml of hydrogen peroxide is added and the reaction is started. The reaction is assessed by the addition of 2 ml of dichromate acetic acid reagent.

Standard hydrogen peroxide in the range of 4 to 2 mM are taken and treated similarly. The tubes are heated in a boiling water bath for 10 minutes.

The green colour developed is read at 570 nm in a colorimeter. Catalase activity is expressed as m moles of H_2O_2 utilized / min / mg protein under incubation condition[90].

5.10.3.4 Evaluation of Superoxide Dismutase

The evaluation of Superoxide Dismutase is carried as per the steps provided below,

To 0.5 ml of the tissue homogenate, 0.25 ml of absolute ethanol and 0.15 ml of chloroform are added. After 15 minutes of shaking in a mechanical shaker, the suspension is centrifuged and the supernatant obtained constituted the enzyme extract.

The assay mixture for the enzyme contained 2 ml of 0.1M Tris HCl buffer 0.5ml of pyrogallol, 0.5 ml of aliquots of the enzyme extract and water to give a final volume of 4 ml. The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme is noted at 470 nm at on interval of 1 min for 3 min.

The enzyme activity is expressed in terms of units/mg protein and one unit corresponds to the amount of enzyme required to inhibit the auto oxidation reaction by 50 %[91].

5.10.3.5 Evaluation of Glutathione Peroxidase

The evaluation of Glutathione Peroxidase is carried as per the steps provided below,

0.2ml each of EDTA, sodium azide, glutathione (reduced), hydrogen peroxide, 0.4 ml of sodium phosphate buffer and 0.1ml of tissue homogenate are mixed and incubated at 37°C for 10 minutes. The reaction is arrested by the addition of 0.5 ml of TCA and the tubes are centrifuged at 2000 rpm.

To 0.5 ml of supernatants, 4 ml of disodium hydrogen phosphate and 0.5 ml of DTNB were added and the colour developed is read at 420nm, immediately using a colorimeter. Graded concentrations of the standards were also treated similarly.

Glutathione peroxide activity is expressed as mg. of glutathione utilized / min /mg protein under incubation conditions[92].

5.10.3.6 Evaluation of Glutathione -s-transferase

The evaluation of Glutathione-s-transferase is carried as per the steps provided below,

To 1ml of phosphate buffer, 0.1ml of tissue homogenate, 1.7 ml of water and 0.1ml of CDNB are added and incubated at 37°C for 15 minutes. After incubation 0.1ml of reduced glutathione is added.

The increase in optical density is measured against that of the blank at 340 nm using a colorimeter. The enzyme activity is expressed as m moles of CDNB conjugated / min/mg protein under incubation conditions[93].

5.10.4 Study on Effect of EEMB on Diabetic Induce Nephropathy in Male Wistar rats

The induction of Type-II Diabetic Mellitus is explained below,

Streptozotocin is freshly dissolved in (0.1M, pH 4.5) citrate buffer and nicotinamide is dissolved in normal physiological saline and maintained on ice prior to use.

⇩

Non-insulin-dependent diabetes mellitus is induced in overnight fasted rats by an intramuscular injection of 60mg/kg streptozotocin and thereafter 120 mg/kg nicotinamide is injected after 5min.

⇩

The elevated plasma glucose is determined after 3 days of streptozotocin and nicotinamide administration and those rats with fasting glucose levels greater than 250mg/dl were served as diabetic rats and used in the study[94].Nephropathy is noted in rats between 4–8 weeks after the administration of STZ[95].

The experiments of the study is detailed below,

The rats are grouped into four categories and each group contains six rats and hence, n = 6.The details of each group is given below,

Group-1: Normal control (0.3 %w/v CMC in water, p.o) (1ml/100gm)

Group-2: Diabetic control (streptozotocin+nicotinamide) (0.3 %w/v CMC in water, p.o) (1ml/100gm)

Group-3: Diabetic rats treated with EEMB (200mg/kg/day, p.o) in 0.3 %w/v CMC in water (1ml/100gm)

Group-4: Diabetic rats treated with EEMB (400mg/kg/day, p.o) in 0.3 %w/v CMC in water) (1ml/100gm)

The treatment with the extract of plant is commenced from the 4th day following induction of STZ and NAA and sustained for 60 days. The first and ending day's body weight of different categories of groups are noted. On sixteenth

days healing, twenty four hours urine is gathered by means of metabolic cage and the level of urine is recorded[96]. The same is used for the evaluation of following profiles,

- Microalbumin
- Urine creatinine
- Urine urea
- Urine uric acid

Later, the blood sample is collected from the rats of overnight fasted categories using EDTA tubes through cardiac puncture. The same is used for the evaluation of following profiles.

- Blood creatinine levels.
- Blood urea
- Blood uric acid
- Glucose
- Glycosylated hemoglobin

Finally, the rats are sacrificed through cervical dislocation and the kidneys are taken and dissected out immediately, weighed then those are used to investigate the histopathological examination. Sample of ten percentage weight per volume of kidney tissue homogenate is made in ice cold along with ten percentage of potassium chloride solution and it is undergone for centrifuge process for fifteen minutes at four degree Celsius. The supernatant which are gathered is used for the evaluation of the following profiles,

- Catalase
- Glutathione peroxidase
- Glutathione -s-transferase

- Lipid peroxidation
- Superoxide dismutase

5.10.5 Histopathology of Kidney

After completion of sixty days of treatment the animals are sacrificed by cervical dislocation and the obtained kidneys are dissected out and the same is fixed in ten percent of buffered formalin solution which is prepared using 100 ml of formaldehyde, 4 gram of sodium phosphate monobasic, 6.5 gram of sodium phosphate dibasic along with 0.9 liter of water and those samples are subjected to additional dispensation for histopathological estimation.

5.10.6 Statistical Analysis

The obtained values are expressed in mean ± SEM. The obtained data are analysed using one way analysis of variance technique later the effect of different does of selected extracts are compared with control using the statistical tool-Tukey's multiple comparison Test and by using statistical software called Graph Pad Prism in the version of 5.01, the "p" is calculated and if the value "$p<0.05$" is treated as significant otherwise non-significant.

CHAPTER – 6

RESULTS AND ANALYSIS

6.1 Extraction

Different types of extracts of *M. brunoniana* were prepared and the percentage yield was calculated. Among the prepared extracts, maximum yield was obtained from ethanol and water. Percentage yields, colour and nature of the prepared plant extracts are presented in **Table 2.**

Table 2. Color, nature and percentage yields of *Marsdenia brunoniana*

Extract	Color	Nature	Yield
Hexane	Dark greenish	Sticky semisolid	2.6%
Chloroform	Dark greenish Yellow	Solid	1.5%
Ethyl acetate	Greenish yellow	Solid	6.3%
Ethanol	Yellowish brown	Solid	8.6%
Water	Brown	Solid	10.4%

6.2 Qualitative Phytochemical Analysis

The screening of preliminary phytochemical of the extracts of plant - *M. brunoniana* demonstrate the existence of diverse phytochemical components. phyto-sterols, fixed oils and fats were found in hexane extract, whereas chloroform extract restrain phytosterols and gives a very low extractive yield when compared to other extracts of plant. The extract of ethyl acetate consists of glycosides, flavonoids and phenolic compounds. Glycosides, phytosterols, tannins, flavonoids, polyphenolics, proteins& amino acids and carbohydrates were found in ethanol extract. Aqueous extract contains many polar constituents such as phenolic compounds, glycosides, proteins & amino acids, flavonoids, tannins and carbohydrates. Alkaloids, mucilage gums and terpenoids are not present in all the prepared extracts. The results are

more clearly proved that the existence of each phytoconstituents depends upon the phytochemicals solubility in the particular solvents. Many of the constituents were extracted by ethanol. The results are displayed in **Table 3**.

Table 3. Preliminary Phytochemical Studies of *Marsdenia brunoniana*

Constituents	Extract				
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
Carbohydrates	A	A	A	P	P
Phytosterols	P	P	A	P	A
Alkaloids	A	A	A	A	A
Glycosides	A	A	P	P	P
Terpenoids	A	A	A	A	A
Proteins & amino acids	A	A	A	P	P
Saponins	A	A	A	A	A
Tannins	A	A	A	P	P
Phenolic compounds	A	A	P	P	P
Flavonoids	A	A	P	P	P
Fixed oils & Fats	P	A	A	A	A
Gums & Mucilages	A	A	A	A	A

(P) Presence (A) Absence

6.3 *In vitro* Antioxidant Activity

The activity of antioxidant property of various solvent extracts of *M. brunoniana* was investigated through various *in vitro* models. Since, free radicals are of diverse chemical entities, it is necessary to test the extracts against many free radicals to check their antioxidant activity. Hence, a large number of *in vitro* methods were employed for the screening. IC_{50} values acquired were compared with the standards ascorbic acid and rutin.

In the current work, all the prepared extracts apart from hexane extract, exhibiting radical scavenging activity against ABTS radical. The IC_{50} values of ethyl acetate and ethanol extracts were found to be 52.35 ± 2.2 µg/ml and 60.33 ±

5.88 µg/ml which showed strong activity against scavenging of ABTS radical. The order of activity was as follows: 1) Ethyl acetate 2) Ethanol 3) Water, 4) Chloroform 5) Hexane. In DPPH radical scavenging assay, ethyl acetate and ethanol extracts are found to be active. The order of activity was as follows: 1) Ethyl acetate 2) Ethanol, 3) Water 4) Chloroform, 5) Hexane. All the results were compared with the standards. The scavenging activities of the MB extracts were displayed in **Table 4.**

In the assay of superoxide scavenging, the various MB extracts displayed moderate activity. Hexane extract does not exhibit much activity against superoxide radicals. Based on the IC_{50} values, extracts of ethanol and water have an appreciable effect on scavenging superoxide radical. Also in hydrogen peroxide radical scavenging assay, ethanol and ethyl acetate extracts exhibit the IC_{50} values of 97.46 ± 3.91µg/ml and 117.83 ± 11.58 µg/ml and showed appropriate activity when compared to other extracts. The results obtained are displayed in **Table 5**.

In the present work, the hydroxyl radical scavenging activity of different extracts of *M. brunoniana* was analysed by the inhibition of p-NDA bleaching method. Among the solvent extracts, ethanol and water extracts show more appropriate activity with the IC_{50} values of 108.6 ± 3.7 µg/ml and 115.57 ± 2.31 µg/ml when compared to other solvent extracts of MB. The hydroxyl radical scavenging activity of diverse extracts of *M. brunoniana* is presented in **Table 6.**

In the total reducing power assay, the absorbance is increased with increase in concentration. This increase in the absorbance indicates higher reducing activity. Among the tested extracts, ethanol, ethyl acetate and water extracts shows potent reducing activity whereas hexane and chloroform extracts shown least activity. The result is illustrated in **Figure. 3.**

Table 4. Different Extracts of *Marsdenia brunoniana* on ABTS and DPPH Techniques

Extracts	IC_{50} (µg/ml)* by method	
	ABTS	**DPPH**
Hexane	Greater than 1000	Greater than 1000
Chloroform	101.67 ± 10.01	102.59 ± 9.96
Ethyl acetate	52.35 ± 2.20	28.78 ± 5.08
Ethanol	60.33 ± 5.88	30.61 ± 4.15
Water	79.03 ± 3.94	60.99 ± 4.37
Ascorbic Acid	13.63 ± 1.17	7.05 ± 0.76
Rutin	6.61 ± 0.64	8.11 ± 0.42

***Avg. of 3 determinations; Data are mentioned as mean ± SEM**

Table 5. Various Extracts of *Marsdenia brunoniana* on Scavenging of Superoxide Radical and Hydrogen peroxide techniques

Extracts	IC_{50} (µg/ml)* by method	
	Superoxide radical scavenging	**H_2O_2 radical scavenging**
Hexane	Greater than 1000	Greater than 1000
Chloroform	216.7± 17.53	227.4 ±13.27
Ethyl acetate	134.17 ± 5.43	117.83 ± 11.58
Ethanol	91.88± 4.42	97.46 ±3.91
Water	107.74 ± 10.12	123.8 ± 5.95
Ascorbic Acid	187.53 ± 11.93	75.16 ± 3.78
Rutin	55.97 ± 2.21	32.56 ± 1.05

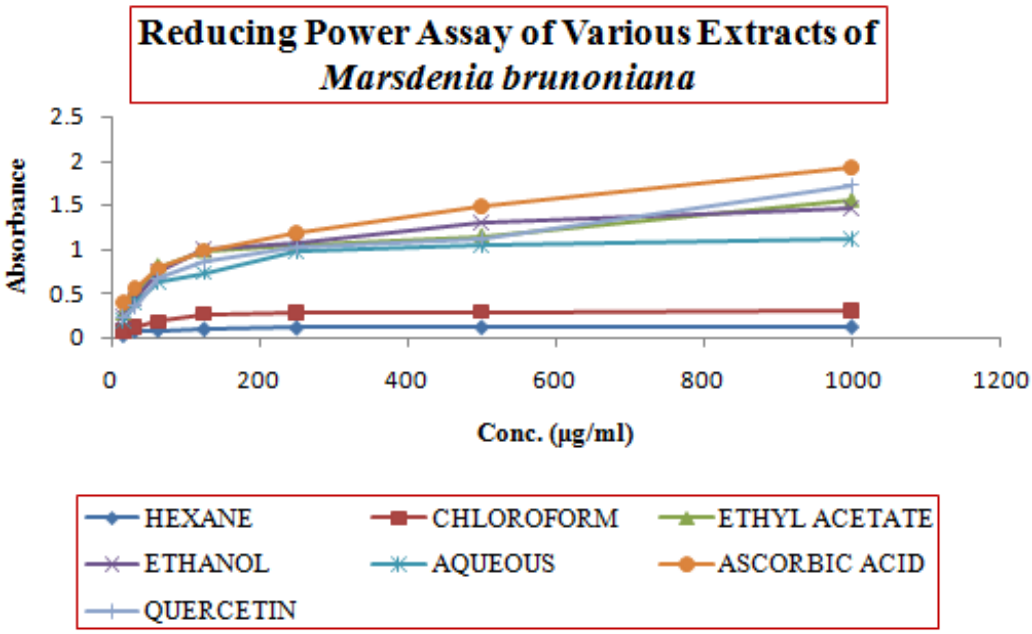
***Avg. of 3 determinations; Data are mentioned as mean ± SEM**

Table 6. Different Extracts of *Marsdenia brunoniana* on Hydroxyl-Radical Scavenging Assay

Extracts	IC_{50} (µg/ml)*
	Hydroxyl Radical Scavenging (p-NDA method)
Hexane	>1000
Chloroform	211.07±6.67
Ethyl acetate	128.8 ±4.16
Ethanol	108.6 ± 3.7
Water	115.57 ± 2.31
Ascorbic Acid	182.5 ± 4.88
Rutin	44.1 ±4.4

***Average of 3 determinations; Data are expressed as mean ± SEM**

Fig. 3.The Total Reducing Power Assay of Various Solvent Extracts of *M.brunoniana*



6.4 *In vitro* antidiabetic activity

6.4.1 *α- glucosidase* inhibitory effect of various solvent extracts of *M. brunoniana*

To assess the antidiabetic potential of the prepared solvent extracts with different concentrations (15.625 – 1000µg/ml) of *M. brunoniana*, alpha glucosidase inhibitory activity was determined *in vitro.* A dose dependent and gradual increase in the percentage inhibition of α-glucosidase was observed with the prepared plant extracts except hexane extract. Among the prepared extracts, IC_{50} value of ethanol extract was noticeable with 85.28 ± 4.86µg/ml which indicates the potent enzyme inhibitory nature. The activities order is follows: 1.Ethanol, 2.Ethyl acetate, 3.water, 4.Chloroform, 5.Hexane. Acarbose at a concentration of 0.15 to 10 µg/ml was employed and served as reference standard drug for α-glucosidase inhibitory activity. The IC_{50} value of various solvent extracts of *M. brunoniana* and acarbose against alpha glucosidase is present in **Table 7.**

6.4.2 *Alpha- amylase* inhibitory effect of various solvent extracts of *M. brunoniana*

Different concentrations (15.625 – 1000µg/ml) of various solvent extracts of *M. brunoniana* were evaluated for α-amylase inhibitory activity. A proportionate increase in the percentage inhibition of α-amylase was noticed for the tested extracts except hexane extract. Among the tested extracts, ethanol extract showed inhibitory concentration value of 87.48 ± 5.34 µg/ml. The concentration of acarbose (standard drug) employed in the test was similar to that of the extracts. The concentration of acarbose required for inhibiting α-amylase enzyme was higher when compared to the plant extracts tested. The order of activity as follows: 1Ethanol, 2Ethyl acetate, 3water, 4Chloroform, 5Hexane. The results are displayed in **Table 7.**

Table 7. Alpha- glucosidase and Alpha- amylase inhibitory action on various extracts of *Marsdenia brunoniana*

Extracts/Standard	IC_{50} Value (µg/ml)*	
	α-glucosidase	***α -amylase***
Hexane	>1000	>1000
Chloroform	153.23 ± 2.11	138.9 ± 3.96
Ethyl acetate	99.63 ± 2.93	100.42 ± 3.43
Ethanol	85.28 ± 4.86	87.48 ± 5.34
Water	120.77 ± 4.1	122 ± 2.63
Acarbose	0.5627 ± 0.053	265.27 ± 12.51

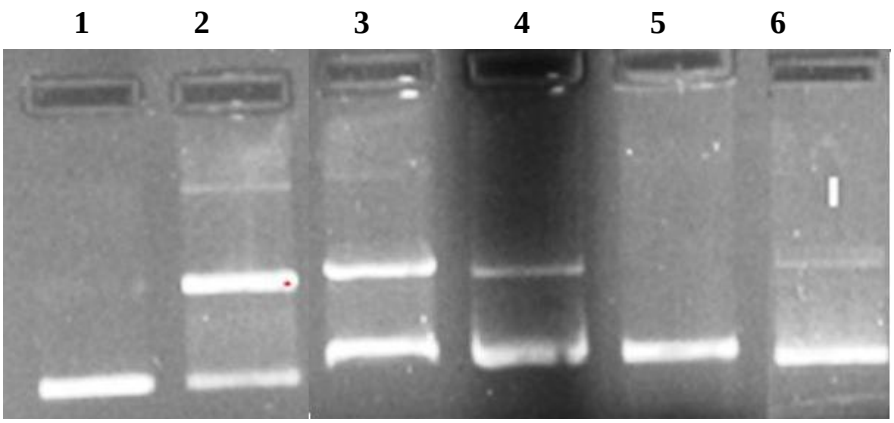
***Avg. of 3 determinations; Data are mentioned as mean ± SEM**

6.5 Solvent extracts selection for further studies of *M. brunoniana*

Based on the yield, preliminary phytochemical analysis, *in vitro* antioxidant activity and *in vitro* enzyme inhibitory activity ethanol extract of *M. brunoniana* was selected for further studies.

6.5.1 DNA Protecting Activity

FIGURE 4: Effect of ethanol extract of *M. brunoniana* on damaged supercoiled pUC18 plasmid DNA. Lane 1: pUC18 DNA + PBS; Lane 2: pUC18 DNA + Fenton's reagent; Lane 3: DNA+ Fenton's reagent+ EEMB (50 µg/ml); Lane 4: DNA+ Fenton's reagent + EEMB (100 µg/ml); Lane 5: DNA+ Fenton's reagent + EEMB (200µg/ml); Lane 6: Mannitol (200 µg/ml).

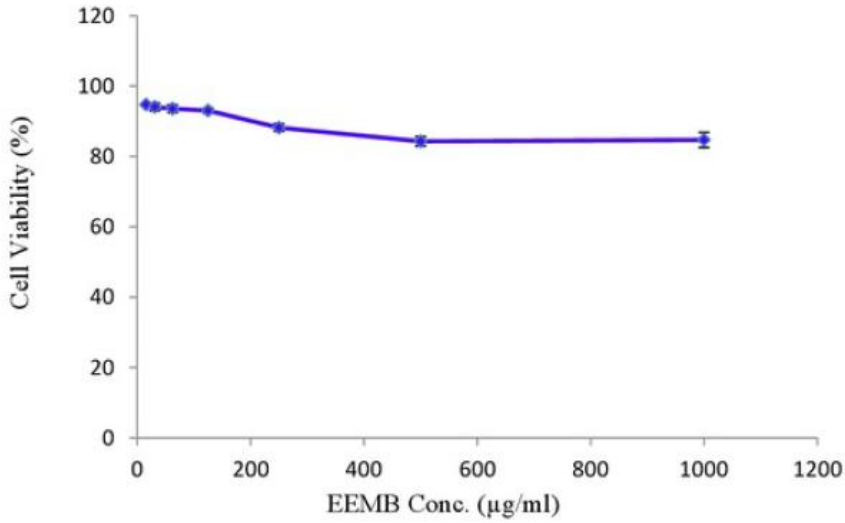


The DNA protection assay estimation is used for testing the ability of plant extracts to protect the plasmid DNA damage due to the hydroxyl radicals' release. Normally the fenton reaction releases hydroxyl radical which cause oxidative induced breaks in the strands of DNA and change the structure to a relaxed and open circular forms. Subsequently free radicals induced reaction over plasma DNA also takes place. The nitrogenous bases of DNA react with the hydroxyl radicals' results in the formation of base radicals and sugar radicals. Again these radicals react with sugar molecules and breaks the nucleic acid structure leads to the strand break. The MB extract added to the fenton reagent protects DNA damage completely and partially at 50,100,200 µg/ml and are displayed in Figure 4.

6.5.2 Cell viability Assay

To examine the cytotoxicity action of ethanol extract of *M. brunoniana*, the *in vitro* MTT- assay on 3T3-L1 preadipocytes cell line was performed and it was proved that the effect of EEMB was non-cytotoxic at various concentrations and it is displayed in **Figure 5.**

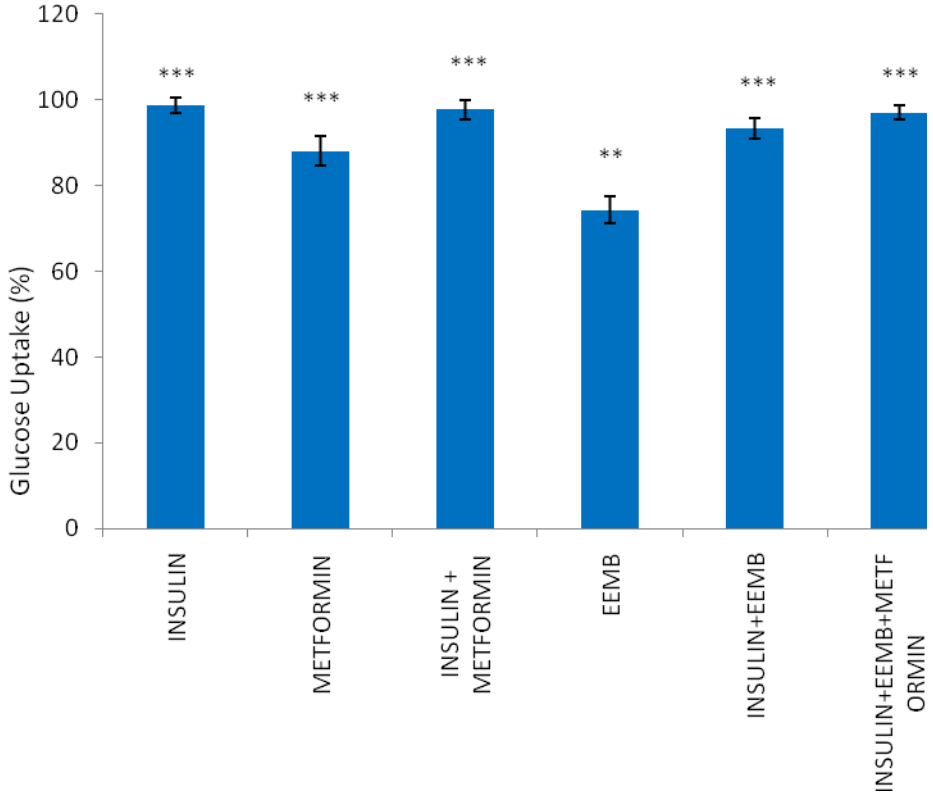
Fig. 5. Cell Viability assay



6.5.3 Glucose uptake assay

Based on the enzyme inhibitory studies, it was found that among all the solvent extracts ethanol extract showed strong α-amylase and α-glucosidase inhibitory action, therefore its activity in utilization of glucose in differentiated 3T3-L1 adipocytes cell line was also studied *in vitro.* The capability of the plant extract to aggregate glucose uptake was checked in different combinations (**Table 1**) and it was found that the ethanol extract of *M. brunoniana* alone showed significant glucose utilization up to 74.3 ± 3.18%, in the presence of insulin it increases up to 93.33 ± 2.33%, and in the presence of both insulin and metformin the glucose uptake was noticeably elevated to 97.67 ± 2.17 % over the value of control. In addition, Insulin and metformin exhibited 98.67 ± 1.76 % and 88.0 ± 3.46 % of glucose uptake action, respectively, while 97.0 ± 1.53 % of glucose uptake was noticed during their synergistic treatment. The results obtained are shown in **Figure 6.**

Fig. 6. Glucose uptake assay



6.6 Quantitative Analysis for Phytochemical

Quantitative determination of major phytochemical constituents in EEMB was done and the results are displayed in the below **Table no.8.** Quantitative phytochemical analysis of ethanol extract of *M. brunoniana* revealed that the extract is rich in phenols, flavonoids, tannins and glycosides. Apart from these constituents, it contains considerable amount of ascorbic acid, which acts as a natural antioxidant.

Table 8. Quantitative Analysis for Phytochemical Constituents of C_2H_5OH extract of plant - *M. brunoniana*

S.No	Phytochemical Constituents	Quantity *
1.	Glycosides (mg/gram of extract)	141.3 ± 4.51
2.	Phenol Content (mg/gram of Gallic acid equivalent)	73.46 ± 1.23
3.	Total flavonoid content (mg/gram of Quercetin equivalent)	24.32 ± 1.26
4.	Tannins (mg/g of extract)	181.92 ± 2.63
5.	Ascorbic acid (mg/g of extract)	16.28 ±0.82

***Avg. of 3 determinations; Data are mentioned as mean ± SEM**

6.6.1 Analysis of EEMB using GC-MS Technique

The GC-MS spectrum analysis of ethanolic extract of *M. brunoniana* is presented in **Figure 7.** The GC-MS analysis results of EEMB exhibit many phytochemical constituents such as, methyl-α-D-Glucopyranoside, 4-C-methyl-Myo-Inositol, n-hexadecanoic acid and phytol which denote the medicinal potency. The phytochemicals identified by GC-MS are presented **Table 9.**

Fig. 7. GC-MS analysis of EEMB

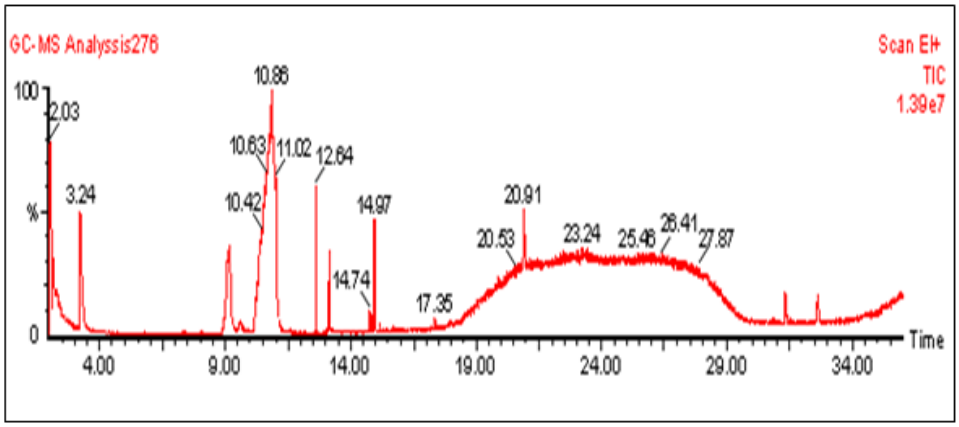


Table 9. GC-MS analysis of EEMB

S.No.	Retention Time	Compound	Molecular Formula	MW	Peak Area %
1.	3.24	1-Butanol, 3-methyl-, format	$C_6H_{12}O_2$	116	4.77
2.	9.17	α-D-Glucopyranoside, methyl	$C_7H_{14}O_6$	94	8.56
3.	10.86	Myo-Inositol, 4-C-methyl-	$C_7H_{14}O_6$	94	59.66
4.	12.64	9-Octadecenoic acid, 12-(acetyloxy)-, methyl ester, [R-(Z)]-	$C_{21}H_{38}O_4$	54	1.68
5.	13.14	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	1.77
6.	14.74	1-Tridecyne	$C_{13}H_{24}$	180	0.26
7.	14.97	Phytol	$C_{20}H_{40}O$	296	1.59
8.	15.18	Cyclopentaneundecanoic acid, methyl ester	$C_{17}H_{32}O_2$	268	0.09
9.	17.35	Ethanol, 2-(2-propenyloxy)-	$C_5H_{10}O_2$	102	0.18
10.	20.91	Vinyl 10-undecenoate	$C_{13}H_{22}O_2$	210	19.42
11.	31.33	11,12-Dihydroxyseychellane	$C_{15}H_{26}O_2$	238	0.97
12.	32.62	6,9,12-Octadecatrienoic acid, phenylmethyl ester, (Z,Z,Z)-	$C_{25}H_{36}O_2$	368	1.06

6.6.2 HPLC Analysis of EEMB

The Ethanolic extract of *M. brunoniana* was subjected to HPLC analysis for the phenolic compounds identification and the peak is represented in **Figure 8.** HPLC peaks of EEMB showed that the extract contains rutin, quercetin, ferulic acid, caffeic acid, and gallic acid. Among these presented compounds, quercetin was found to be one of the major compounds. The results are displayed in **Table 10.**

Fig 8. Chromatogram of EEMB by HPLC technique

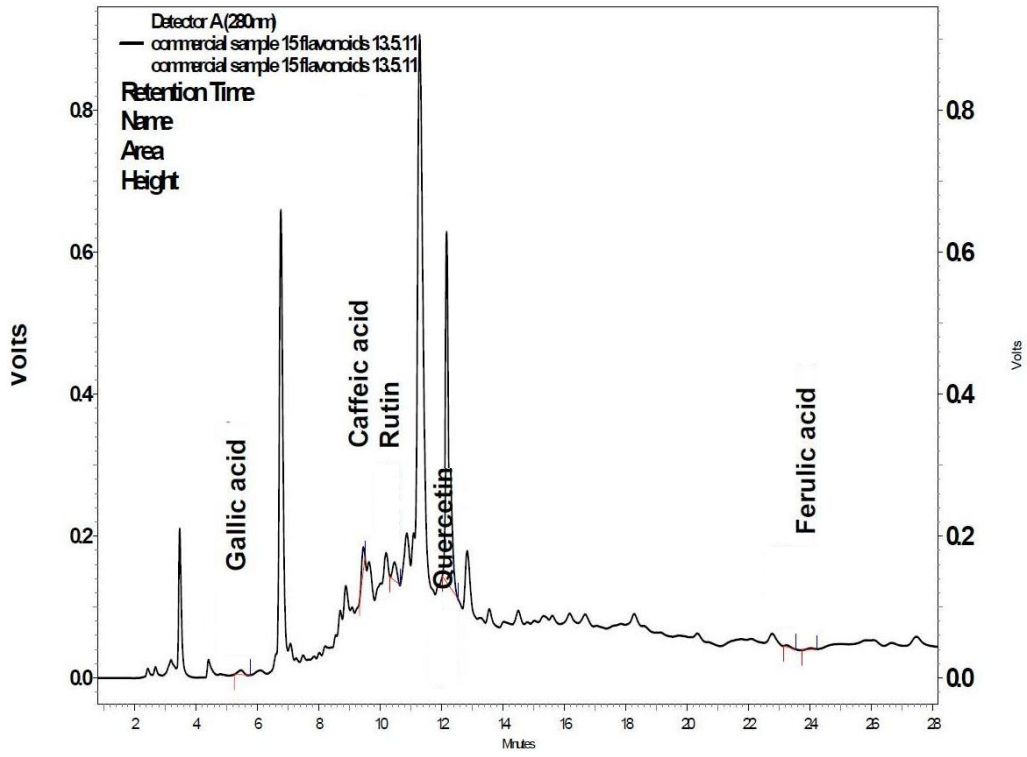


Table 10. Compounds Identified from EEMB through HPLC

RT	Area	Height	Name
5.482	74050	6195	Gallic acid
9.471	199628	31326	Caffeic acid
10.514	246847	26414	Rutin
12.172	3247073	291087	Quercetin
24.205	26483	1907	Ferulic acid

6.6.3 HPTLC Analysis of EEMB

Ethanolic extract of *M. brunoniana* was treated to HPTLC technique for the identification of different phytochemicals. HPTLC fingerprint of EEMB display twelve peaks. Amongst the resulted peaks, R_f value of 0.87 and 0.94 were observed to have larger area such as 6578.7 and 10685.4. The area is directly proportional to the quantity of compound present in the plant extract. The HPTLC fingerprints & peak display of EEMB was shown in Figure 9 and 10 and the results are displayed in **Table 11.** Ethanolic extract of *M. brunoniana* was further subjected to the identification of flavonoid substances by HPTLC method using quercetin as standard. The results showed that 4 flavonoids were present in EEMB.

Fig 9. HPTLC fingerprints of EEMB

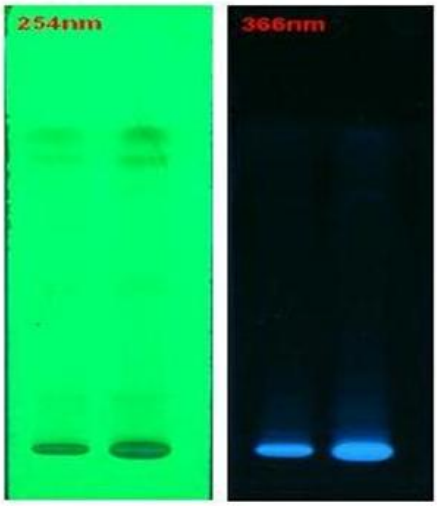


Fig 10. Display of Peak -EEMB at 254 nm

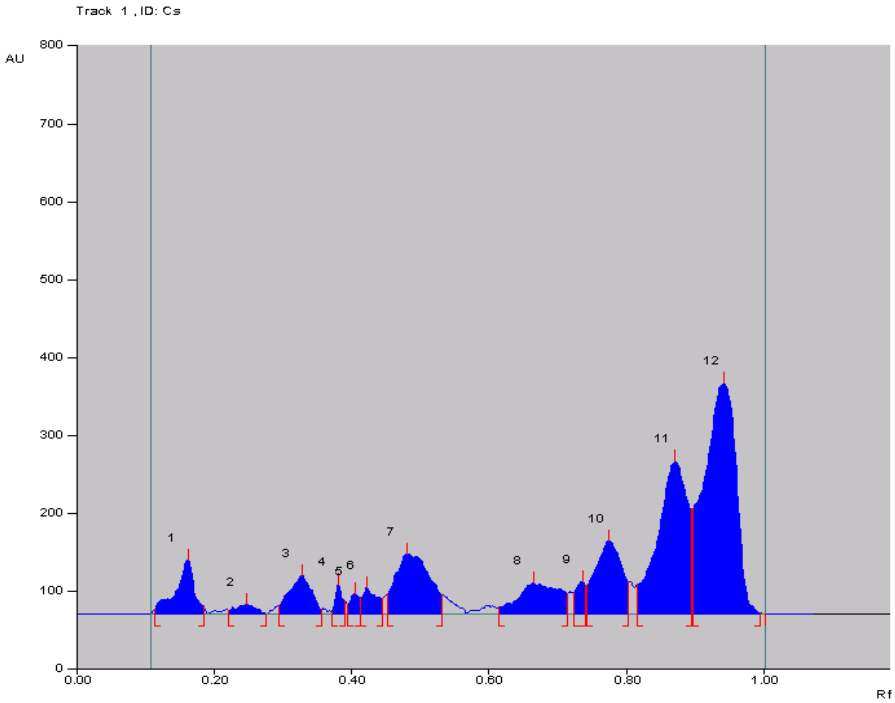


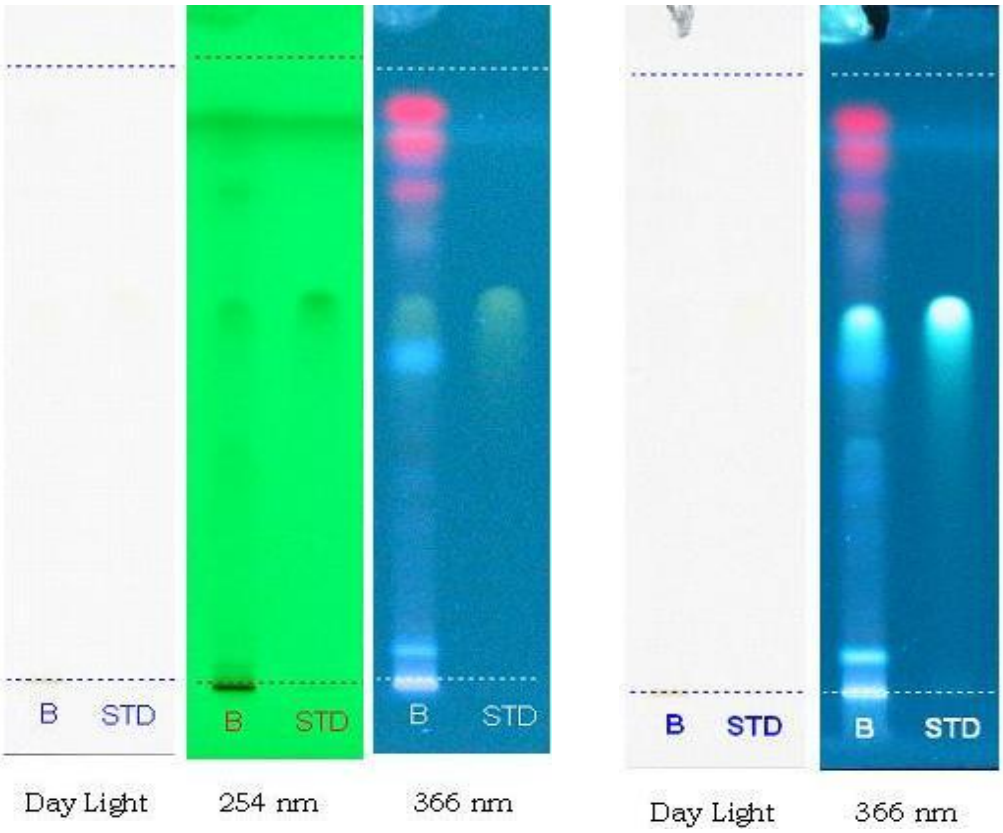
Table 11. Finger prints of EEMB - HPTLC

Peak No.	R_f Value	Area
1	0.16	1438.8
2	0.25	283.2
3	0.33	1253.5
4	0.38	297.4
5	0.40	275.4
6	0.42	543.6
7	0.48	2958.7
8	0.67	1861.4
9	0.74	471.3
10	0.77	2808.5
11	0.87	6578.7
12	0.94	10685.4

6.6.4 HPTLC Analysis of EEMB for Flavonoid Profile

The analysis of EEMB for its flavonoid profile using High Performance thin layer chromatography was performed. The peaks of the EEMB were compared with the peaks of flavonoid standard Quercetin. The HPTLC Finger print analysis s of EEMB for flavonoid profile was displayed from **Figure 11 to 15** and the obtained results are illustrated in **Table 12**.

Fig 11. Analysis of EEMB for Flavonoid profile- HPTLC



Before Derivatization **After Derivatization**

Fig 12. Baseline display of Flavonoid standard (Quercetin) (Scanned at 254 nm)

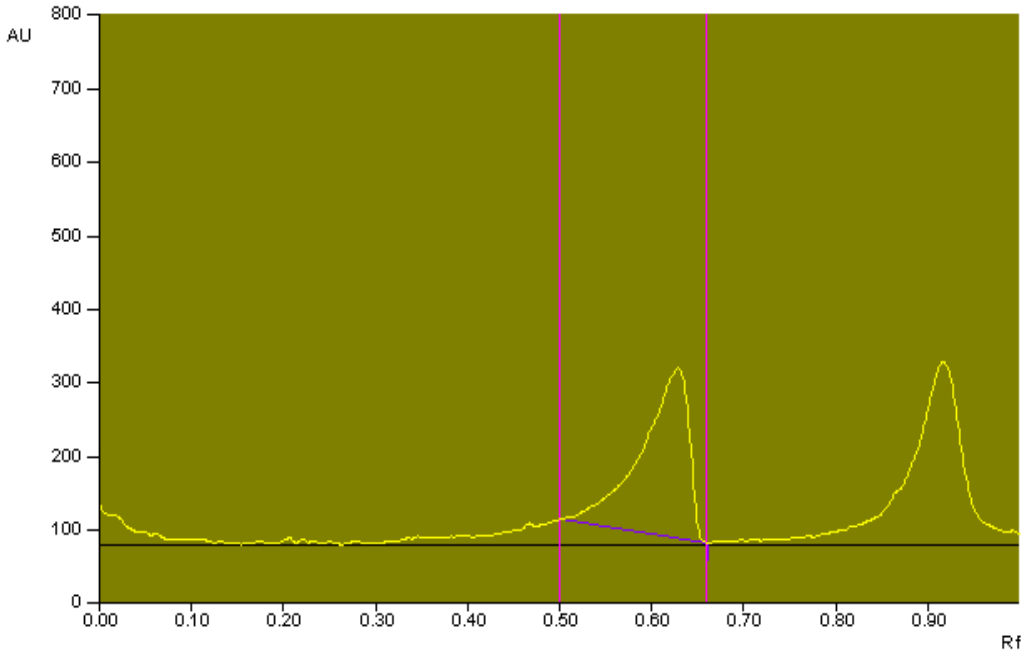


Fig 13. Peak densitogram display of Flavonoid standard (Quercetin) (Scanned at 254 nm)

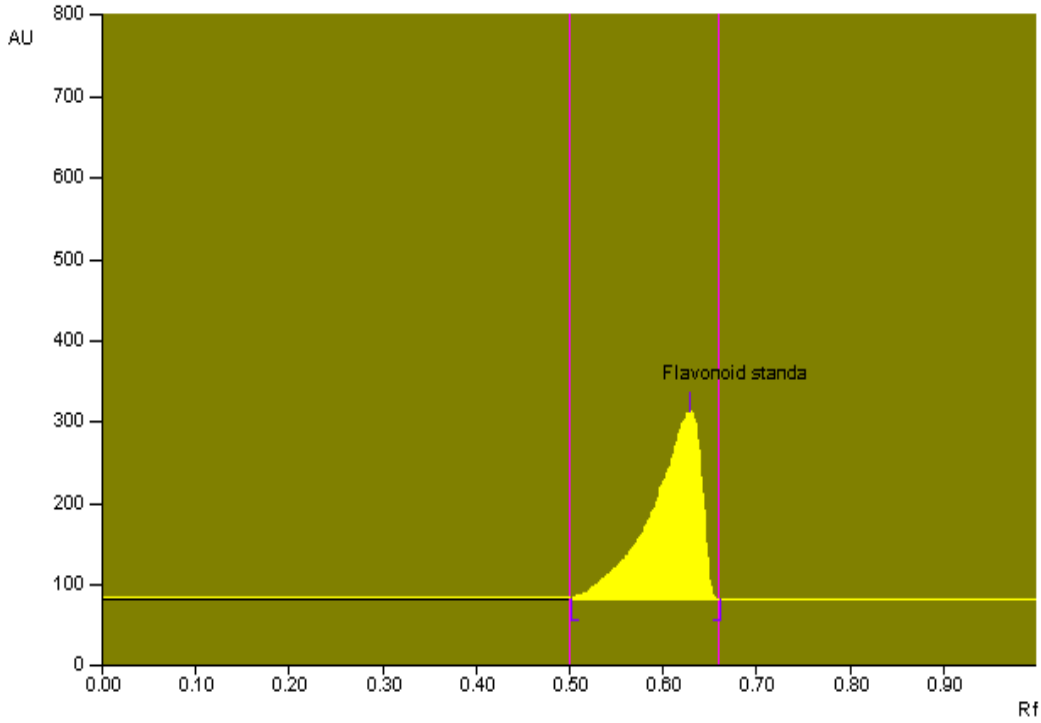


Fig 14.EEMB Baseline display (Scanned at 254 nm)

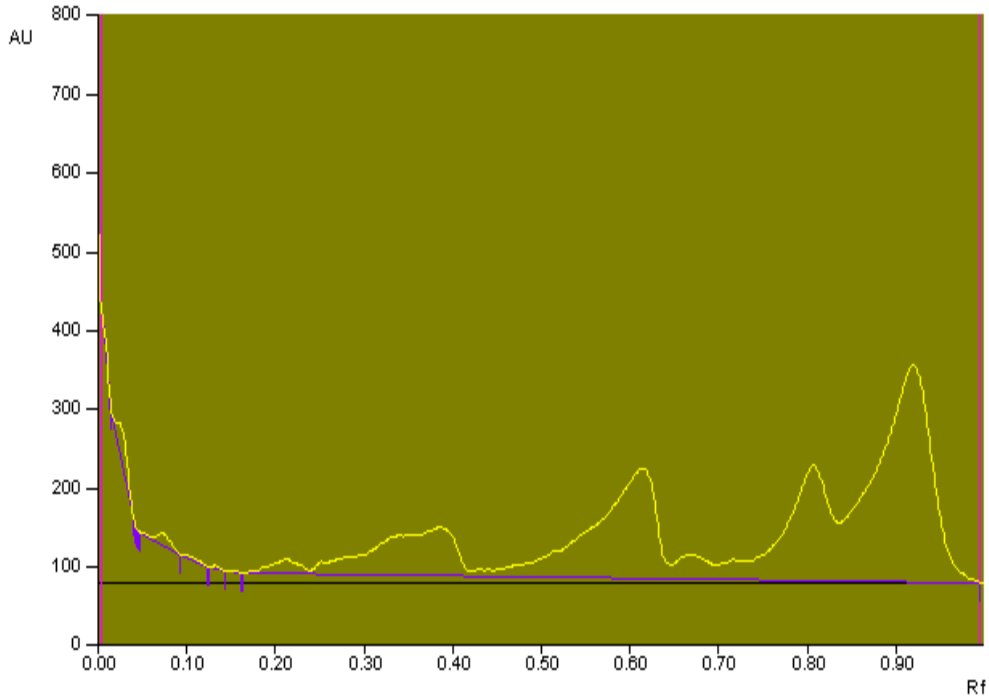


Fig 15.EEMB Peak densitogram display (Scanned at 254 nm)

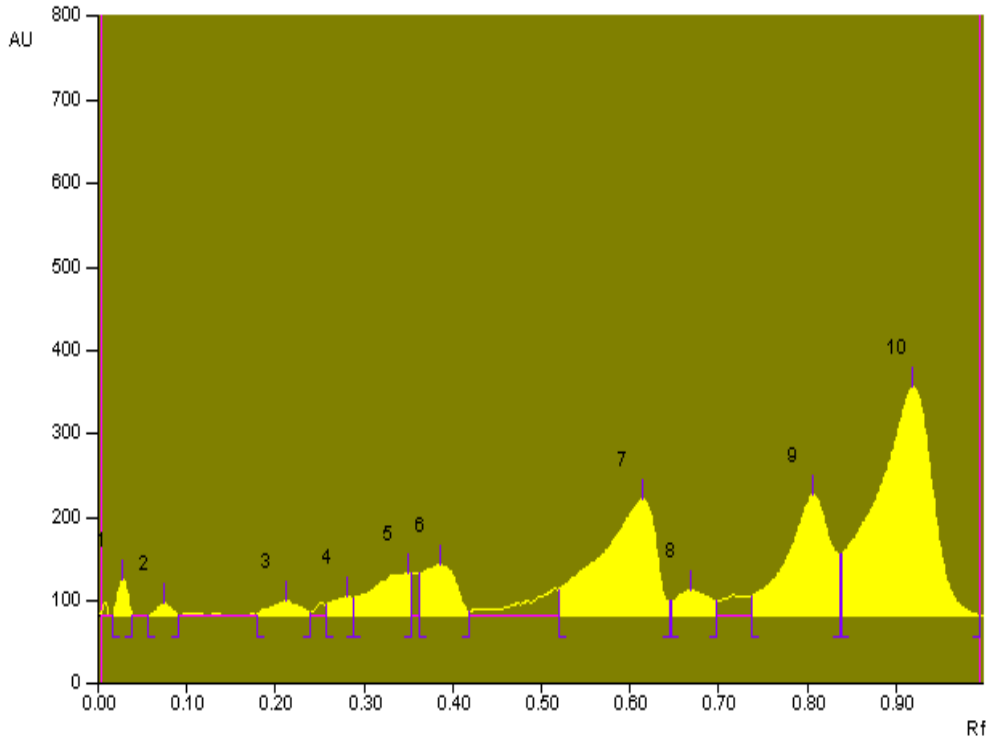


Table 12. HPTLC of EEMB for Flavonoid Profile

Track	Peak	Rf	Height	Area	Assigned substance
STD	1	0.66	234.8	13459.6	Quercetin (Standard)
EEMB	1	0.04	43.4	424.1	Unknown
EEMB	2	0.06	15.3	206.1	Flavonoid 1
EEMB	3	0.22	17.4	515.3	Unknown
EEMB	4	0.27	21.6	487.6	Unknown
EEMB	5	0.36	52.4	2037.4	Flavonoid 2
EEMB	6	0.40	60.8	2109.4	Flavonoid 3
EEMB	7	0.62	136.7	8034.5	Flavonoid 4
EEMB	8	0.66	31.4	992.6	Unknown
EEMB	9	0.83	147.6	6467.8	Unknown
EEMB	10	0.93	278.4	15620.7	Unknown

6.7 Toxicological Studies

6.7.1 Acute Toxicity Study

Based on the extractive yield, preliminary phytochemical analysis, *in vitro antioxidant* and antidiabetic activity, the ethanol extract of *M. brunoniana* was taken for the *in vivo* studies. Hence, the ethanolic extract of *M. brunoniana* (EEMB) was allowed to study the toxicity studies include acute and sub acute study. In the acute toxicity study, the ethanol extract of *M. brunoniana* did not show any lethality in mice up to the maximum dose 2000 mg/kg. The tested doses produced any gross apparent effect on general locomotor activity, muscular weakness, fecal output, food behaviors during the observation period. This denotes that the extract was found to

be safe at the tested dose range. So 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of this dose were selected for the further *in vivo* studies.

6.7.2 Sub-acute Toxicity Studies

The body weight, food consumption and the food intake was taken in to account after the 28 days of experiment period and the tested groups showed significant results when compared with the control group which is shown in **Table 13, 14 &15**. At various dose levels from up to 200 to 1000mg/kg the extract treated group did not show any deviation in the body weight, food intake and water intake. No statistical significant changes was noted in the tested groups at the end of the study in the mean hemoglobin content, WBC RBC and differential cell counts when compared with the normal group **(Table 16)**. The biochemical parameters also did not show any statistically significant changes **(Table 17 & 18).**The excretion pattern of the tested groups were also found to be normal **(Table 19)**

The gross examination of the isolated organ from the various test groups does not show any abnormalities on the gross study examination. The important organs are weighed for the determination and it did not reveal any significant changes on the weight of the vital organs .The results are displayed in Table 20. The liver and kidney histological studies did not show any pathological changes and are found to be normal and the obtained figure is displayed in **Figure 16 to 23.**

Table 13. Effect of EEMB on Body Weight

Treatment	0 Day	7 Day	14 Day	21 Day	28 Day
Control	149.7 ± 11.7	157.5 ± 11.9	166.2 ± 12.3	173.4 ± 13.3	182.8 ± 14.8
EEMB 200	152.9 ± 12.4	161.3 ± 12.3	169.1 ± 12.8	177.3 ± 13.5	186.1± 14.6
EEMB 400	154.0 ± 13.1	163.2 ± 13.2	171.4 ± 13.5	179.1 ± 14.2	189.5 ± 15.7
EEMB 1000	149.5 ± 11.6	156.9 ± 12.0	165.8 ± 12.6	173.1 ± 13.4	183.4 ± 14.5

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 14. Effect of EEMB on Food Intake

Treatment	Food consumption(g/d)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Control	9.8 ± 1.81	11.5 ± 2.54	13.2 ± 2.42	14.1 ± 2.84	15.2 ± 2.72
EEMB 200	10.1 ± 3.32	12.2 ± 3.41	14.1 ± 3.63	15.1 ± 2.53	16.0 ± 3.23
EEMB 400	9.7 ± 2.21	11.4 ± 2.55	13.5 ± 2.61	14.4 ± 3.11	15.5 ± 2.32
EEMB 1000	10.3 ± 3.32	12.6 ± 3.63	14.4 ± 3.84	15.3 ± 2.72	16.2 ± 3.43

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 15. Effect of EEMB on Water Intake

Treatment	Water intake (ml/d)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Control	24.3 ± 1.2	25.4 ± 1.7	26.2 ± 1.4	27.0 ± 1.6	28.2 ± 1.4
EEMB 200	24.1 ± 0.9	25.3 ± 1.5	26.5 ± 1.6	27.3 ± 1.3	28.5 ± 1.6
EEMB 400	25.0 ± 1.4	26.1 ± 1.9	27.0 ± 1.8	27.9 ± 1.5	29.1 ± 1.8
EEMB 1000	24.7 ± 1.5	25.6 ± 1.6	26.4 ± 1.7	27.6 ± 1.2	28.7 ± 1.5

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 16. Effect of EEMB on Hematological Parameters

Treatment	RBC (million/mm^3)	Hb (g/dl)	WBC (million/mm^3)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Lymphocytes (%)	Monocytes (%)
Control	9.0 ± 1.2	12.1 ± 1.3	8.9 ± 1.3	23.46 ± 6.2	2.73 ± 1.1	0.18 ± 0.05	75.33 ± 3.7	3.24 ± 1.5
EEMB 200	9.2 ± 1.1	12.3 ± 1.2	8.7 ± 1.1	22.38 ± 8.3	2.43 ± 1.4	0.19 ± 0.08	73.66 ± 2.4	4.06 ± 1.3
EEMB 400	8.9 ± 0.9	12.1 ± 1.1	8.6 ± 1.2	21.58 ± 6.4	2.28 ± 1.2	0.16 ± 0.07	76.65 ± 4.5	3.14 ± 1.6
EEMB 1000	9.3 ± 1.2	12.2 ± 0.9	9.0 ± 1.0	24.56 ± 7.8	2.74 ± 1.1	0.17 ± 0.06	74.45 ± 4.2	2.66 ± 1.4

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 17. Effect of EEMB on Serum Hepatic Parameters

Treatment	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total cholesterol (mg/dl)	Bilirubin	
					Total(mg/dl)	Direct (mg/dl)
Control	192.3 ± 1.51	82.5 ± 1.12	233.1 ± 1.12	121.6 ± 2.6	1.23 ± 0.31	0.24 ± 0.02
EEMB 200	190.6 ± 1.81	78.2 ± 1.49	236.0 ± 4.24	123.2 ± 3.4	1.24 ± 0.23	0.22 ± 0.01
EEMB 400	192.2 ± 1.49	81.3 ± 1.51	234.2 ± 1.10	119.9 ± 3.8	1.22 ± 0.21	0.25 ± 0.03
EEMB 1000	191.9 ± 1.55	82.1 ± 1.10	232.5 ± 1.78	122.1 ± 4.2	1.25 ± 0.31	0.23 ± 0.02

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 18. Impact of EEMB on Serum Renal Parameters

Design of treatment	Urea (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)	Total Protein (gm/dL)	Albumin (gm/dL)
Control	57.5 ± 3.14	26.78 ± 1.48	0.75 ± 0.022	6.92 ± 0.094	4.067 ± 0.06
EEMB 200	53.3 ± 2.33	24.88 ± 1.09	0.733 ± 0.033	7.083 ± 0.06	4.183 ± 0.079
EEMB 400	62.67 ± 3.13	29.23 ± 1.48	0.817 ± 0.03	7.133 ± 0.049	4.217 ± 0.07
EEMB 1000	58 ± 5.020	27.1 ± 2.3	0.8 ± 0.025	6.983 ± 0.047	4.083 ± 0.07

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 19. Urine Analysis of EEMB Treated Animals

Design of treatment	Appearance	Specific gravity	pH	Glucose	Protein	Ketone bodies	Blood cells
Control	Straw Yellow	1.0283 ± 0.003	6.42 ± 0.03	NIL	NIL	NIL	NIL
EEMB 200	Straw Yellow	1.025 ± 0.0034	6.43 ± 0.021	NIL	NIL	NIL	NIL
EEMB 400	Straw Yellow	1.038 ± 0.003	6.467 ± 0.02	NIL	NIL	NIL	NIL
EEMB 1000	Straw Yellow	1.0217 ± 0.004	6.47 ± 0.021	NIL	NIL	NIL	NIL

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Table 20. Impact of EEMB on Weight of Vital Organs

Treatment	Liver	Kidneys	Heart	Lungs	Spleen
Control	4.34 ± 0.098	1.035 ± 0.049	0.633 ± 0.0095	1.043 ± 0.0088	0.34 ± 0.022
EEMB 200	4.865 ±0.095	1.1783 ± 0.047	0.64 ± 0.026	1.173 ± 0.038	0.39 ± 0.013
EEMB 400	4.725 ± 0.13	1.103 ± 0.044	0.621 ± 0.025	1.1717 ± 0.069	0.408 ± 0.022
EEMB 1000	4.623 ± 0.20	1.157 ± 0.040	0.5983 ± 0.011	1.067 ± 0.029	0.377 ± 0.018

N=6; Values were expressed as Mean ± SEM; Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Histopathology - Liver

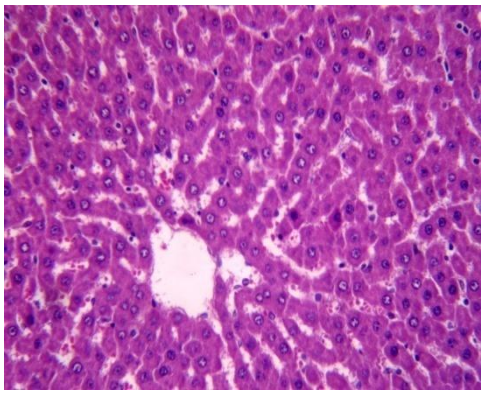


Fig. 16. Normal group illustrating normal pattern of architecture of hepatic cords (H&E x 400)

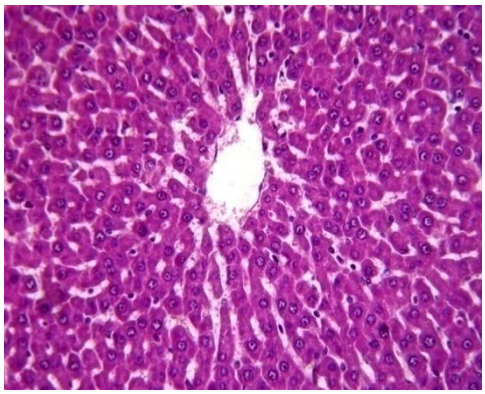


Fig 17. EEMB-200 group illustrating normal pattern of architecture of hepatic cords around portal triad (H&E x 400)

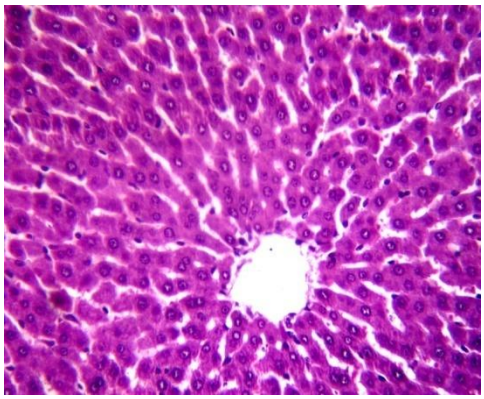


Fig 18. EEMB- 400 group illustrating normal pattern of architecture of hepatic cords around portal triad (H&E x 400)

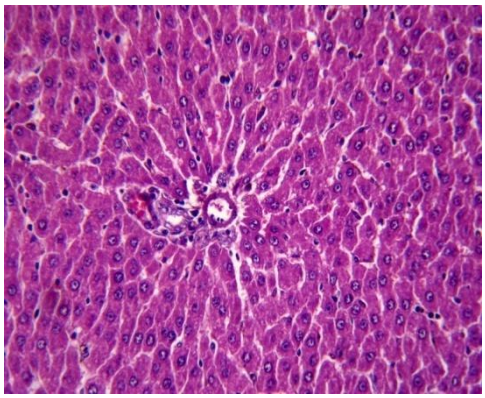


Fig 19. EEMB-1000 group illustrating normal pattern of architecture of hepatic cords (H&E x 400)

Histopathology - Kidney

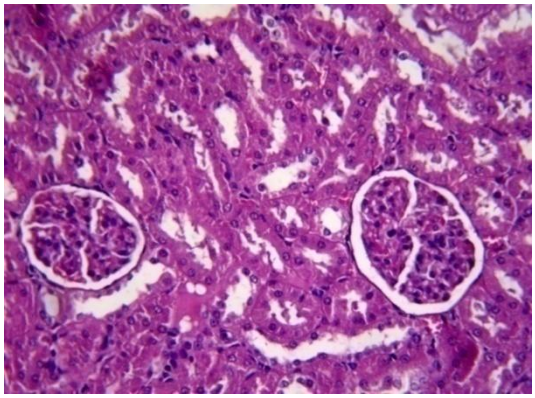


Fig 20. Control group illustrating normal architectural pattern of glomerulus and tubules (H&E x 400)

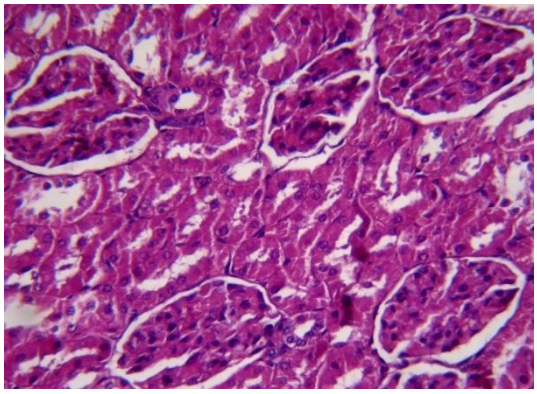


Fig 21. EEMB - 200 group illustrating normal architectural pattern of glomerulus and proximal tubules with brush borders (H&E x 400)

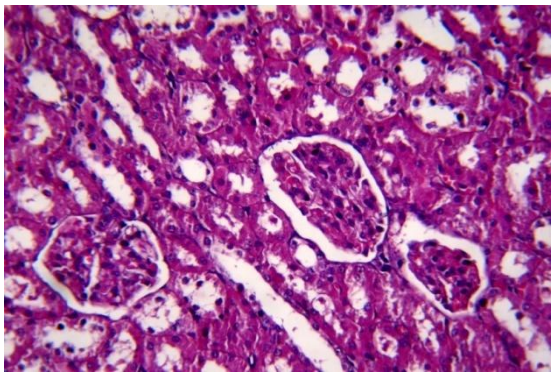


Fig 22. EEMB 400 group showing normal architectural pattern of glomerulus and tubules (H&E x 400)

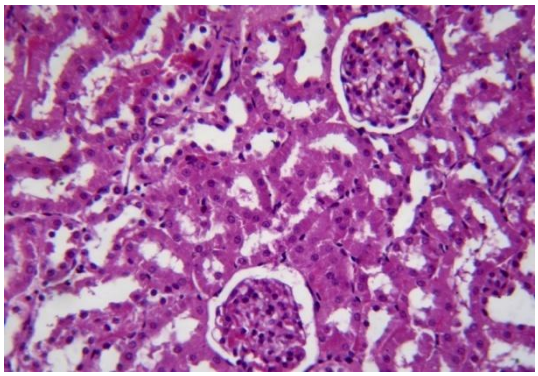


Fig 23. EEMB 1000 group showing normal pattern of architecture of glomerulus and tubules (H&E x 400)

6.8 Screening of EEMB for anti-diabetic activity

Ethanol extract of *M. brunoniana* was evaluated for its *in vivo* antidiabetic, antihyperlipidemic and antioxidant activities on streptozotocin - nicotinamide induced type II DM in rats. Various parameters including body weight, serum glucose levels, hemoglobin, glycosylated hemoglobin and protein content was estimated. In diabetes induced rats, a highly significant reduction in the body weight is indicated by $p < 0.001$, whereas the extract treatment significantly protects the animals from weight loss which is commonly observed in diabetic animals. After the drug treatment, the glucose content in the serum was found to be near normal at both 200mg/kg and 400 mg/kg & $p < 0.001$ denotes the potency of the plant extract as antidiabetic. The results are represented in Table **21 & 22 and Figure 24 to 25.**

A statistically significant reduction in hemoglobin was noted in diabetic animals whereas it reversed to normal in extract treatment group. Glycosylated hemoglobin (HbA1C) is a very good indicator to assess the glycemic control of any drug. The glycosylated hemoglobin level was found to be 12.7 ± 0.87% higher in diabetic animals, but the extract treatment significantly reduces the glycosylated hemoglobin amount to 4.71 ± 0.36% at 400 mg/kg dose level & $p < 0.001$ indicates the therapeutic potential of the ethanol extract of *M. brunoniana* as antidiabetic drug. The protein level was found to be 7.92 ± 0.68g/dL and the treatment group at 400 mg/kg improved the protein level to 6.71 ± o.51g/dL. Protein levels are also bought back to normal which shows the protective role of the extract and its phytochemical constituents on inner organs. Further to evaluate the antidiabetic potential of the plant extract, the serum insulin level was also estimated. The extract treatment shows significantly increased serum insulin level to 11.96 ± 1.12 IU when correlated with diabetic control animals with the serum level of 5.2 ± 0.74 IU. Among the tested dose levels 400 mg/kg dose level showed good activity when compared to its lower dose. All the results obtained were comparable with glimepride which is used as a standard. The results obtained are displayed in **Table 23 to 24 and Figure 26 to 27.**

Table 21. Effect of EEMB on Body weight

Design of Treatment	Body weight (g) / Day(s)				
	0	7	14	21	28
Control	220.7 ± 11.8	252 ± 14.2	258 ± 12.4	272.4 ± 10.4	279.2 ± 7.42
Diabetic control	213.7 ± 8.74	193.4 ± 8.54[b]	184.1 ± 8.7[a]	173.2 ± 5.21[a]	166.3 ± 6.14[a]
Glimepride 5mg/kg	217.4 ± 9.42	243.4 ± 11.5[e]	258.4 ± 10.9[d]	262.3 ± 8.62[d]	267.4 ± 9.42[d]
EEMB 200	215.4 ± 6.69	219.3 ± 7.16	227.2 ± 7.56[e]	235.4 ± 7.84[d]	237.3 ± 6.86[b,d]
EEMB 400	217.4 ± 7.45	226.5 ± 11.7	232.3 ± 9.42[e]	236.7 ± 8.32[d]	246.4 ± 5.64[c]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001, [b]P<0.01, [c]P<0.05 vs Normal; [d]P<0.001, [e]P<0.05 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.24. Effect of EEMB on Body weight

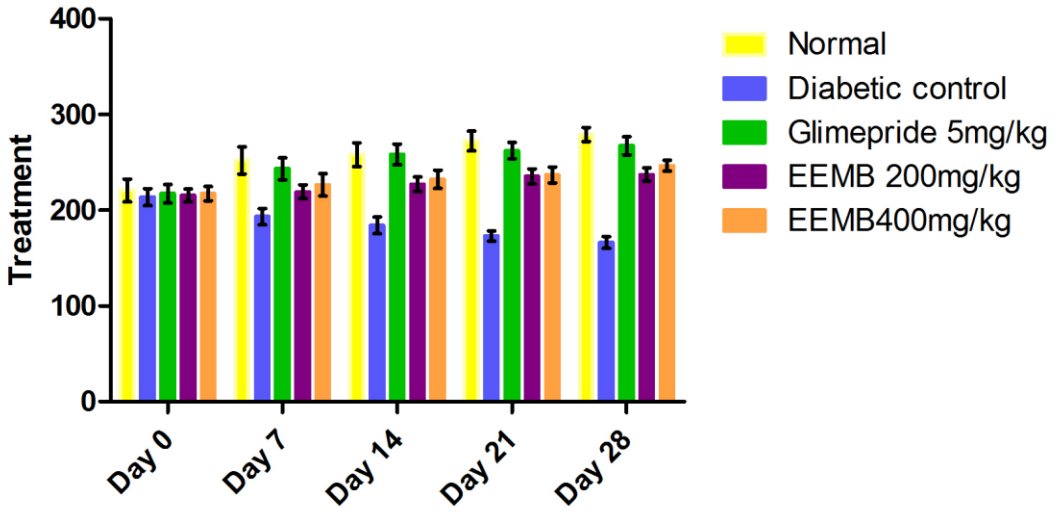


Table 22. Effect of EEMB on Serum Glucose level

Design of Treatment	Serum glucose level (mg/dL)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Normal	73.4 ± 4.35	69.7 ± 2.429	70.2 ± 2.52	72.7 ± 3.27	72.8 ± 1.93
Diabetic control	286.7 ± 13.91[a]	328.3 ± 12.64[a]	328.3 ± 7.84[a]	324.6 ± 12.17[a]	326.4 ± 8.92[a]
Glimepride 5mg/kg	272.4 ± 12.4[a]	226.47 ± 8.44[a,b]	224.2 ± 5.67[a,b]	187.2 ± 9.27[a,b]	164.7 ± 6.24[a,b]
EEMB 200	274.3 ± 11.9[a]	286.4 ± 9.63[a,b]	197.2 ± 9.94[a,b]	163.4 ± 8.94[a,b]	146.1 ± 8.3[a,b]
EEMB 400	276.8 ± 13.61[a]	227.4 ± 14.1[a,b]	191.7 ± 11.4[a,b]	154.7 ± 8.12[a,b]	132.5 ± 6.18[a,b]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.001 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.25 Effect of EEMB on Serum Glucose level

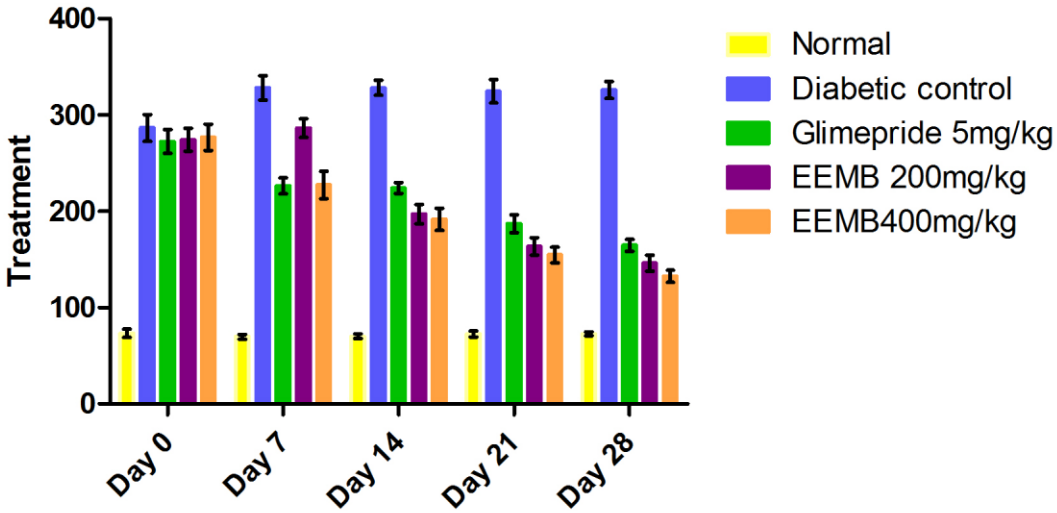


Table 23. Action of EEMB on hemoglobin, Glycosylated hemoglobin (HbA_1C) and total protein level

Design of Treatment	Hemoglobin Level (g/dL)	Glycosylated Hemoglobin Level (%)	Total protein (g/dL)
Normal	13.42 ± 0.76	4.04 ± 0.81	7.92 ± 0.68
Diabetic control	7.56 ± 0.46[a]	12.71 ± 0.87[a]	4.96 ± 0.51[a]
Glimepride 5mg/kg	13.17 ± 0.36[d]	4.65 ± 0.41[b]	6.41 ± 0.34[c,e]
EEMB 200	10.41 ± 0.66[a,d]	6.51 ± 0.64[a,b]	5.73 ± 0.31[b]
EEMB 400	12.76 ± 0.42[d]	4.71 ± 0.36[b]	6.71 ± 0.51[d]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001, [b]P<0.05, [c]P<0.01 vs Normal; [d]P<0.001, [e]P<0.05 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.26. Action of EEMB on hemoglobin, Glycosylated hemoglobin (HbA_1C) and total protein level

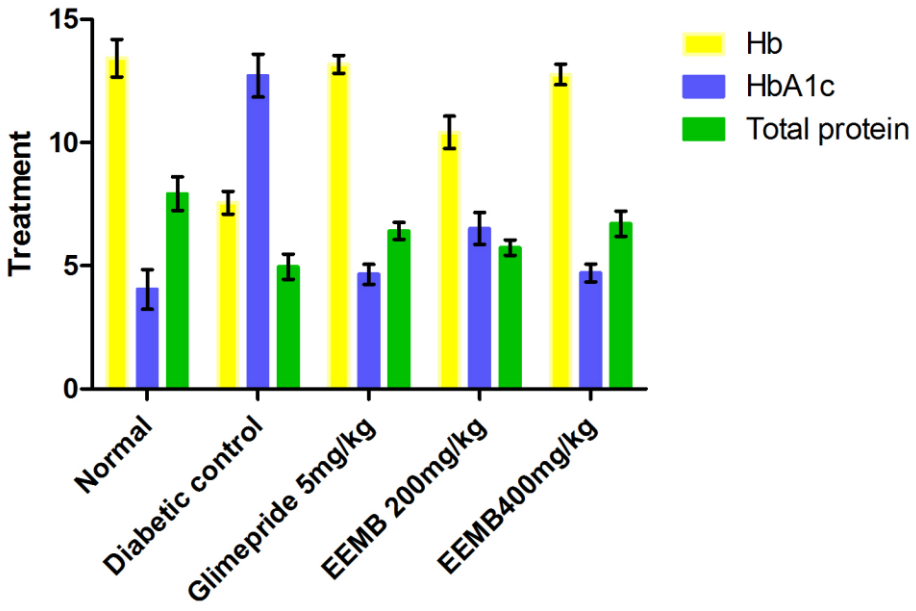
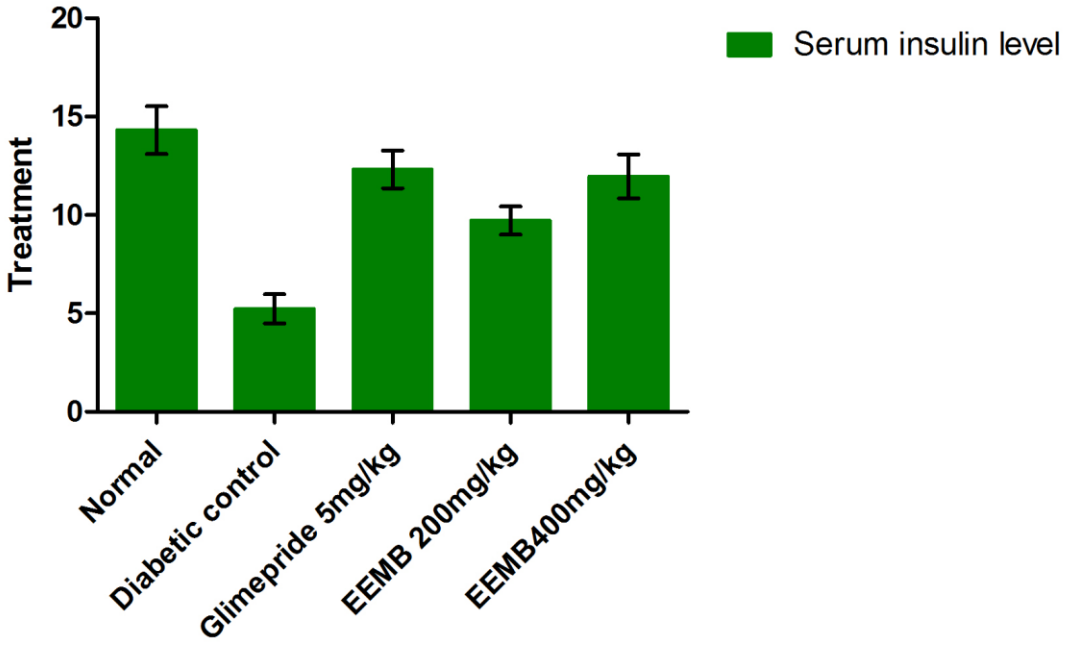


Table 24. Effect of EEMB on Serum Insulin level of Normal and diabetic animals

Design of Treatment	Serum Insulin Level (IU)
Normal	14.31 ± 1.21
Diabetic control	5.23 ± 0.74[a]
Glimepride 5mg/kg	12.32 ± 0.96[c]
EEMB 200	9.72 ± 0.72[b]
EEMB 400	11.96 ± 1.12[c]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001, [b]P<0.05 vs Normal; [c]P<0.001 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.27. Effect of EEMB on Serum Insulin level of Normal and diabetic animals



6.9 Antihyperlipidemic activity of EEMB on Diabetic rats

The ethanol extract of *M. brunoniana* was studied upon serum lipid profile of the diabetes induced animals. A significant raise in LDL, VLDL and triglyceride

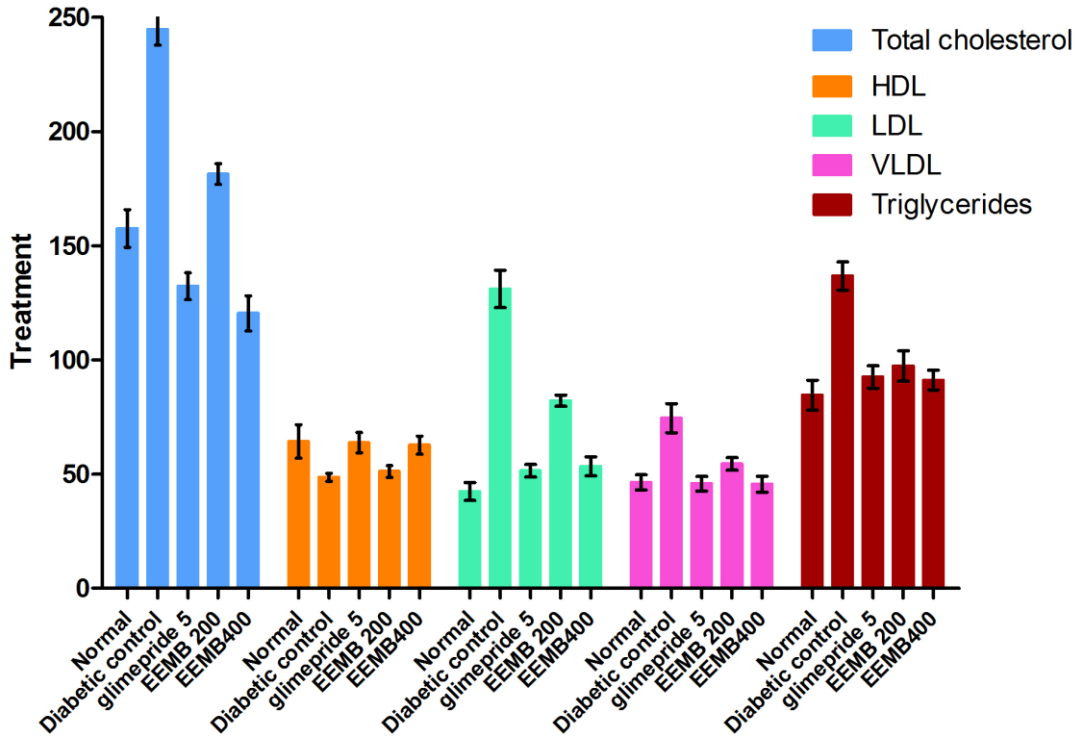
levels was observed in diabetic animals whereas the HDL level was significant reduced. The animals treated with ethanol extract of *M. brunoniana* significantly reduced the elevated LDL, VLDL and triglyceride levels to 82.2 ± 2.4, 54.5 ± 2.7 and 97.4 ± 6.6 at 200mg/kg respectively. Also there was an increase in the HDL level to 51.2 ± 2.55 at 200mg/kg and 62.7 ± 3.9 at 400 mg/kg.The results were comparable with that of the standard, glimepiride used. Among the tested doses, 400 mg/kg exhibit better activity when compared to 200 mg/kg. The results obtained are displayed in **Table 25 and Figure 28.**

Table 25. Effect of EEMB on Serum Lipid level of Normal and diabetic animals

Design of Treatment	Total Cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	VLDL (mg/dL)	Triglycerides (mg/dL)
Normal	157.6 ±12.3	64.3 ± 7.3	42.4 ± 3.9	46.4 ± 3.3	84.6 ± 6.6
Diabetic control	244.7 ± 6.8[a]	48.6 ± 1.8[a]	131.1 ± 8.2[a]	74.5 ± 6.4[a]	136.8 ± 6.2[a]
Glimepride 5mg/kg	132.3 ± 5.84[b]	63.8 ±4.41[b]	51.53 ± 2.75[b]	45.8 ± 3.2[b]	92.6 ± 4.92[b]
EEMB 200	181.4 ± 4.56[b]	51.2 ± 2.55[b]	82.2 ± 2.4[a,b]	54.5 ± 2.7[b]	97.4 ± 6.6[b]
EEMB 400	120.4 ± 7.7[a,b]	62.7 ± 3.9[b]	53.4 ± 4.2[b]	45.6 ± 3.5[b]	91.2 ± 4.3[b]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.001 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.28. Effect of EEMB on Serum Lipid level of Normal and diabetic animals



6.10 *In vivo* antioxidant and anti-lipid per oxidative effects of EEMB on diabetes induced animals

To evaluate the antioxidant and anti-lipid per oxidative potential of the EEMB, the antioxidant enzyme levels such as CAT, SOD, GPx & GST in liver tissue homogenate was estimated. Further, the lipid peroxidation level was also estimated. The results obtained revealed that the lipid peroxidation level was drastically elevated in diabetic animals, whereas, the antioxidant enzyme levels were significantly reduced. The extract treatment significantly declines the elevated lipid peroxidation level to13.74 ± 1.21at 200mg/kg and 8.92 ± 0.53 at 400mg/kg in rat liver homogenate. At the same time, the levels of antioxidant enzyme namely superoxide dismutase, catalase, GPX and GST were found to be increased to 1.32 ± 0.12, 32.81 ± 1.69, 11.69 ± 0.69 and 61.31± 2.56 respectively at 200 mg/kg and 1.57 ± 0.14, 57.72 ± 1.21, 12.74 ± 0.71 and 78.82 ± 2.17 at 400mg/kg respectively

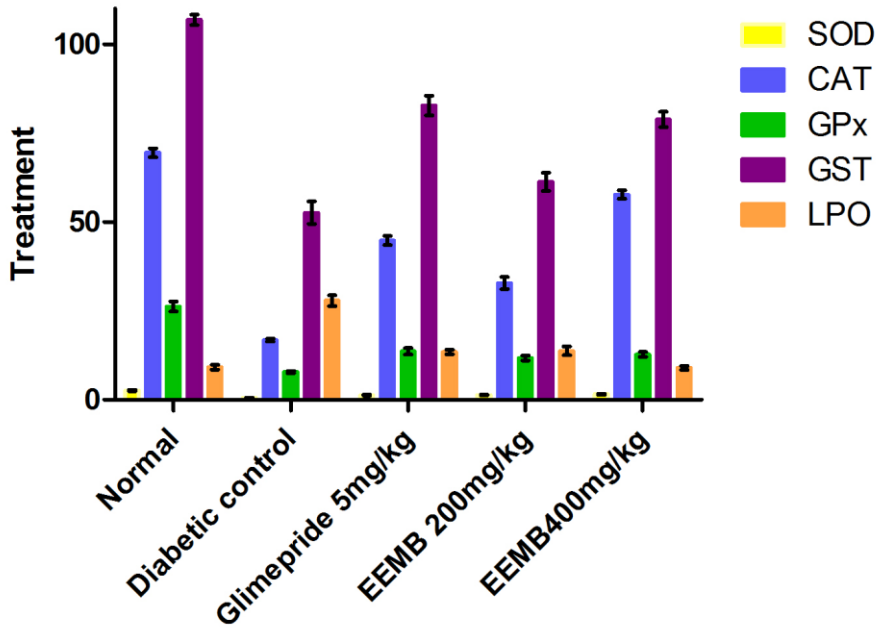
in the extract treated groups. The results obtained indicate that the extract protects the inner organs and restores the antioxidant enzyme system in diabetic rats. Same kind of results also observed in the group treated with standard drug, glimepride, which indicates that the extract is equipotent to the standard drug. The results are displayed **Table 26 and Figure 29**.

Table 26. Effect of EEMB on antioxidant enzyme levels and Lipid peroxidation level

Design of treatment	Antioxidant enzymes				LPO
	SOD	CAT	GPx	GST	
Normal	2.63 ± 0.16	69.52 ± 1.24	26.21 ± 1.45	106.8 ± 1.47	9.2 ± 0.68
Diabetic control	0.56 ± 0.04[a]	16.74 ± 0.41[a]	7.81 ± 0.31[a]	52.6 ± 3.14[a]	27.87 ± 1.51[a]
Glimepride 5mg/kg	1.57 ± 0.13[a,c]	44.81 ± 1.27[a,c]	13.6 ± 0.91[a,c]	82.74 ± 2.77[a,c]	13.42 ± 0.61[c]
EEMB 200 mg/kg	1.32 ± 0.12[a,c]	32.81 ± 1.69[a,c]	11.69 ± 0.69[a,d,e]	61.31 ± 2.56[a,e]	13.74 ±1.21[b,c]
EEMB 400 mg/kg	1.57 ± 0.14[a,c]	57.72 ± 1.21[a,c,e]	12.74 ± 0.71[a,c]	78.82 ± 2.17[a,c]	8.92 ± 0.53[c]

N=6; Data are expressed as Mean ± SEM; [a] p<0.001; [b] p<0.01 vs Normal. [c]p<0.001; [d] P<0.05 vs Diabetic control. [e] p<0.001 vs glimepride. Data were analysed by using Tukey-Kramer multiple comparison test.

Fig. 29. Effect of EEMB on antioxidant enzyme levels and Lipid peroxidation level



6.11 Nephroprotective activity of EEMB

Renal damage and diabetic nephropathy are the common problems associated with diabetes mellitus. Most of the antidiabetic drugs do not protect the renal system from damage. The natural products or plant extracts which are rich in polyphenolics and flavonoids may protect the renal system. Hence an attempt was made to evaluate the renoprotective activity of the ethanol extract of *M. brunoniana* on chronic diabetes induced rats. Body weight of the diabetes induced animals was significantly reduced to 185 ± 8.06 g when compared to the normal animals weight 271.6 ± 6.01 g. The extract treatment significantly increases the body weight to 255 ± 14.32 g & 261.66 ± 10.14 g at 200 mg/kg & 400mg/kg respectively, the results clearly indicates the protective and beneficial effect of extract on diabetic animals. A significant increase in the kidney weight was observed in diabetic control animals. But it was retained to near normal in test group treated with extract. Among all tested dose levels, 400 mg/kg was shown to produce good effects when compared to lower dose level. Mean blood sugar level and Hba1c levels was

increased to 438 ± 17.48mg/dl and 14.18 ± 0.39 % which indicates the uncontrolled sugar level in diabetic control group. But the extract treatment significantly decreased the elevated blood glucose level and HbA1C to 141.26 ± 11.26 mg/dl and 5.88 ± 1.10% respectively which expose the antidiabetic potential of the plant extracts. The results are displayed from **Table 27 to 29 and Figure 30 to 33.**

Table 27. Effect of EEMB on body weight

Design of Treatment	Body weight (g)		Kidney weight (g/100g body weight)
	Initial	Final	
Normal	226.6 ± 8.43	271.6 ± 6.01	0.65 ± 0.037
Diabetic control	223.33 ± 8.03	185 ± 8.06[a]	1.18 ± 0.052[a]
EEMB200mg/kg	225 ± 9.92	255 ± 14.32[b]	0.73 ± 0.09[b]
EEMB400mg/kg	226.6 ± 7.61	261.66 ± 10.14[b]	0.69 ± 0.043[b]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.001 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.30. Effect of EEMB on body weight

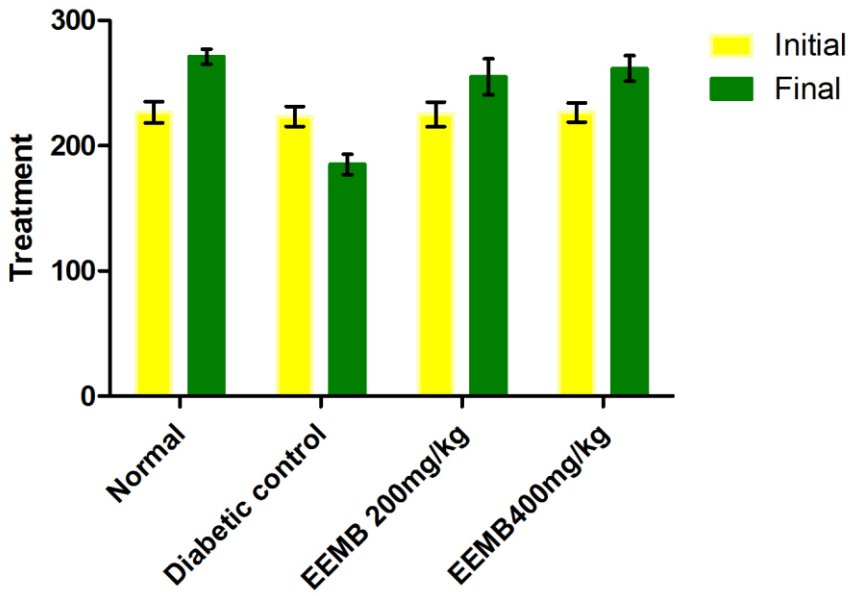


Fig.31. Effect of EEMB on kidney weight

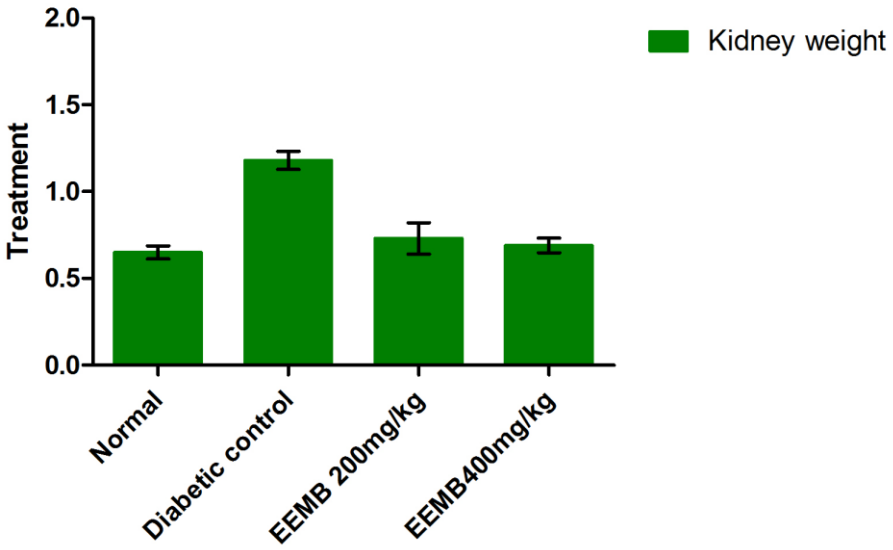


Table 28. Effect of EEMB on blood glucose levels

Design of Treatment	Mean blood glucose levels (mg/dL)	
	Initial	Final
Normal	90.83 ± 5.39	85.3 ± 7.58
Diabetic control	317 ± 29.45	438 ± 17.48[a]
EEMB 200mg/kg	322.5 ±23.51	158.9 ± 12.26[b,d]
EEMB 400 mg/kg	349.33 ± 27.01	141.26 ± 11.26[c,d]

N=6; Data were expressed as Mean ± SEM; [a]P<0.001, [b]P<0.01, [c]P<0.05 vs Normal; [d]P<0.001 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.32. Effect of EEMB on blood glucose levels

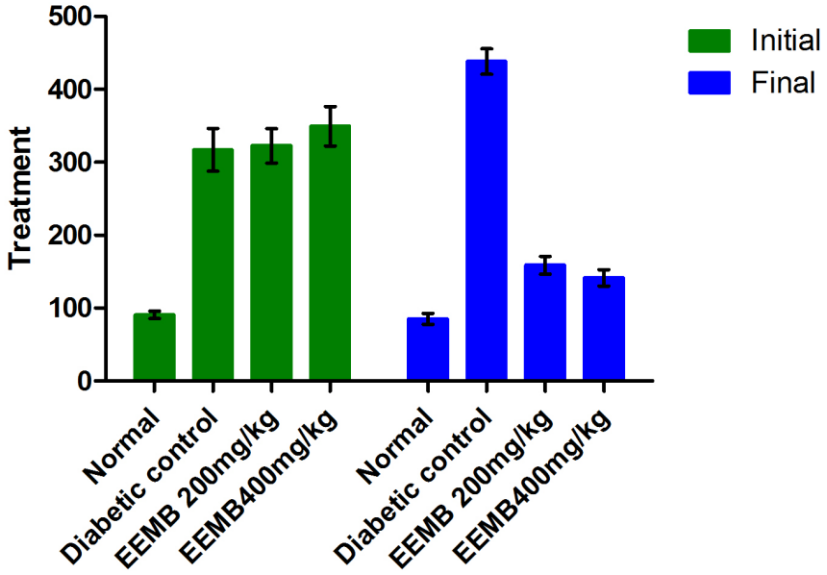
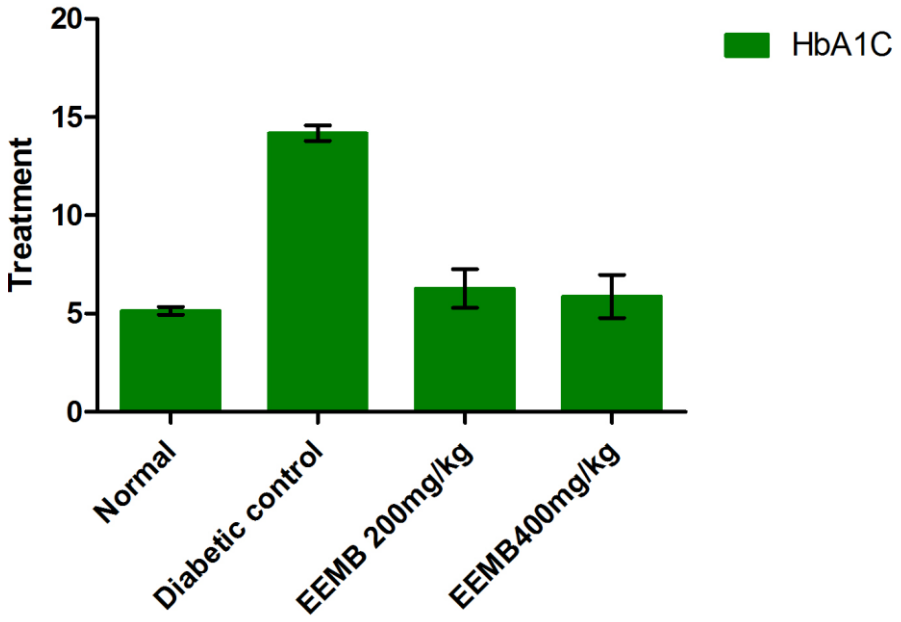


Table 29. Effect of EEMB on Glycosylated hemoglobin (HbA1C) level

Design of Treatment	Glycosylated hemoglobin level (%)
Normal	5.14 ± 0.20
Diabetic control	14.18 ±0.39[a]
EEMB 200 mg/kg	6.27 ± 0.98[b]
EEMB 400 mg/kg	5.88 ± 1.10[b]

N=6; Values are expressed as mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.001 vs diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.33. Effect of EEMB on Glycosylated hemoglobin



6.11.1 Effect of EEMB on Blood Urea, Creatinine Levels and uric acid

To evaluate the nephroprotective effect of EEMB, serum urea, creatinine levels were analysed. A significant rise in the levels of serum urea, creatinine & uric acid was shown to be 63.73 ± 5.15, 0.69 ± 0.058 and 1.62 ± 0.16 respectively in diabetic control animals when compared to normal which clearly indicate the renal damage. But the extract treated group at the tested dose level of 400mg/kg significantly reduced from elevated levels of urea to 41.85 ± 1.31 and creatinine to 0.36 ± 0.023 in the blood of diabetic induced animals. Even the uric acid levels have been reduced to 0.93 ± 0.088 from 1.62 ± 0.16. Thus the results confirmed the renoprotective effect of the plant extract. The results obtained are displayed in **Table 30 and Figure 34 to 35.**

Table 30. Effect of EEMB on blood Creatinine and Urea levels

Design of Treatment	Blood creatinine level (mg/dL)	Blood urea level (mg/dL)	Blood uric acid level (mg/dL)
Normal	0.49 ± 0.02	38.55 ± 1.49	1.02 ± 0.18
Diabetic control	0.69 ± 0.058[a]	63.73 ± 5.15[a]	1.62 ± 0.16[a]
EEMB 200 mg/kg	0.43 ± 0.032[b]	43.16 ± 4.49[b]	1.05 ± 0.099[b]
EEMB 400 mg/kg	0.36 ± 0.023[b]	41.85 ± 1.31[b]	0.93 ± 0.088[b]

N=6; Values are expressed as mean ± SEM; [a]P<0.001vs Normal; [b]P<0.001 vs diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig. 34. Effect of EEMB on blood Creatinine and Uric acid level

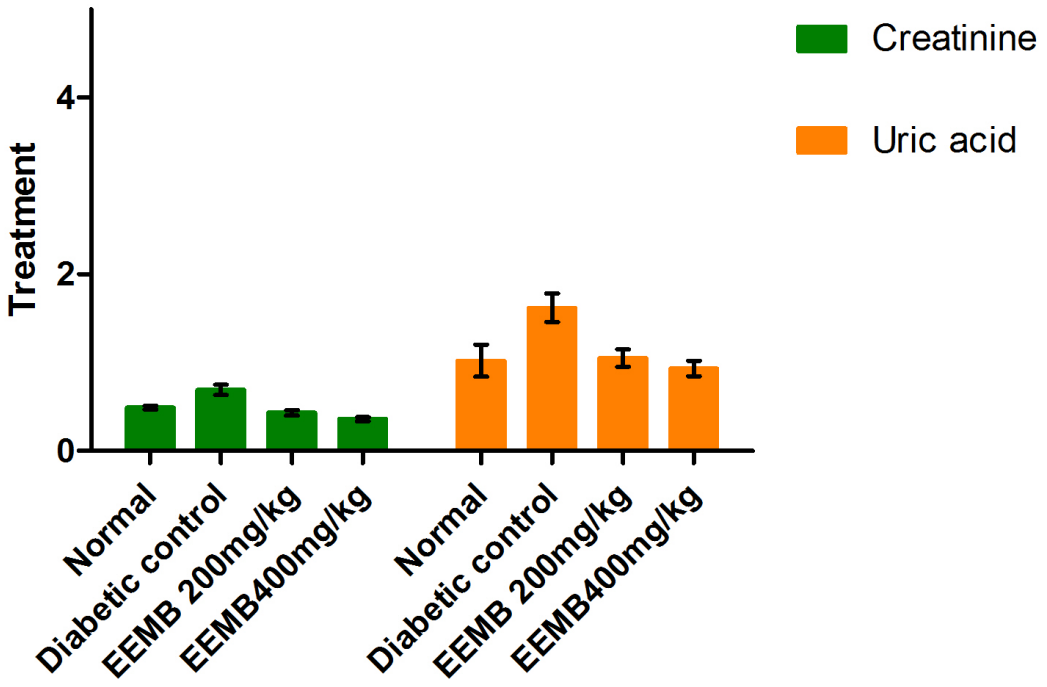
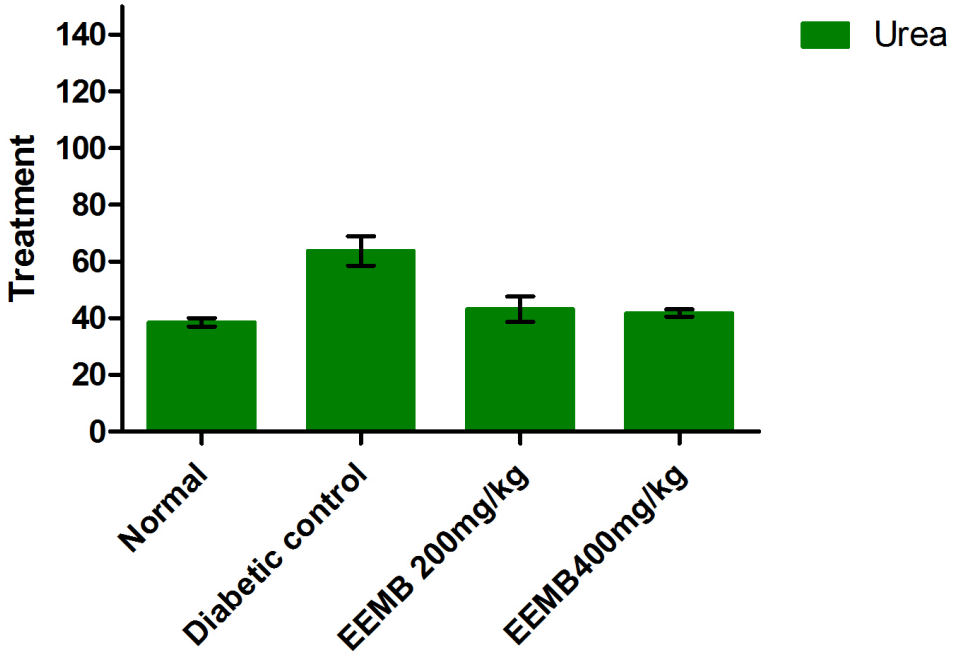


Fig. 35. Effect of EEMB on blood urea



6.11.2 Effect of EEMB on urine Urea, Creatinine, Uric acid and Microalbumin Levels.

To assess the nephroprotective potential of the plant extract, urine of the normal, and diabetic and extract treated animals was collected and it was analysed for urea, creatinine and uric acid levels. Urine output is significantly increased in both diabetic control and extract treated animals from the normal animals. But when correlated with the diabetic control group, the urine output is significantly reduced to 11.5 ± 1.07 and 9.58 ± 0.78 at 200 mg/kg and 400 mg/kg respectively in tested groups. Among tested dose levels, 400 mg/kg shows moderate activity when compared to lower dose.

Further the levels of urea, creatinine and uric acid were evaluated. Renal clearance of creatinine and uric acid significantly reduced in diabetic animals which clearly indicate the renal damage. At the same time the uric acid excretion and creatinine excretion was significantly elevated to 21.61 ± 1.27 and 18.6 ± 1.15 at 400 mg/kg with the EEMB treatment. In case of urea, there was no significant

change at the low dose level, but at high dose, the extract treatment produced moderate activity.

Presence of proteins in urine also indicates the renal damage. To assess the nephroprotective activity, the ethanol extract of *M. brunoniana* was subjected to the urine microalbumin analysis Microalbumin level was significantly increased to 0.8 ± 0.036µg/dl in the urine of diabetic control group animals. But the extract treatment group at 400 mg/kg significantly reduced the excretion of microalbumin to 0.35 ± 0.06 in urine. Among the tested doses, 400 mg/kg significantly reduced the risk of renal damage whereas at 200mg/kg moderately protects the kidneys. The results are displayed from **Table 31 to 35 and Figure 36 to 40.**

Table 31. Effect of EEMB on urine volume

Design of Treatment	Volume of urine (ml)
Normal	4.93 ± 0.28
Diabetic control	19.88 ± 1.24[a]
EEMB 200 mg/kg	11.5 ± 1.07[a,c]
EEMB 400 mg/kg	9.58 ± 0.78[b,c]

N=6; Values are expressed as mean ± SEM; [a]P<0.001, [b]P<0.01 vs Normal; [c]P<0.001 vs diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.36. Effect of EEMB on urine volume

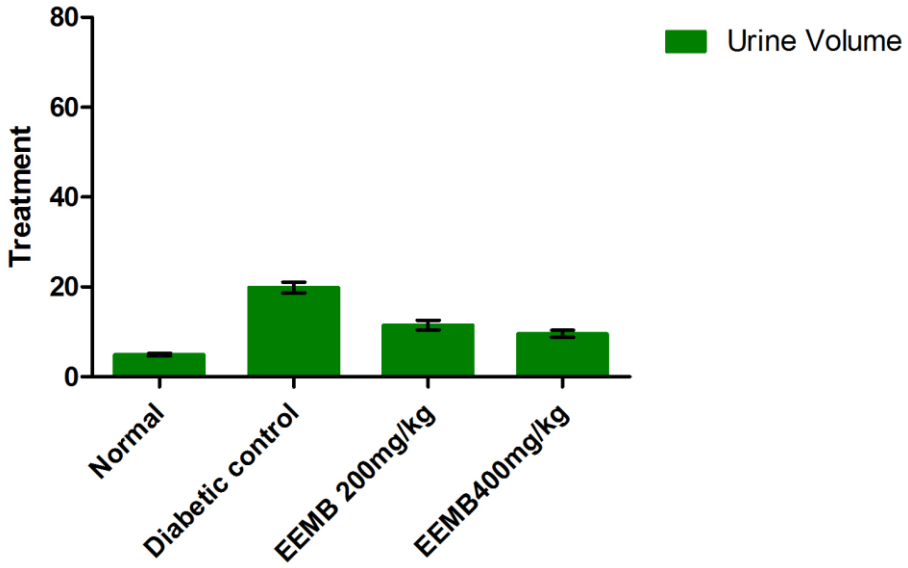


Table 32. Effect of EEMB on urine Creatinine levels

Design of Treatment	Urine creatinine level (mg/dL)
Normal	66.76 ± 2.89
Diabetic control	35.41 ± 1.85[a]
EEMB 200 mg/kg	57.15 ± 1.96[b,c]
EEMB 400 mg/kg	62.56 ± 2.01[c]

N=6; Values are expressed as mean ± SEM; [a]P<0.001, [b]P<0.05 vs Normal control; [c]P<0.001 vs diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig. 37 Effect of EEMB on urine Creatinine levels

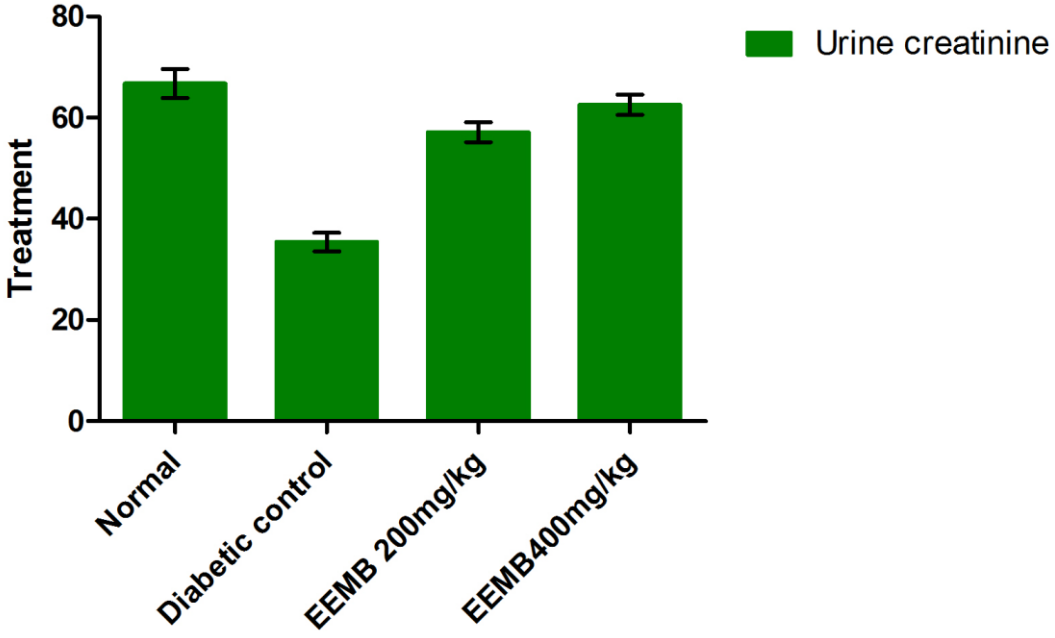


Table 33. Effect of EEMB on urine urea levels

Design of Treatment	Urine urea level (mg/dL)
Normal	83.01 ± 1.58
Diabetic control	97.06 ± 1.17[a]
EEMB 200 mg/kg	90.91 ± 2.88
EEMB 400 mg/kg	86.93 ± 2.13[b]

N=6; Values are expressed as mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.05 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.38. Effect of EEMB on urine urea levels

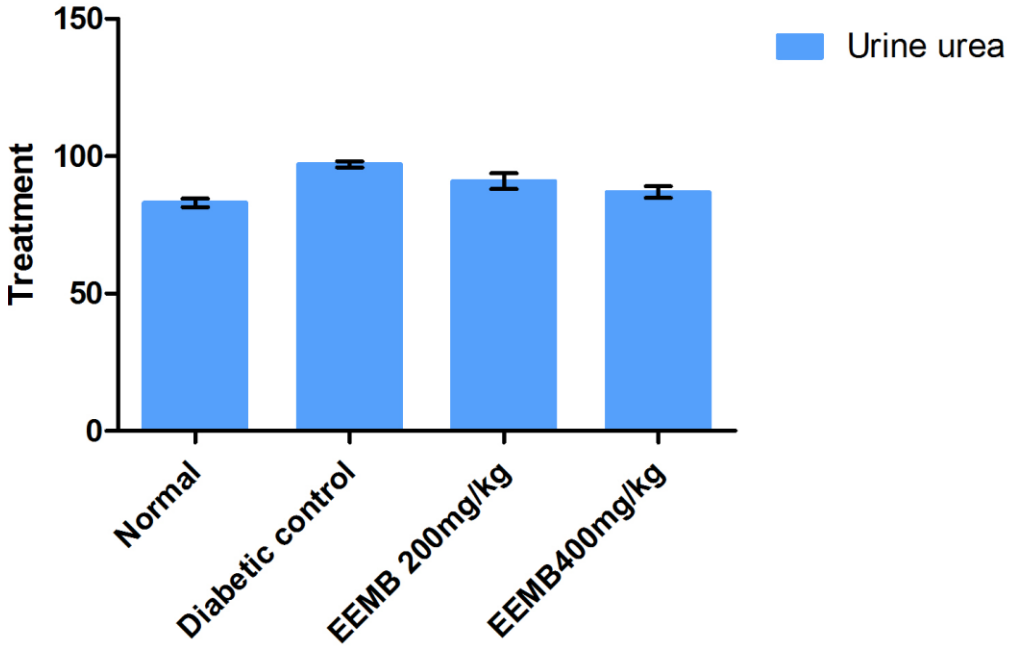


Table 34. Effect of EEMB on urine uric acid levels

Design of Treatment	Urine uric acid level (mg/dL)
Normal	24.33 ± 0.86
Diabetic control	11.73 ± 1.05[a]
EEMB 200 mg/kg	18.6 ± 1.15[b,d]
EEMB 400 mg/kg	21.61 ± 1.27[c]

N=6; Values are expressed as mean ± SEM; [a]P<0.001, [b]P<0.01 vs Normal; [c]P<0.001, [d]P<0.01 vs Diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig 39. Effect of EEMB on urine uric acid levels

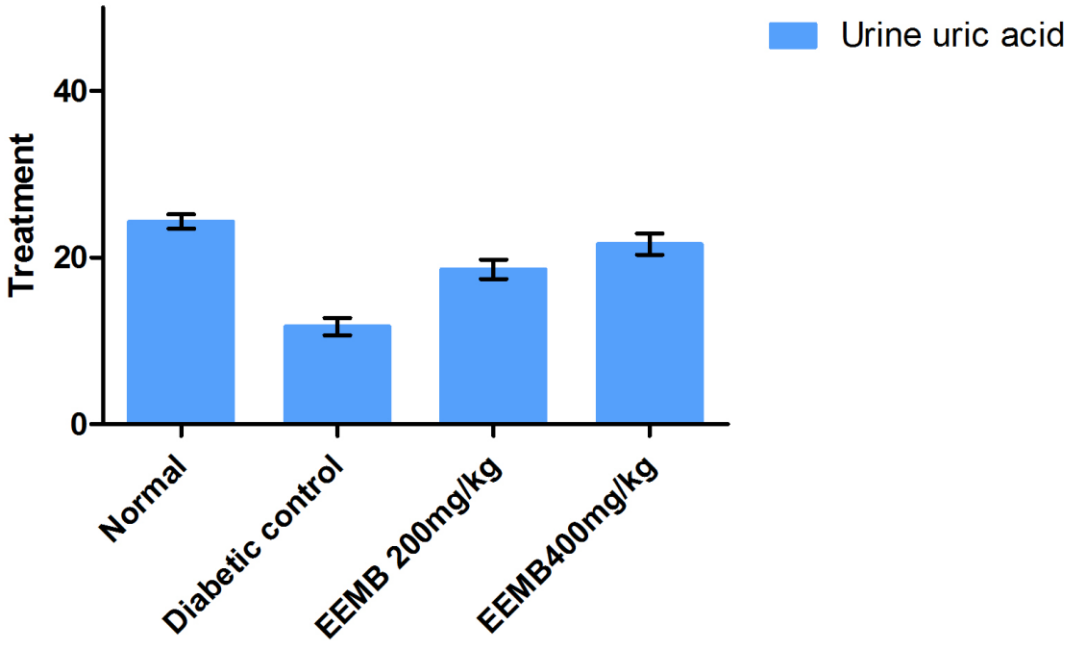
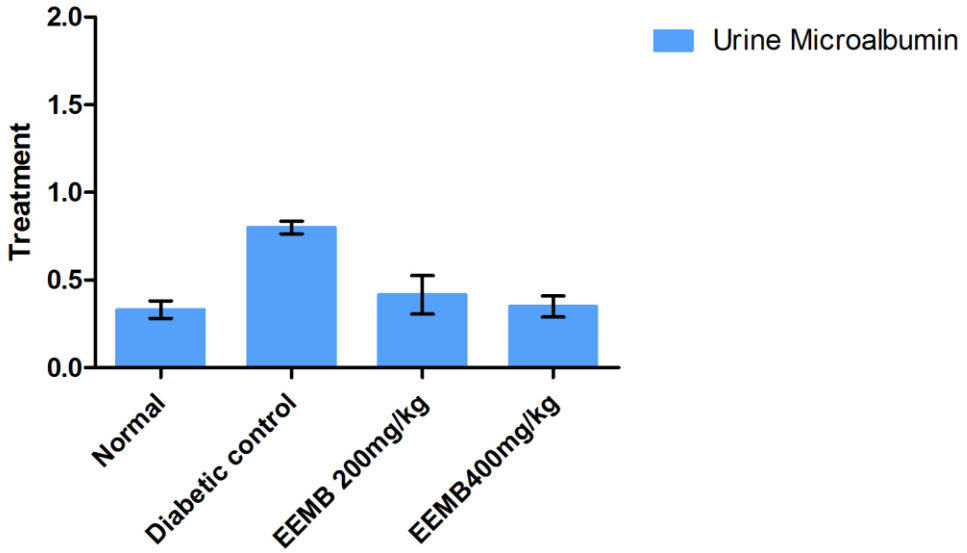


Table 35. Effect of EEMB on urine microalbumin levels

Design of Treatment	Urine microalbumin levels (µg/dL)
Normal	0.33 ± 0.05
Diabetic control	0.8 ± 0.036[a]
EEMB 200 mg/kg	0.416 ± 0.11[c]
EEMB 400 mg/kg	0.35 ± 0.06[b]

N=6; Values are expressed as mean ± SEM; [a]P<0.001 vs Normal; [b]P<0.001, [c]P<0.01 vs diabetic control; Data were analyzed by using One way ANOVA followed by Tukey-Kramer Multiple Comparison Test.

Fig.40. Effect of EEMB on urine microalbumin levels



6.12 Effect of EEMB on Antioxidant Enzyme Levels

In order to assess the nephroprotective effect of ethanol extract of *M. brunoniana*, the antioxidant enzyme levels and lipid peroxidation levels were analysed in the kidney tissues. A 10% kidney tissue homogenate was prepared and it was utilized for the evaluation of antioxidant enzyme levels and lipid peroxidation. Lipid peroxidation, glutathione peroxidase and glutathione-s-transferase levels were found to be significantly increased to 29.54 ± 1.6, 47.95 ± 1.8 and 0.46 ± 0.01respectively in diabetic control animals. Other antioxidant enzyme such as catalase and superoxide dismutase was significantly reduced to 16.9 ± 0.53 and 0.16 ± 0.01 compared with normal animals. After the EEMB treatment there was an significant reduction in the levels of Lipid peroxidation, glutathione peroxidase and glutathione-s-transferase to 12.78 ± 0.67, 20.8 ± 0.76 & 0.28 ± 0.008 at 400mg/kg and at the same time the levels of SOD & CAT was significantly increased to 0.32 ± 0.01 & 25.84 ± 0.59 at 400 mg/kg. The altered parameters were found to be reversed to normal after the treatment and the results clearly represents the restoration of kidney tissues. The results also indicate the ethanol extract of *M.brunoniana* has

nephroprotective activity against streptozotocin-nicotinamide induced diabetic mellitus in animals. The results are represented in the **Table 36 and Figure 41 to 42.** Histopathological observations also supports the results obtained and were displayed in **Figure 43 to 46**.

Table 36. Effect of EEMB on LPO and anti-oxidant enzyme levels - kidney tissue

Treatment	LPO	SOD	CAT	GPx	GST
Normal	7.36 ± 0.52	0.43 ± 0.006	36.54 ± 1.02	15.65 ± 0.38	0.19 ± 0.02
Diabetic control	30.14 ± 1.8[a]	0.18 ± 0.02[a]	17.1 ± 0.54[a]	48.16 ± 1.2[a]	0.42 ± 0.02[a]
EEMB 200	21.21 ± 1.02[a,c]	0.23 ± 0.01[a,c]	22.14 ± 0.81[a,d]	25.32 ± 1.1[a,d]	0.30 ± 0.01[a,c]
EEMB 400	12.84 ± 0.67[b,d]	0.30 ± 0.01[a,c]	24.86 ± 0.56[a,c]	21.4 ± 0.72[b,c]	0.26 ± 0.008[a,c]

N=6; Data are expressed as mean ± SEM; [a]P<0.001; [b]P<0.05 *vs* Normal; [c]P<0.001;[d]P<0.01 *vs* Diabetic control; Data were analyzed by Tukey-Kramer multiple comparison test.LPO, µ moles of MDA/min/mg protein; SOD, units/min/mg protein; CAT, µ mole of H_2O_2 consumed/min/mg protein; GPx, µ moles of GSH oxidized/min/mg protein; GST, µ moles of CDNB conjugation formed/min/mg protein.

Fig.41. Effect of EEMB on SOD & GST Levels

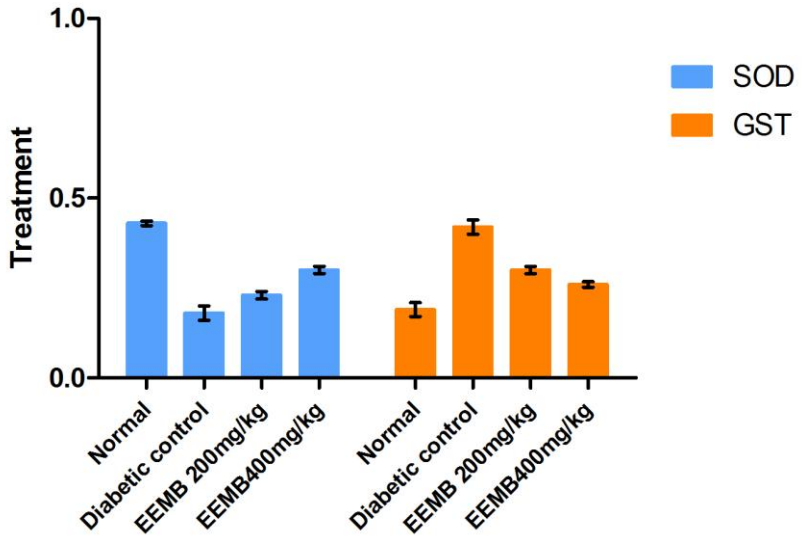
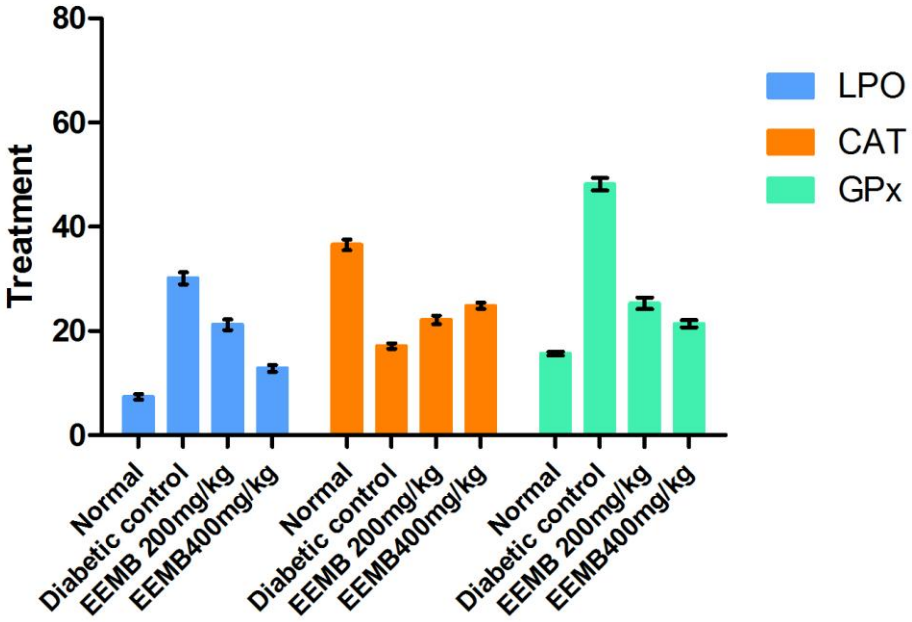


Fig.42. Effect of EEMB on LPO, CAT & GPx Levels



HISTOPATHOLOGY – KIDNEY

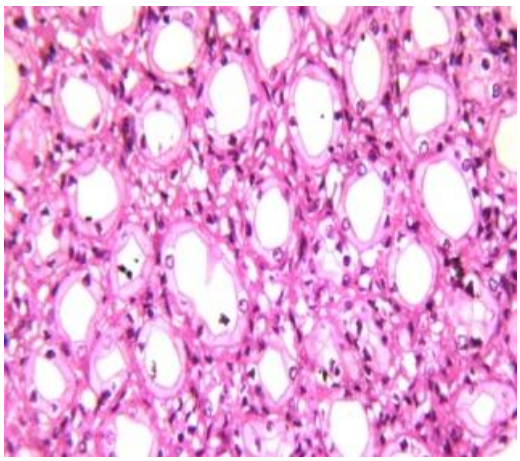


Fig.43. Control group Illustrating normal architectural pattern of glomerulus and tubules (H&E x 400)

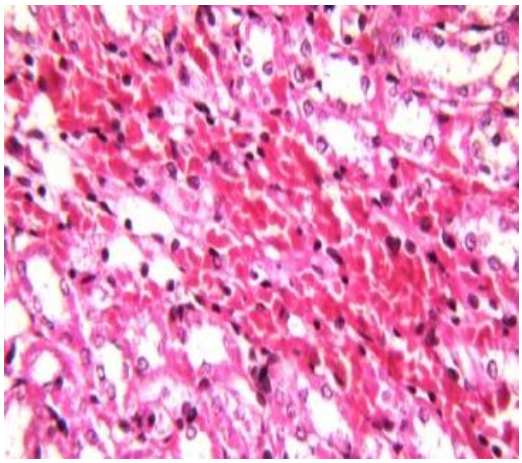


Fig.44. Diabetic control animals Illustrating disrupted tubules, degeneration and necrosis of epithelial cells and intertubular haemorrhage (H&E x 400)

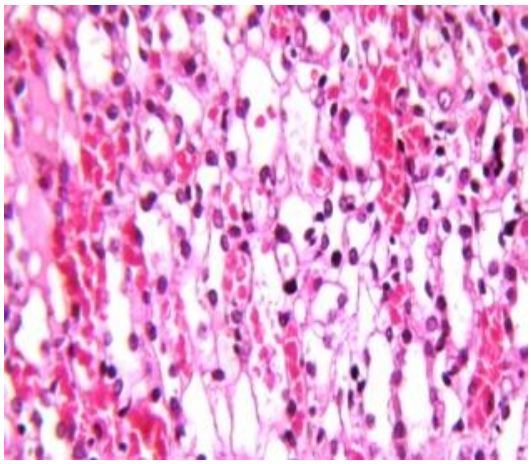


Fig.45. EEMB- 200 treated group Illustrating regenerating tubular epithelium and moderate intertubular haemorrhage (H&E x 400)

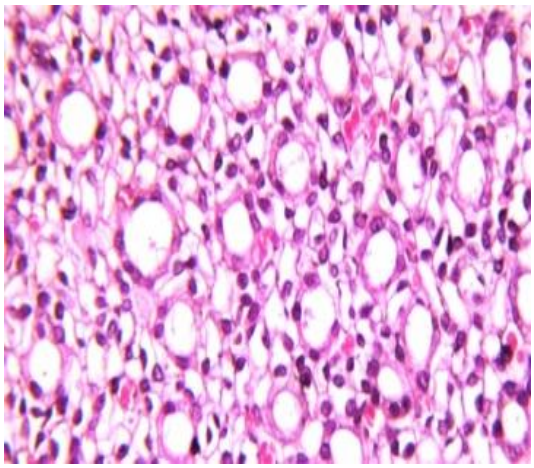


Fig.46. EEMB-400 treated group Illustrating normal tubules with intact epithelium and presence of few RBCs in-between tubules (H&E x 400)

CHAPTER – 7

DISCUSSION

DM is a metabolic disorder characterised by hyperglycemia due to improper secretion in insulin, insulin action or both[1]. Currently, there are 40 million peoples surviving with diabetes in India and estimated to rise about 70 million by 2025[97]. Hyperglycemia is the most significant factor in the initiation and progress of diabetic complications mainly by producing oxidative stress[98]. Altered cellular metabolism is caused by diabetes and it has been suggested that it will play an important role in increasing the risk of cardiovascular, renal, ophthalmic and neurological complications. In diabetes, beside hyperglycemia, an abnormality in the lipid level is also directly correlated with accelerated atherosclerosis and subsequent cardiovascular disorder is the major causes of lethality in the world. It has been found that the chronic elevation in blood sugar is associated with increasing in generation of free radicals and several hypotheses for their genesis have been reported which includes oxidation of glucose, constant increase in the formation of glucose derived products and degradation of glycated protein. Membrane lipid peroxidation and protein oxidation are significantly increased in diabetic condition and it is clearly signifying the increased free radical generation[99]. Augmented free radical levels or inefficient free radical scavenging leads to tissue damage and it is assessed by the measurement of lipid peroxidation level[100]. Moreover, disturbance of antioxidant defense system lead to alteration in enzymatic and non-enzymatic antioxidants like impaired glutathione metabolism[101] that are consider to be the major cause of vascular complications including accelerated form of atherosclerosis due to endothelial dysfunction and microangiopathy of retinal vessels[102]. It has been found that in the developing countries where the resources are insufficient, the best source for the management of diabetes is phytomedicine[103]. Natural products, like extract of various plants, prepared as pure compounds or as standardized preparation, provide extended opportunities for new antihyperglycemic, antihyperlipidemic drug together with antioxidant property because of the unmatched availability of chemical diversity[104]. The contributed antihyperglycemic activity of these plants are because of their ability to retaine the

function of cells of pancreas by causing an elevation in insulin release or reduced intestinal absorption of glucose[56]. Apart from that, hyperglycemia induced oxidative complications are treated and managed by supportive therapies that include the use of antioxidants and natural products. The present study explores the antihyperglycemic, hypolipidemic, antioxidant and nephroprotective potential of leaf extract of *M. brunoniana* .

The main aim of the extraction is to isolate the major biologically active compounds from the plant. Normally few milligrams of the substance which is isolated will be used for the structure elucidation or for the various animal experiments.

The present study utilized the shade dried leaves of *Mardenia brunoniana* and extracted with various solvents with respect to its increasing order of polarity. Preliminary phytochemical analysis revealed that the plant extracts of *M. brunoniana* contains a variety of phytochemical compounds like phytosterols, glycosides, polyphenolics, flavonoids and tannins. Terpenoids, alkaloids and mucilages are not present in all the solvent extracts. All the obtained compounds show antidiabetic, antioxidant, antihyperlipidemic and anticancer activities.

In the biological systems reactive oxygen species and reactive nitrogen species play an important and dual acion .It can be helpful and also harmful in someways.[106] Reactive oxygen species involve in the physiological roles in the cellular duties for instance it fight against the agents which cause infection and involves in the signaling systems of the cells. Other important function of the ROS is it induces the mitogenic responses at low concentration. At high concentrations it act as a mediator in the cell structure damage including its membranes of lipid & proteins and nucleic acids coined as oxidative stress.[107] Oxidative stress is a major cause for many of the diseases like inflammation, diabetes, neurodegenerative disorders and aging processes.

The oxidative processes are delayed by the antioxidants and helps in managing the oxidative stress and restore the normal activities.[108] Most of the antioxidant enzymes prevents or balances the harmful effects of ROS. Eventhough

the cells antioxidants fight against the oxidative damages caused by ROS, sometimes it react within the cell and damage the cellular components such as proteins, lipids and DNA. These damages develop many age dependent diseases namely, arteriosclerosis, arthritis, neurodegenerative disorders cancer etc.[105]

Considering the importance of antioxidants the free radical scavenging capacity of various solvent extracts of *Marsdenia burnoniana* was evaluated using *in vitro* models. Free radicals are made with diverse chemical structures so it is made compulsory to test the plant extracts to ensure its antioxidant capacity.

ABTS method is widely used due to its capability in both organic and aqueous solvents and more over its pH stability nature. For estimation of antioxidant activities this method is most acceptable even in the mixtures such as food products, plant extractions and biological solutions.[66] the ethyl acetate and ethanol extracts of *Marsdenia brunoniana* showed potent antioxidant activity and the results were compared with standards ascorbic acid and rutin. The presence of phenolic compounds in the extracts may play a role in the free radical scavenging activity.

The widely used other method is the DPPH method due to is stable free radical nature. It is involved in the estimation of free radical scavenging acitivity of newer antioxidants.[109] It turns to a a stable diamagnetic molecule by accepting the electron radical or hydrogen radical.[110] The antioxidant capability of the substance is evaluated by the reduction in the capacity of the DPPH radical which is measured at absorbance 517nm. From the obtained results it is notified that the ethanolic extract of MB have free radical scavenging property. Most of the deadly chronic diseases like cancer, cardiovascular diseases and diabetes are evolved because of the release of free radicals.[111] the ability of the extract in scavenging free radicals is dose dependent. The substance like glutathione, cysteine, ascorbic acid, flavonoids, and tocopherols has the property of reducing and decolorizing the radical DPPH by donating hydrogen. The ethanolic leaf extract of *M. brunoniana* have radical scavenging property probably by the presence of poyphenolics and flavanoids.

Superoxide radical is considered to a highly reactive species since it activates and promotes large number of reactive substances.[105] it is formed inside the body and by dismutation reaction it produces hydrogen peroxide. These hydrogen peroxide and superoxide together converted in to reactive substances. The scavenging property of the extract was found to be highly active in the DMSO system. When compare with the other extracts of the plant the ethylacetate and ethanol extract shows good activity on scavenging superoxide radicals.

Elimination of the hydrogen peroxide is regarded as an important reaction in the antioxidant defence mechanism in the systems of cells. Hydrogen peroxide is not so reactive but it produce hydroxyl radical inside the cell at sometimes.[112] The polyphenols and orthohydroxy phenolic compounds such as quercetin, gallic acid, caffeic acid and catechin protects the cells of mammals from the damage due to hydrogen peroxide.[113]In this concern our extract may act as a potent scavenger of hydrogen peroxide since it also possess phenolic compounds.

Hydroxy radical is the most powerful reactive species and causes damage to the biomolecules.[114] The Scavenging capacity of leaf extracts of *M. brunoniana* was analysed by its inhibition capacity of p-Nitroso dimethyl aniline bleaching method. Hydroxyl radical is produced by Fenton reaction and in this reaction hydrogen peroxide react with the iron-EDTA complex to form hydroxyl radical in presence of ascorbic acid which bleaches p-NDA. The scavenging activity of the leaf extracts is by restricting the discoloration of p-NDA. The various phytoconstituents like polyphenols and flavanoids in the extract might be responsible for the scavenging action.

In the reducing ability assays the measurement accounts from the transformation of Fe^{3+} - Fe^{2+}. The reduction of Fe^{3+} is used as a denoter of electron donating activity which is counted as a most efficient pathway of phenolic antioxidants. The same mechanism would be compared with other antioxidant activities.[115] The presence of reductones in the plant extracts are responsible for the reducing properties which act by donating the hydrogen atom lead to break up of free radical chain results in the antioxidant action.[116] The results observed from the

present work denotes the potent antioxidant activity of the extract.Various mechanism may be involved in the antioxidants such as management of chain formation, transition metal ion catalyst binding, hydrogen abstraction prevention radical scavenging property and also peroxide decomposition. As like the activity of antioxidants the extracts reducing ability also rises with the increase in concentration.[117]

From the literatures related to our study reveal that the content of phenolic substances in the plants is the important cause for the antioxidant nature or its free radical scavenging property. Hence it is evident to screen the total phenol and flavanoid content in the plant taken for the research. The natural phenolic compounds like flavonoids are essential substances and it possess diverse chemical and biological activities adjoining with scavenging property. The results show that the extract contains enormous phenol and flavonoid substances. The earlier literatures proved that the antioxidant activity rises with the quantity of phenol and flavonoids and has a linear relationship within them and its antioxidant property.[118]

The plant which is categorized as edible and medicinal plants have contains phenolic compounds and various journals have stated that it possesses diverse biological activities which add its antioxidant capacity. The antioxidant activity of the phenolic compound is due to its redox activities which act by free radicals neutralization, quenching of singlet and triplet oxygen or peroxide decomposition.[119] the ethanolic extract of *M. brunoniana* has potent antioxidant activity among the various extracts. The activity may be due to the phenolic compounds of the extract. The compounds which is having the free radical scavenging property will delay or prevent the biomolecule oxidation and reduce the oxidative stress.[120-121]

The antioxidant and radical scavenging potential of *Marsdenia brunoniana* was reported for the first time up to our knowledge.

A major aim in the treatment of DM is to regulate the blood glucose levels to normal level in the fasting rate as well in the postprandial state[122] The one therapeutic aim to reduce postprandial blood sugar is to diminish the synthesize or absorption of glucose from the GIT through the restriction of *alpha-amylase* or

alpha-glucosidase enzymes[123-126].*α-amylase* catalyzes polysaccharides into various oligosaccharides and disaccharides. Disaccharides produced by *α-amylase* are hydrolyzed further by *α-glucosidases* to produce glucose and other monosaccharides, which are readily absorbed in the small intestines[127]. Preclinical and clinical studies have shown that inhibitors of both *α-amylase* and *α-glucosidase* can diminish the synthesize and retention of glucose in small intestine[128]. Further, some inhibitors of *α–amylase* and *α–glucosidase* such as phaseolamin, acarbose and vogiblose are currently used to suppress postprandial glucose levels in diabetic patients[123]. Plants are well known producers of variety of *glucosidase* inhibitors which provide protection and fight against insects and microbial organisms[129-130]. Therefore, the prepared plant extracts are subjected for *α-amylase* and *α-glucosidase* inhibitory properties.

In type-II diabetes mellitus, large amount of glycogenolysis and gluconeogenesis in the hepatic system is linked with reduced utilization of glucose by tissues is the fundamental mechanism underlying hyperglycemia[131].The inhibition of *α-1, 6-glucosidase* is done by the glucosidase inhibitors in the liver and also diminish the glycogen stores.[132-133.] Inhibition of alpha glucosidase and alpha amylase enzymes reduces the levels of glucose in the blood of diabetic patients and also show profound decrease in the glycosylated hemoglobin. On the basis of the mechanism mentioned in the earlier studies, it was hypothesized in the current study that various solvent extracts of leaves of *M. brunoniana* may also have the similar inhibitory nature against either *α-amylase* or *α-glucosidase* or both *in vitro*

The ethanolic extract of *M. brunoniana* shows strong *in- vitro α-amylase* inhibitory activity and reveals the significant antidiabetic action in *in vitro* studies apart from other extracts. The binding site of carbohydrate on α-1, 4 glucosidic linkage in starch and related polysachharides are may be the binding site of the extract may probably the underlying mechanism for the prevention postprandial diabetes. *α-amylase* is the enzyme responsible for the break down of starch in to maltose and further to glucose before absorption. *α-amylase* play a major role in starch breakdown in humans and animals, such action of these inhibitors in the food

supplements involves in the impaired digestion of starch.[134-135] Thus inhibitors of alpha amylase have become a novel agents in the antidiabetic medication.

In the epithelial cells of small intestine, the acarbose restricts the enzyme alpha glucosidase, have been proposed to reduce the postprandial blood glucose level and it promotes the metabolism of glucose without altering the secretion of insulin. But acarbose does not inhibit alpha amylase on the same assay conditions. From the earlier results it is stated that the acarbose either have less potent inhibitory action or nil inhibitory action over *α-amylase*[136-137] . Among the solvent extracts, ethanolic extract of *M. brunoniana* was shown better inhibitory action over alpha *amylase* than the reference drug, an indication that the ethanol extract may be more beneficial in the prevention of postprandial hyperglycemia than acarbose[123-124].

The reference drug acarbose inhibit *α-glucosidase* present in the epithelium of small intestine, have been demonstrated to decrease PPDM and improve impaired glucose metabolism without promoting insulin secretion. But acarbose was not a potent inhibitor of *α-amylase* under the current study assay conditions. This is consistent with other reports that either described a very weak inhibitory activity of acarbose or no inhibition of *α-amylase.*[136-137]

Phytochemical investigation of the plant extracts revealed the presence of phenols, flavonoids and tannins. Considering the earlier reports of the studies related to the *α-glucosidase* inhibitory potential of the plant extracts stating its action might due to the presence of flavanoids, polyphenols and also their glycoside constituents, [138-140]it is rational to suggest the mechanism for the present study on EEMB and can be proclaimed that the action may be due to the activity of flavonoids and phenolic compounds.

We, for the first time, have explored the extracts of *M. brunoniana* as potent anti-hyperglycemic agents and denoted the targets of the plant extracts as alpha glucosidase and alpha amylase which are considered to be the main cause behind the condition of diabetes. Future studies on the plant may promote it as herbal medication in controlling and preventing type 2 DM.

Based on the extractive yield, preliminary phytochemical analysis, *in vitro* anti-oxidant and anti-diabetic activity, ethanol extract of *M. brunoniana* (EEMB) was selected for further studies.

Many in vitro animal studies revealed that the emerge of free radicals in the cells can definitely cause damage to the DNA molecules and lead to various major defects in mutagenesis, carcinogenesis, diabetes .[141-142] Thus, on the basis of the *in vitro* antioxidant studies, it has been concluded that the EEMB have potent antioxidant activity when compared to other solvent extracts.To confirm further an attempt was made to secure DNA from damage induced by free radical.The single and double strand break was resulted due to the formation of hydroxyl radical when the plasmid DNA gets damaged. Many reports supported that the plant extracts have potent activity in defending against the damage of DNA.[142] The results of our study clearly indicates that the damage of DNA caused by hydroxyl radical was highly suppressed by EEMB and the results were compared with standard Mannitol. The mechanism involved in the above reaction was probably due to the antioxidant potency of the EEMB, which might have guarded the Fe2+ ions & H_2O_2 reaction or by giving up of hydrogen atom and arresting ROS. The results have become evident in proving the antioxidant capacity of the extract and also extend the use of the extract in various ROS induced diseases especially in the case of diabetes.

Postprandial blood glucose level is known to be regulated by glucose uptake, a rate limiting step for glucose metabolism. In the present work we have utilized the differentiated 3T3-L1 cell lines where the glucose uptake takes place at high rate than the undifferentiated cell, the reason may be the expression of GLUT4.[143]For the first time, and our results of EEMB have indicated that it upgrades the glucose uptakes in to the tissue. Metformin and insulin were used as markers, since these two substances translocate the GLUT4 to the surface of the cell and supports actively the uptake of glucose. The basic mechanism involved in this may be postulated that it could be passed to insulin transmitted glucose transporter pathway where many proteins like PPAR, PPI3 and proein kinase c take part in it.[143-144]Following this GLUT4 translocation takes place to the membrane of plasma to promote the uptake of glucose from blood stream to cells.

Thus, the occurrence of polyphenolic compounds in ethanol extract may be the underlying cause in promoting the signaling proteins and regulation of these proteins, which are authoritative for its glucose uptake activity. Further, the ethanol extract of *M. brunoniana* was subjected to 3T3-L1 preadipocytes cell line assay and it was shown that EEMB was non-cytotoxic at different levels of concentrations.

The bioactive compounds responsible for the mentioned actions of EEMB, quantitative phytochemical analysis was performed and the results revealed the presence of considerable amount of glycosides, phenolics, flavonoids, tannins and ascorbic acid. Many of these compounds possess free radical scavenging activity, thus the anti-oxidant and anti-diabetic activity of the extract may be due to these kinds of compounds. To detect the phytochemicals present in EEMB, GC-MS analysis was performed. It was found that the ethanol extract of *M. brunoniana* contains many phytochemical constituents namely methyl-α-D-Glucopyranoside, 4-C-methyl-Myo-Inositol, n-hexadecanoic acid and phytol which contribute to the medicinal activity of the extract. HPLC and HPTLC technique results of the extract also revealed the existence of many flavonoidal and phenolic compounds.

In the toxicity studies, animals treated with EEMB did not exhibit any symptoms of toxicity or death when treated with the maximum dose of 2000 mg/kg orally. This shows that the extract was proven to be safe at the tested levels. So the dose level of 200 mg/kg and 400 mg/kg was taken for the *in -vivo* studies.

The long term safety level of a compound can be predicted from acute or even shorter than sub acute studies[145-146].

Acutely nontoxic compounds may be found toxic when it is exposed for a prolonged period even at low doses due to accumulation, enzyme level alterations and irregularities in the physiological& biochemical homeostasis.

The 28 days study period results showed that there was no changes in the extract treated animals including its weight and other parameter such as red blood cells, white blood cell, counts of ganulocytes and hemoglobin content. Even there were no observed changes in the various serum tests like AST, ALT, ALP and

protein content. The cholesterol level and the bilirubin levels in the extract treated animal group are also normal. The result of urine analysis was not having any change from normal pattern.

The above resuls are evident to prove that EEMB treatment is effective and possesess nutritional value on the weight of vital organs with no adverse effects on the systems of liver and kidney. There was no lethality observed on the treatment of EEMB even at maximum dose levels and denotes its harmless nature.

Biotransformation of drug takes place mostly in the liver and kidney cells. So it is essential to involve the liver and kidney tissues of the treated animals for the histologoical studies. The impacts of the study imply no hepatic fat infilteration condition and no degeneration. There were no defects like portal tract inflammations and accumulated central veins. The kidney of the extract treated animals is found regular glomerular cells with usual tubular cells when correlated with the normal group of animals. The gross examination results showed no malformation in the kidney and liver cells. It is clear from these results that EEMB is free from hepatic and renal toxicity.

DM is a major metabolic disorder ends in hyperglycemia. Hyperglycemia may be attributed to defects in pancreatic β- cells, insulin secretion, hepatic glucose output, glucose uptake of peripheral tissues and immune function[147]. Liver is the mainly concerned organ in regulating normal levels of glucose in the blood by restoring glucose in the form of glycogen and synthesize the glucose molecules from the glycogen.[148] The STZ-nicotinamide induce DM model is an commonly used one and resemble as human hyperglcemic nonketonic diabetes mellitus .[149]

For the chronic diabetic studies STZ-nicotinamide model is more appropriate method since it is producing similar hyperglycemic conditions as humans.Beta cells are relatively defended by nicotinamide from the streptozotocin influenced cell toxicity. The mechanism behind the nicotinamide action is protecting the Nicotinamide Adenine Dinucleotide by substituting as a prosubstance of Nicotinamide Adenine Dinucleotide or suppressing the action of poly Adenosine di

phosphate –ribose synthetase which is an NAD consuming enzyme activated by streptozotocin[150].

Plant derived substances such as flavonoids and phenolic compounds suppress the hyperglycemic condition by regulating the lipid peroxidation level and decreases the utilization of internally secreted insulin in DM patients.[151] In the current study, the in vivo antidiabetic study have been performed using STZ-Nicotinamide induced diabetic model.

When compared with the initial weight of the body of each rats there was an extremely difference with weight gain. It exhibits the nutritive content of the plant *Marsdenia brunoniana*. As usual there was a reduction in the weight of the diabetic animals from its earlier weight. . In EEMB treated diabetic groups at tested dose levels, there was significant increase in body weight when compared with initial body weight. Elevated muscle wasting in diabetes is the main cause for the reduction in the body weight of STZ-Nicotinamide administered group.[152] The improved body weight of the treated animals may be because of the prevention of muscle wasting that is reversing the process of gluconeogenesis.

The fasting blood glucose level of the diabetic control group was not declined remarkably from the initial period even after 30 days of study time. But the blood glucose level of the extract treatment notably declined the initial blood glucose level. This is indicating that ethanol extract of *M. brunoniana* have antihyperglycemic activity. The effect of *M.brunoniana* as antidiabetic may probably by its action over the rise in secretion of insulin suggested as insulinogenic effect from the available beta cells results in enhanced plasma insulin level. The possible mechanism underlying this may be the secretion of insulin by shunting the channels of K^+-ATP, depolarization of membranes and improvement in the influx of ca^+ ions, considered as basic process in the insulin secretion. Further the antidiabetic activity may be because of the regeneration of beta cells and reverting the pancreas normal histoarchitecture in an appreciable manner[49, 153]

The significant elevation of triglycerides was observed in the diabetic control group when compared with the normal. At the same time it is observed that the

EEMB treated diabetic group shown significant reduction in the levels of triglyceride with that of the normal control group.

The glycosylated hemoglobin levels are also compared between the normal control group and diabetic group in which diabetic group have elevated glycosylated hemoglobin levels. After the extract treatment it reveals that the diabetic condition is suppressed to a notable level. Glycosylated hemoglobin acts as a marker in the degree of control of diabetes since it is involved in the development and progression of various vital organ disfunctions in the diabetic patient. The reaction is nonenzymatic and involves condensation of glucose with the N-terminal aminoacids of hemoglobin molecules.[154] Glycosylated hemoglobin tests are much supportive in controlling and monitoring the blood glucose level as well to prevent the microvascular and macrovascular ailments in the diabetic patients.[155]

In the present study, the hemoglobin level of diabetic control group showed significant change when compared to normal control group. EEMB treated groups produced significant change when compared to diabetic control group which show the protective effect of the extract on the hemopoietic system. The serum insulin levels of diabetic animals were found to be less when compared with the normal. But the extract dosed animals exhibited significant increase in the serum insulin level indicates the insulin secretogogue effect of the extract.

LPO is the cause for the cell injuries in animals as well as plants and act as a marker of oxidative stress in the cells.It is more unstable and forms many complex substances like carbonyl compound. When it undergoes decomposition it releases MDAand hydroxyalkenals. Estimation of above compounds indicate LPO.[156]

The present study shows that the LPO levels have significantly reduced in the extract treated animals than the diabetic control animals.Our results also correlated with the results of previous researchers, revealed that lipid peroxide levels have been significantly increased in diabetic control group than the normal control group[157-158].

The barrier function of the lipid membrane and its fluidity was affected by lipid peroxidation. The protein synthesis, blood macrophage action was altered by the products of lipid peroxides such as hydrogen peroxides and their aldehyde derivatives. These derivatives also alter the chemotactic signals and enzymatic activities.

In the present study, lipid peroxides were measured by using TBARS assay.LPO and oxidative stress both are interrelated. Oxidative stress is also one of the mediators for the cause of DM. It is also stated that the release of oxygen radicals might occur in STZ administration and leads to liver damage, which involves in the rise in blood sugar in animals.[59] in our study, EEMB have a strong anti-oxidant activity and lower the lipid peroxide levels in liver. This may be the possible mechanism of action of the plant extract for its anti-diabetic activity. The plant extract also significantly increases the antioxidant enzyme levels in the diabetic animals.

Diabetic nephropathy is one of the major microvascular complications in DM and is the only reason of last-stage kidney dysfunction worldwide[159].It is considered as a important ailment causes predeaths in diabetic patients adjoined with cardiovascular complaints and renal dysfunction.[160] STZ administration along with nicotinamide reported to resemble human DM.[149] Administration of STZ (60 mg/kg) to rats for 4-8 weeks leads to nephropathic condition. Streptozotocin damages the DNA of the pancreatic-β cells and triggers multiple pathways, including activation of protein kinase-C, poly (ADP-ribose) polymerase and nicotinamide adenine dinucleotide phosphate-oxidase, with consequent generation of reactive oxygen species (ROS) and advanced glycation end products resulting in renal damage and nephropathy[95].

The present study results show that the kidney weight increased in the diabetic control than the normal control group. The extract treated group were significantly reduced in the kidney weight than the other groups.The weight of the kidney signifies the kidney enlargement which is the important feature exhibited during the early stage changes of DM. Initial stages of nehropathy along with diabetes turns the kidney size and its glomerular filtration rate in experimental

animals as well as in diabetic patients.[161] The degree of renal enlargement is directly propotional to glycemic level and it explores the importance of glycemic control in the management of diabetes.

In the diabetic control group, the fasting blood glucose level significantly increased after the 60 days treatment when compared with its initial blood glucose level. The EEMB treated groups significantly reduces the glucose level and HbA1c level when compared to initial levels. This is indicating that the plant extract has anti-hyperglycemic activity. The percentage reduction in the HbA1c levels reduces the microvascular complications, myocardial infarctions and even deaths due to diabetes.[162]

Creatinine is produced inside the body and delivered into body fluids. Its clearance indicates the glomerular filtration rate.In hyperglycemic condition the creatinine level in the serum found to be increased and leads to osmotic diuresis and draining of extracellular fluid.[163-164] Creatinine levels in the diabetic control group was found to be higher significantly when compared with normal group.In EEMB treated animals the creatinine level and urea level was significantly decreased at both dose levels.

The increase in the production or reduction in elimination of uric acid is termed as hyperuricemia.[165] here, the study shows that the extract of the EEMB have reduced the uric acid levels in the blood than the diabetic control group.

The excretion of glucose is done along with the excretion of water which leads to osmotic diuresis.Water loss cause an elevation in serum polarity and activates the thirst centre hypothalamus.[166] the urine volume of the diabetic control group significantly increased when compared to normal control group whereas the EEMB treated group much significantly decreases the urine volume.

The urine creatinine levels of diabetic control significantly reduced than the normal group. Extract treated group there was no significant results regarding the alteration in the levels of creatinine. At the same time the EEMB treated group show a slight significant change in the urine urea levels at maximum dose of extract used

in the study.The uric acid levels also declined in the extract treated group when compared to normal control group.

The proteins released from the kidney as a result of its normal cell process and biotransformation condition leads to increased microalbumin levels.These proteins level will be enhanced when the kidney cells deviate from its function as in the case of diabetes.[167] The microalbumin levels are also determined in the study and the results declared that at the maximum dose of the extract used produce a significant decrease in the urine microalbumin levels when compared to diabetic control group.

Histopathological study revealed that the control group animals have normal kidney epithelial cells and tubules whereas the diabetic control group exhibits damaged tubules with degeneration and epithelial declines with intertubular disruption. The EEMB treated diabetic group showed much improvement by regenerating the tubular cells with slightly moderate intertubular bleeding. From the results of EEMB treated diabetic rats it is evident that the kidney regain the normal tubules with unimpaired epithelial cells with less intertubular hemorrhage.

Thus, on the basis of above discussion, it has been concluded that among the different solvent extracts of *M. brunoniana*, ethanol extract exhibited the most potent antioxidant, genoprotective, *alpha-glucosidase* and *alpha-amylase* enzyme inhibitory activity. The extract also illustrated the enhanced glucose uptake action in pre-adipose 3T3-L1 cell line and was found to be non cytotoxic. In addition, the GC-MS, HPLC and HPTLC analysis of this extract revealed several bioactive compounds which possess the above activities. The combined results were undoubtedly provides scientific confirmation and evidence for the use of ethanol extract of *M. brunoniana* as effective/powerful/strong antihyperglycemic, antihyperlipidemic and nephroprotective agents. The above research of this plant is coupled with their potent antioxidative property, can suggest additional values in prevention of oxidative stress. Hence the plant extract has plenty and extreme benefits in the prevention and treatment of nephropathy, retinopathy, and diabetes linked hyperlipidemia and other cardiovascular diseases.

CHAPTER – 8

SUMMARY AND CONCLUSION

The various solvent extracts of *Marsdenia brunoniana* was prepared and evaluated for its in vitro antioxidant, free radical scavenging and *in vitro alpha glucosidaseand alpha amylase* inhibitory activities. Amongst the various solvent extracts of *M.brunoniana*, ethanol extract shows more pronounced activity while comparing with remaining solvent extracts. Hence, the ethanolic extract of *Marsdenia brunoniana* has been studied for the toxicological, antidiabetic, antihyperlipidemic and nephroprotective activities in STZ-NAA induced diabetic animals. And the acute toxicity results revealed that the extract did not produce any motility to toxic effects even at maximum dose administered.

Subacute toxicity study also explored the non-toxic nature of the extract. Further, the extract was subjected to various studies like glucose uptake assay, DNA protection assay and *in vitro* cytotoxic assay. The results obtained in these studies revealed that the extract is significantly increases the glucose uptake and it protects the DNA and it also did not produce any cytotoxic activity at the tested dose levels.

The Preliminary phytochemical analysis, HPLC, HPTLC and GC-MS analysis of EEMB also revealed the presence of many phytochemical compounds such as flavonoids, phenols, methyl-α-D-Glucopyranoside, 4-C-methyl-Myo-Inositol, n-hexadecanoic acid, phytol, rutin, quercetin, ferulic acid, caffeic acid, gallic acid and which are having pronounced biological effects.

The EEMB significantly reduces the elevated level of glucose in blood and level of lipid profile in diabetic persuaded animals. At the same time it is able to increase the insulin secretion, restore the antioxidant enzyme system and prevent the weight loss in diabetic animals.

It also has the incredible effect on reducing the glycosylated hemoglobin level in diabetic induced animals.

In the nephroprotective activity, the extract significantly declines the HbA1C level and microalbumin level in diabetic rats.

Nevertheless, it increases the excretion of urea and uric acid through urine. All these finding in the research work concludes/emphasized that the ethanol extract of *Marsdenia brunoniana* has potent antidiabetic, antihyperlipidemic and nephroprotective activities and it can be used as an effective antidiabetic agent to treat type 2 diabetic mellitus, which is the first claim in this respect.

CHAPTER – 9

RECOMMENDATIONS FOR FURTHER WORK

- The present research work is studied on leaf part of unexplored plant *Marsdenia brunoniana wight & arn* and illustrated the important phytoconstituents present in the plant's leaf as well as its antidiabetic potential with nephroprotective activity. Further work can be preceded with other parts of the plant in order to establish its enormous and valuable potential or therapeutic effectiveness.

- Various advanced analytic techniques may be used to put forth its natural bioactive compounds and also to strengthen the importance of *Marsdenia brunoniana.*

- Since the various species of this genus have been reported for treating many ailments, *Marsdenia brunoniana* also can be screened for therapeutic effects.

- Combined effect of *Marsdenia* species exerted component(s) in their activity could study for medicinal property.

- Many medicinal plants are in the list with strong ethano-botanical background to screen for its activities, if further research done it may be useful for the world population to get rid of disorders without any adverse effects.

भारत सरकार / GOVERNMENT OF INDIA
पर्यावरण एवं वन मंत्रालय / MINISTRY OF ENVIRONMENT & FORESTS
भारतीय वनस्पति सर्वेक्षण / BOTANICAL SURVEY OF INDIA

दक्षिणी क्षेत्रीय केन्द्र / Southern Regional Centre
टी,एन.ए.यू कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003

टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
टेलीफक्स/ Telefax: 0422- 2432835
ई-मैल /E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2013-14/Tech. /1002 दिनांक/Date: 18th September 2013

सेवा में / To

Ms. M. Sudha
Lecturer
Department of Pharmacology
J.K.K. Nattraja College of Pharmacy
Komarapalayam – 638 183
Namakkal Dist.
Tamil Nadu

महोदया/Madam,

The plant specimen brought by you for identification is identified as *Marsdenia brunoniana* Wight & Arn. - ASCLEPIADACEAE

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

18/9/13

(डॉ. एम. पलनिसामी/Dr. M. Palanisamy)
वैज्ञानिक ' सी ' प्रभारी/Scientist 'C'-In-Charge

वैज्ञानिक 'सी' / SCIENTIST 'C'
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयंबत्तूर / Coimbatore - 641 003

J.K.K.NATTRAJA College of Pharmacy, Komarapalayam, Tamil Nadu.

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

Institutional Animal Ethics Committee (IAEC)

REG. NO: 887/ac/05/CPCSEA

CERTIFICATE

Title of the Project	: **PHYTOCHEMICAL INVESTIGATION AND PHARMACOLOGICAL SCREENING ON AN UNEXPOSED PLANT-*Marsdenia brunoniana* Wight and Arn**
Department	: Pharmacology, JKKNCP
Proposal Number	: JKKNCP/PhD/01/2013-14
Approval date	: 04-03-2013
Animals	: Wistar rats (Male)
No. of animals sanctioned	: 54
Expiry date (Termination of the project)	: 03-03-2017

Dr. R. SAMBATH KUMAR
Chairman IAEC

Dr. R. SHANMUGA SUNDRAM
Biological Scientist

Dr. A. PRAKASH
Animal House In-charge

Dr. M. VIJAYABASKARAN
Scientist I

Dr. N. VENKATESWARA MURTHY
Scientist II

Dr. A. SATHISH
Veterinarian

Sudha M. *et al.* / *International Journal of Phytopharmacology.* 7(1), 2016, 34-41.

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF VARIOUS SOLVENT EXTRACTS OF *MARSDENIA BRUNONIANA*

Sudha M[1,*], Muthusamy P[2] and Senthil Kumar R[3]

[1] Research Scholar, The Tamil Nadu Dr.M.G.R. Medical University, Chennai, Tamil Nadu, India.
[2] College of Pharmacy, Madras Medical College, Tamil Nadu, India.
[3] Swamy Vivekanandha College of Pharmacy, Tiruchengode, Tamilnadu, India.

ABSTRACT

In the present study, the antioxidant and free radical scavenging activities of various solvent extracts of leaves of *Marsdenia Brunoniana* was evaluated by different *in vitro* antioxidant assay models. The plant extracts exhibited strong antioxidant and radical scavenging activity on ABTS radical cation, DPPH free radical, hydrogen peroxide, superoxide radical and hydroxyl radical. The leaf extracts showed beneficial activity in total reducing power assay. The antioxidant and free radical scavenging properties of the extracts were comparable to standard antioxidants such as ascorbic acid and rutin. The extracts had esssential phenol and flavonoid contents. The antioxidant and radical scavenging activity of the plant extracts may be due to the rich amount of phenols and flavonoids. Therefore, the plant could be considered as a very good antioxidant source with strong therapeutic efficacy.

Key words: *Marsdenia brunoniana*, Antioxidant, Free radical scavenging activity, Flavonoids, Total phenol content, Total flavonoid content.

INTRODUCTION

A free radical (FR) can be defined as a chemical species possessing an unpaired electron. FR can be positively charged negatively charged or electrically neutral. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders. Oxidative damage to cellular biomolecules such as lipids, proteins and DNA is thought to play a crucial role in the incidence of several chronic diseases. Flavonoids are a group of polyphenolic compounds found abundantly in the plant kingdom. Interest in the possible health benefits of flavonoids and other polyphenolic compounds has increased in recent years owing to their potent antioxidant and free-radical scavenging activities. The effects of free radicals on human beings are closely related to toxicity, disease and aging. Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS). Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process. The antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Habibur *et al.*, 2013).

Antioxidants are chemical substances that donate an electron to the free radical and convert it to a harmless molecule. They may reduce the energy of the free radical or suppress radical formation or break chain propogation or repair damage and reconstitute membranes.

Recent investigations suggest that the plant origin antioxidants with free-radical scavenging properties may have great therapeutic value and

Corresponding Author

Sudha M
Email: sudhasivakkumar@gmail.com

importance. Antioxidants are vital substances which provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (Ghosal *et al.*, 1996; Ozsoy *et al.*, 2008). Several anti-inflammatory, antinecrotic, neuroprotective, chemopreventive and hepatoprotective drugs have recently been shown to have free radical scavenging mechanism as part of their activity (Lin and Huang, 2000; Repetto and Llesuy, 2002). There is an increased interest in natural antioxidants present in medicinal and dietary plants, which might help to prevent oxidative damage (Silva *et al.*, 2005).

Marsdenia brunoniana is a plant of the genus *Marsdenia* belongs to Asclepiadaceae family. It is a rare medicinal twining shrub found in Tamil Nadu and Karnataka states of Peninsular India (Natarajan, 2004). It has long been used by tribes and native medical practitioners to treat various chronic disorders including diabetes. Literature review revealed that no phytochemical and pharmacological studies have been carried out in this plant. Based on these details, the present study is aimed to evaluate the antioxidant and free radical scavenging potential of various solvent extracts of leaves of *Marsdenia brunoniana* using various *in vitro* assay models.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Sigma Aldrich Co., St. Louis, USA. Rutin and p–nitroso dimethyl aniline (*p*–NDA) were obtained from Acros Organics, New Jersy, USA. Ascorbic acid and nitro blue tetrazolium (NBT) were obtained from S.D. Fine Chem, Ltd., Biosar, India. 2- Deoxy–D-ribose was from Hi–Media Laboratories Ltd., Mumbai. All other chemical used were of analytical grade.

Collection and Extraction

The leaves of *M. brunoniana* was collected from Sirumalai Hills, near Dindugal, Tamilnadu in the month of January 2012 and the authenticity of the plant was confirmed from Botanical survey of India, Coimbatore. The shade dried coarse powder of *M.brunoniana* (1.5 kg) was extracted with various solvents by increasing order of polarity viz. Hexane, chloroform, ethyl acetate and ethanol by using soxhlet extractor for 72 h. After completion of extraction, each extract was filtered, concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50 °C). The residues were then stored in vacuum dessicator. The residue obtained after extraction with ethanol was extracted with water by cold maceration process for 72 h. The extractive values and nature of the extracts of *M. brunoniana* were tabulated.

Preliminary phytochemical screening

Prepared plant extracts of *M. brunoniana* were analyzed for the presence of various phytochemical constituents employing standard procedures (Wagner *et al.*, 1984). Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

Preparation of test and standard solutions

The plant extracts of *M. brunoniana* and the standard antioxidants (ascorbic acid and rutin) were dissolved in distilled dimethyl sulfoxide (DMSO separately and used for the *in vitro* antioxidant assays except the hydrogen peroxide method because it interferes with the method. For hydrogen peroxide method, the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain the lower concentrations.

In vitro antioxidant activity

Various extracts of *M. brunoniana* was tested for their *in vitro* antioxidant activity using the standard methods. In all these methods, a particular concentration of the extracts or standard solution was used which gave a final concentration of 1000-15.625 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standards, but without the reagents. A control test was performed without the extract or standards. Percentage scavenging and IC_{50} values were calculated.

ABTS radical scavenging activity

In a final volume of 1 ml, the reaction mixture comprised 950 µl of $ABTS^*$ solution and 50 µl of the plant extracts at various concentrations. The reaction mixture was homogenized and incubated for 20 min. Absorbances of these solutions were measured spectrophotometrically at 734 nm (Re *et al.*, 1999).

DPPH radical scavenging activity

The DPPH assay method depends on the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazine and the remaining DPPH which showed maximum absorption at 517 nm was measured. About 2 ml of various concentrations of the plant extracts or standards were added to 2 ml of DPPH solution (0.1 mM, 2 ml). After 20 min of incubation at 37 °C in the dark, the absorbance was recorded at 517 nm (Shirwaikar *et al.*, 2006).

Superoxide radical scavenging activity by alkaline DMSO method

In this method, superoxide radical is generated by the addition of sodium hydroxide to air saturated dimethyl sulfoxide. The generated superoxide remains stable in solution, which reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature and that can be measured at 560 nm. Briefly, to the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the extracts in freshly distilled DMSO at various concentrations, added 0.1 ml of NBT (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm (Elizabeth and Rao, 1990).

Hydrogen peroxide radical scavenging method

In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm. To 1 ml of various concentrations of extracts or standard in methanol was added to 2 ml of hydrogen peroxide (20 mM) in phosphate buffer saline. After 10 min the absorbance was measured at 230 nm (Jayaprakasha *et al.,* 2004).

Hydroxyl radical scavenging activity by *p*-NDA method

Various concentration of the extracts or standards in 0.5 ml of distilled DMSO were added to a solution mixture containing 0.5 ml of ferric chloride (0.1 mM), 0.5 ml of EDTA (0.1 mM), 0.5 ml of ascorbic acid (0.1 mM), 0.5 ml of hydrogen peroxide (2 mM) and 0.5 ml of *p*-NDA (0.01 mM) in phosphate buffer (pH 7.4, 20 mM) to produce a final volume of 3 ml. Absorbance was measured spectrophotometrically at 440 nm
qualitative analysis report data is displayed in Table 2.

ABTS and DPPH Radical Scavenging Assay

ABTS and DPPH radical scavenging activity of various solvent extracts of *M. brunoniana* are shown in Table 3. The extracts showed potent radical scavenging activity in concentration dependent manner. The various extracts of the leaf of *M. brunoniana* exhibited good radical scavenging activity against the tested models. Among the extracts tested ethanol extract showed potent activity when compared to other extracts. The results obtained were comparable with the standards used.

Superoxide and Hydrogen peroxide radical Scavenging Activity

Superoxide radical scavenging activity of leaf extracts of *M. brunoniana* were assessed by alkaline DMSO method. The plant extracts moderately inhibit the superoxide radical generation. In hydrogen peroxide (Jayaprakasha *et al.,* 2004).

Total reducing power assay

To 1 ml of the plant extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. After incubation period, 2.5 ml of 10% trichloroacetic acid was added and the reaction mixture was centrifuged at 1000 rpm for 10 min. The upper 2.5 ml layer was mixed with 2.5 ml of deionized water and 0.5 ml of ferric chloride and thoroughly mixed. The absorbance was measured spectrophotometrically at 700 nm. A higher absorbance indicates a higher reducing power (Oyaizu, 1986).

RESULTS

Preliminary Qualitative Analysis

Colour, nature and extractive yields of prepared extracts of *M. brunoniana* are presented in Table 1. Ethanol and water extracts showed highest extractive yield when compared to other solvents. Ethyl acetate gives moderate extractive yield. Preliminary qualitative analyses of the leaf extracts of *M. brunoniana* were undergone for the detection of various phytochemical constituents. The plant extract showed the presence of a variety of phytochemicals. Hexane extracts showed the presence of phytosterols whereas chloroform extract contains phytosterols and alkaloids. Ethyl acetate extract showed the presence of phenolic and flavonoid compounds. Ethanol extract showed the presence of majority of the phytochemicals present in the plant. Aqueous extract contains more polar compounds like carbohydrates, proteins, tannins and glycosides. Terpenoids, saponins and gums and mucilages were found to be absent in all the prepared extracts. The preliminary radical scavenging assay, the extracts were found to be equipotent with ascorbic acid but less potent when compared to rutin. The values were tabulated in Table 4.

Hydroxyl radical Scavenging Assay

Hydroxyl radical scavenging activity of leaf extracts of *M. brunoniana* was measured by p-NDA method. In this method, the ethanol and aqueous extracts showed potent activity when compared to standards used. The IC_{50} values were presented in Table 5.

Total Reducing Power Assay

In this assay model, an increase in absorbance was observed when the concentration of extracts increased. This indicates the antioxidant potential of the prepared plant extracts. Among the prepared extracts, ethanol extract showed potent antioxidant activity and the results obtained were comparable with the standards used. The results obtained were displayed in Fig.1.

Table 1. Color, nature and extractive yields of various extracts of *Marsdenia brunoniana*

Name of the Extract	Colour	Nature	Yield (%)
Hexane	Dark green	Sticky semisolid	2.6
Chloroform	Dark greenish yellow	Solid	1.5
Ethyl acetate	Greenish yellow	Solid	6.3
Ethanol	Yellowish brown	Solid	8.6
water	Brown	Solid	10.4

Table 2. Preliminary Phytochemical Studies of various extracts of *Marsdenia brunoniana*

Phytochemical constituents	Name of the Extract				
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
Carbohydrates	+	+	+	+	+
Phytosterols	+	-	-	+	-
Alkaloids	-	+	-	-	-
Glycosides	-	-	-	+	+
Terpenoids	-	-	-	-	-
Proteins & aminoacids	-	-	-	+	+
Saponins	-	-	-	-	-
Tannins	-	-	-	+	+
Phenolic compounds	-	-	+	+	+
Flavonoids	-	-	+	+	+
Fixed oils & Fats	+	-	-	-	-
Gums & Mucilages	-	-	-	-	-

(+) Presence (-) Absence

Table 3. Effect of the leaf extracts of *Marsdenia brunoniana* on ABTS and DPPH Method

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	ABTS	DPPH
Hexane	>1000	>1000
Chloroform	101.67 ± 10.01	102.59 ± 9.96
Ethyl acetate	52.35 ± 2.20	28.78 ± 5.08
Ethanol	60.33 ± 5.88	30.61 ± 4.15
Water	79.03 ± 3.94	60.99 ± 4.37
Ascorbic Acid	13.63 ± 1.17	7.05 ± 0.76
Rutin	6.61 ± 0.64	8.11 ± 0.42

*Average of three determinations; Data are expressed as mean ± SEM

Table 4. Effect of Various Extracts of *Marsdenia brunoniana* on Superoxide Radical Scavenging and Hydrogen peroxide Scavenging Methods

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	Superoxide radical scavenging	H_2O_2 radical scavenging
Hexane	>1000	>1000
Chloroform	216.7± 17.53	227.4 ±13.27
Ethyl acetate	134.17 ± 5.43	117.83 ± 11.58
Ethanol	91.88± 4.42	97.46 ±3.91
Water	107.74 ± 10.12	123.8 ± 5.95
Ascorbic Acid	187.53 ± 11.93	75.16 ± 3.78
Rutin	55.97 ± 2.21	32.56 ± 1.05

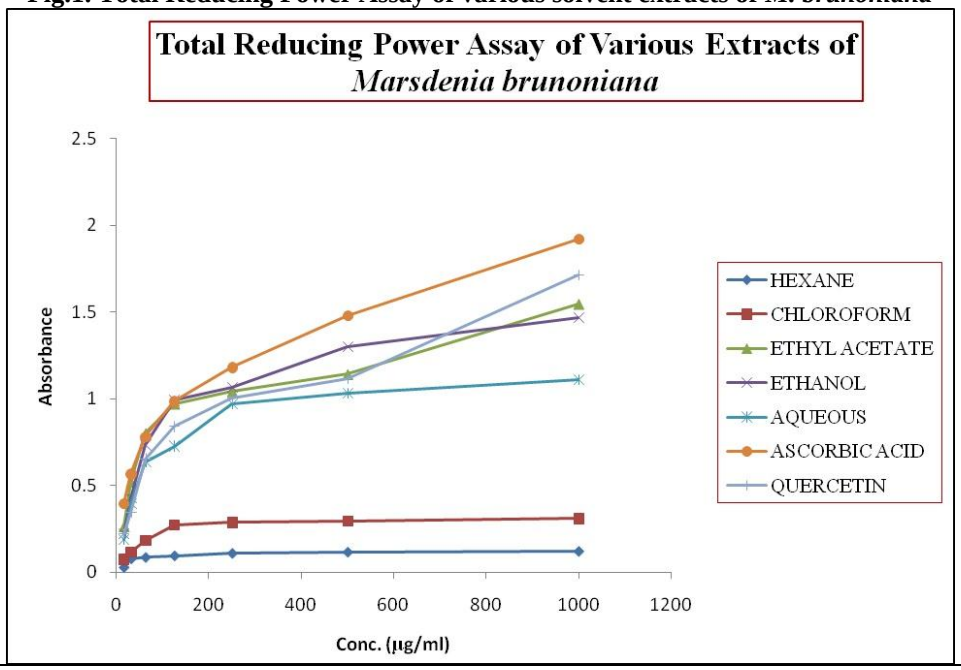
*Average of three determinations; Data are expressed as mean ± SEM

Table 5. Effect of leaf *extracts of Marsdenia brunoniana* on Hydroxyl Radical Scavenging Assay

Extracts/ Standards	IC_{50} (µg/ml)*
	Hydroxyl Radical Scavenging (p-NDA method)
Hexane	>1000
Chloroform	211.07±6.67
Ethyl acetate	128.8 ±4.16
Ethanol	108.6 ± 3.7
Water	115.57 ± 2.31
Ascorbic Acid	182.5 ± 4.88
Rutin	44.1 ±4.4

*Average of three determinations; Data are expressed as mean ± SEM

Fig.1. Total Reducing Power Assay of various solvent extracts of *M. brunoniana*



DISCUSSION

The biochemistry of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen is important in aerobic metabolism of the cell mostly reactive nitrogen species are well recognized for playing dual function as both dangerous and beneficial species. Overproduction of ROS from mitochondrial electron transport chain leakage or excessive stimulation of xanthine oxidase and other oxidative enzymes results in oxidative stress, a process that can be an important mediator of damage to cell structure and function, lipids, proteins, carbohydrates and DNA(Eboh Abraham., 2014).

In contrast, beneficial effects of ROS/RNS occur at very low concentrations and involve physiological roles in cellular responses in defense against infectious agents, gene expression, cellular growth, in the function of a number of cellular signaling pathways, hypoxia and respiratory burst. In the past and present years, progress has been made in the recognition and understanding of the roles of reactive oxygen species in many diseases. The body protects itself from the potential damages of reactive oxygen species, by utilizing antioxidant enzymes and non-antioxidant enzymes e.g. superoxide dismutases, glutathione peroxidases, glutathione reductase and catalase (Eboh Abraham., 2014).

Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant phytoconstituents of the plant materials act as radical scavengers and helps in converting the reactive free radicals to less reactive species. Natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers,

peroxide decomposers, enzyme inhibitors, and synergists. The most current research on antioxidant action focuses on phenolic compounds such as flavonoids. Fruits and vegetables contain different antioxidant compounds, such as vitamin C, vitamin E and carotenoids, whose activities have been established in recent years. Flavonoids, tannins and other phenolic constituents.

The antioxidant assays used in this study measured the oxidative products at the early and final stages of oxidation. The antioxidant and free radical scavenging activity of the leaf extracts of *M. brunoniana* was investigated against various *in vitro* models. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity. Hence, a large number of *in vitro* methods were used for the screening. IC_{50} values obtained were compared with the standards used, that is, ascorbic acid and rutin.

ABTS radical scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS ⋅ + for the estimation of antioxidant activity (Nenadis *et al.*, 2004). The extracts showed potent antioxidant activity in ABTS method which is comparable to the standard used. Here, the extracts radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

The electron donation ability of natural products can be measured by 2, 2 -diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Pratap Chandran *et al.*, 2013).

The experimental data of the extracts revealed that the extracts are likely to have the effects of scavenging free radicals. From the result we observe that a dose dependent relationship in the DPPH radical scavenging activity. The involvement of free radicals, especially their increased production, appears to be a feature of most of the human diseases including cardiovascular diseases and cancer (Deighton *et al.*, 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherols, flavonoids, tannins and aromatic amines reduce and decolorize the DPPH by their hydrogen donating ability. Flavonoids and phenolic compounds of leaf extracts of *M. brunoniana* are possibly involved in its radical scavenging activity.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2007). The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. The extracts are found to be an efficient scavenger of superoxide radical generated in alkaline DMSO system. The result clearly indicates that the plant extracts have a noticeable effect as scavenging superoxide radical.

Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Therefore, removing of hydrogen peroxide is very important for antioxidant defense in cell system. Polyphenols have also been shown to protect mammalian cells from damage induced by hydrogen peroxide, especially compounds with the orthohydroxy phenolic compounds like quercetin, gallic acid, caffeic acid and catechin (Nakayama, 1994). Therefore, the phenolic compounds of the leaf extracts of *Marsdenia brunoniana* may probably be involved in scavenging hydrogen peroxide.

Among the oxygen radicals, hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka *et al.*, 2005). In the present study, the hydroxyl radical scavenging activity of leaf and bark extracts of *M. brunoniana* was assessed by the inhibition of p-NDA bleaching method and deoxyribose degradation method. In p-NDA method, the hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical which can bleach p-NDA specifically. The extracts show potent scavenging activity by inhibition of bleaching of p-NDA. The scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in the extracts.

In the measurement of the reducing ability, it has been investigated from the Fe^{3+} - Fe^{2+} transformation. Fe^{3+} reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action and can be strongly correlated with other antioxidant properties (Dorman *et al.*, 2003). The reducing properties of the plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The data obtained in the present study suggest that it is likely to contribute significantly towards the observed antioxidant effects. However, the antioxidant activity has been attributed by various mechanisms, like

prevention of chain initiation, binding of transition metal ion catalysts, prevention of continued hydrogen abstraction, reductive capacity, radical scavenging activity and decomposition of peroxides. Like the antioxidant activity, the reducing power of the extracts increases with increasing concentration.

The systemic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Therefore, it is necessary to determine the total amount of phenols and flavonoids in the plant extracts chosen for the study. Flavonoids are the most diverse and widespread group of natural compounds and are proposed to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The extracts contains considerable amount of total flavonoids and phenols. Previous literatures showed that high phenol and flavonoid content increases the antioxidant activity (Holasova *et al.*, 2002) and there is a linear relation between the phenol and flavonoid contents and antioxidant activity (Gheldof and Engeseth, 2002).

Phenolic compounds are commonly found in both edible and medicinal plants and they have been reported to have various biological effects including antioxidant activity. The antioxidant activities of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Osawa, 1994). The leaf extracts of *Marsdenia brunoniana* showed strong antioxidant activity in various *in vitro* systems tested. The antioxidant effect of *M. brunoniana* is may be due to the phenolic compounds present in it. To our knowledge this is the first report on the antioxidant and radical scavenging potential of *Marsdenia brunoniana*.

CONCLUSION

The results from various free radicals scavenging systems reveal that various solvent leaf extracts of *Marsdenia brunoniana* have significant antioxidant activity. The extracts are found to have different levels of antioxidant activity in all the methods tested. IC_{50} values obtained were comparable with that of the standards used, that is, ascorbic acid and rutin. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity. Hence, a large number of *in vitro* methods were used for the screening. However, the difference in the activity in extracts may be due to the different chemical entities of the free radicals and the diverse chemical nature of the extracts. According to this study, a significant and linear relationship was found between the antioxidant activity, total phenol and flavonoid contents, indicating that these compounds could be major contributors to antioxidant activity. Further studies are in progress for the isolation and identification of phytochemical compounds and to ensure the most important medicinal properties of the plant *in vivo* in our laboratory to correlate with its antioxidant activity.

ACKNOWLEDGEMENT: None

CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

REFERENCES

Deighton N, Brennan R, Finn C and Davis HV. Antioxidant properties of domesticated and wild *Rubus* species. *J Sci Food Agric.*, 80, 2000, 1307-1313.

Dorman HJD, Peltoketo A, Hiltunen R and Tikkanen MJ. Characterization of the antioxidant properties of deodorized aqueous extracts from selected Lamiaceae herbs. *Food Chem.*,83, 2003, 255-262.

Eboh Abraham Sisein. Biochemistry of Free Radicals and Antioxidants. *Sch. Acad. J. Biosci.*, 2, 2014, 110-118.

Elizabeth K and Rao MNA. Oxygen radical scavenging activity of curcumin. *Intl J Pharm.*, 58, 1990, 237.

Fejes S, Blazovics A, Lugasi A, Lemberkovics E, Petri G and Kery A. *In vitro* antioxidant activity of *Anthriscus cerefolium* L. (Hoffm.) extracts. *J. Ethnopharmacol.*, 69, 2007, 259-265.

Gheldof N and Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of *in vitro* lipoprotein oxidation in human serum samples. *J Agric Food Chem.*, 50, 2002, 3050-3055.

Ghosal S, Tripathi VK and Chauhan S. Active constituents of *Emblica officinalis*. Part I. the chemistry and antioxidant effects of two new hydrolysable tannins, emblicanin A and B. *Indian J Chem.*, 35B, 1996, 941-948.

Gordon MH. The mechanisms of antioxidant action *in vitro*. In BJF Hudson (Ed.) Food antioxidants. *Elsevier Applied Sceince*, London, 1990, 1-18.

Habibur R, Manjula K, Anoosha T,Nagaveni K, M.Chinnaeswaraiah and Dipankar Bardala. *In vitro* Antioxidant activity of *Citrulllus lanatus* seed extracts. Asian *J Pharm clin Res.*, 6, 2013, 347-351.

Halliwell B and Gutteridge JMC. Free radicals in biology and medicine, 4th Edition, Oxford UK, 2007.

Halliwell B. Reactive oxygen species in living systems: Source, Biochemistry and role in human diseases. *Am J Med.*, 91, 1991, 14-22.

Harborne JB. Methods of plant analysis. In Phytochemical Methods, Chapman and Hall, London, 1973, 1-32.

Holasova M, Fiedlerova V, Smrcinova H, Orsak M, Lachman J and Vavreinova S. Buck wheat – the source of antioxidant activity in functional foods. *Food Res Intl.*, 35, 2002, 207- 211.

Huong NTT, Malsumato K, Kasai R, Yamasaki K and Watanabeth. *In vitro* antioxidant activity of Vietnamese ginseng saponin and its components. *Biol Pharm Bull.*, 21,1998, 978–981.

Jayaprakasha GK, Jaganmohan Rao L and Sakariah KK. Antioxidant activities of flavidin in different in vitro model systems. *Bioorg Med Chem.*, 12, 2004, 5141–5146.

Kokate CK. Practical Pharmacognosy 4th Edition, Vallabh Prakashan, Delhi, 1994, 107-111.

Lin CC and Huang PC. Antioxidat and hepatoprotective effects of *Acanthopanax senticosus*. *Phytother Res.*, 14, 2000, 489-494.

Nakayama T. Suppression of hydroperoxide induced cytotoxicity by polyphenols. *Cancer Res.*, 54, 1994, 1991s-1993s.

Natarajan D. Identification of conservation priority sites using remote sensing and GIS-A case study from Chitteri hills, Eastern Ghat, Tamil nadu. *Curr. Sci*.. 86, 2004, 1316-1323.

Nenadis N, Wang L, Tsimidou M and Zhang H. Estimation of scavenging activity of phenolic compounds using the ABTS assay. *J Agric Food Chem.*, 52, 2004, 4669–4674.

Osawa T. Novel natural antioxidants for utilization in food and biological systems. In. Uritani I, Garcia VV, Mendoza EM (eds.) Postharvest biochemistry of plant food materials in the tropics. Japan Scientific Societies Press, Tokyo, Japan, 1994, 241-251.

Oyaizu M. Studies on products of browning reaction: anti-oxidative activity of products of browning reaction prepared from glucosamine. *Jpn J Nutr.*, 44, 1986, 307-315.

Ozsoy N, Can A, Yanardag R and Akev N. Antioxidant activity of *Smilax excelsa* L leaf extracts. *Food Chem.*, 110, 2008, 571-583.

Pratap Chandran R. *In vitro* free radical scavenging activity of aqueous and methanolic leaf extracts of *Aegle tamilnadensis* (Rutaceae). *Intl J Pharm Pharm Sci.*, 5, Suppl 3, 2013, 3847-3851.

Re R, Pellegrini M, Proteggente A, Pannala A, Yang M and Rice EC. Antioxidant activity applying an improved ABTS radical cation decolorisation assay. *Free Radic. Biol. Med*., 26, 1999, 1231–1237.

Repetto MG and Llesuy SF. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. *Braz J Med Biol Res.*, 35, 2002, 523-534.

Sakanaka S, Tachibana Y and Okada Y. Preparation and antioxidant properties of extracts of Japanese Persimmon leaf tea (Ka-Kiniha-Cha). *Food Chem.*, 89, 2005, 569-575.

Sheng-Xiang Qiu, Si-Qi Luo, Long-Ze Lin and Geoffrey A. Cordell (1996) Further polyoxypregnanes from *Marsdenia tenacissima* (Roxb.) Moon. *Phytochemistry*, 41, 1996, 1385-1388.

Silva BA, Ferreres F, Malva JO and Dias ACP. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food Chem.*, 90, 2005, 157-167.

Wagner H, Bladt S and Zgainski EM. Plant Drug Analysis, Springer – Verlag, Berlin, 1984, pp. 298-334.

International Journal of Pharmacotherapy

www.ijopjournal.com

ISSN 2249 - 7765
Print ISSN 2249 - 7773

ALPHA GLUCOSIDASE AND *ALPHA AMYLASE* INHIBITORY ACTIVITIES OF VARIOUS SOLVENT EXTRACTS OF *MARSDENIA BRUNONIANA*

Sudha M[1,*], Muthusamy P[2] and Senthil Kumar R[3]

[1] Research Scholar, Faculty of Pharmacy, The Tamilnadu Dr.M.G.R. Medical University, Chennai, Tamilnadu, India.
[2] College of Pharmacy, Madras Medical College, Chennai, Tamilnadu, India.
[3] Swamy Vivekanandha College of Pharmacy, Thiruchengode, Tamilnadu, India.

ABSTRACT

Diabetes mellitus is caused by the abnormality of carbohydrate metabolism which is linked to low blood insulin level or insensitivity of target organs to insulin. Glucose metabolizing enzyme inhibitors are class of compounds that help in managing diabetes mellitus. Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The herbal drugs with antidiabetic activity are yet to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine. The Present study deals with screening of various solvent extracts of leaves of *Marsdenia brunoniana* for their efficacy to inhibit *α-glucosidase* and *α-amylase* in the *in vitro* systems. Potent and dose dependent inhibition of these carbohydrate digestive enzymes was observed and the results were comparable to standard drug acarbose. This enzyme inhibition provided a strong *in vitro* evidence for confirmation of various leaf extracts of *Marsdenia brunoniana* as excellent antidiabetic remedy. These results provide an intense rationale for further *in vivo* and clinical study.

Key words: *Marsdenia brunoniana*, *α-glucosidase* inhibitory, *α-amylase* inhibitory, Antidiabetic activity.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorders with one common manifestation-hyperglycemia. Chronic hyperglycemia causes damage to eyes, kidneys, nerves, heart and blood vessels. It is caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by the ineffectiveness of the insulin produced. It results either from inadequate secretion of hormone insulin, an inadequate response of target cells to insulin, or a combination of this factors [1]. In spite of the introduction and extensive utilization of hypoglycemic agents, diabetes and the related complications continue to be a major health problem worldwide, which is affecting nearly 10% of the population all over the world and considered as a major cause of high economic loss which can in turn impede the development of nations. It is projected to become one of the world's main disablers and killers within the next 25 years [2]. India is one of the leading countries with high number of people with diabetes mellitus and it is estimated that around 57 million people will be suffering from diabetes mellitus by the year 2025 and would become the diabetes capital of the world [3]. In recent years, herbal medicines have started to gain importance as a source of hypoglycemic agents. More than the ethnobotanical information reports about 1000 plants that may possess antidiabetic potential however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances which

Corresponding Author:- **Sudha M** Email: sudhasivakkumar@gmail.com

demonstrate alternative and safe effects on diabetes mellitus [4].

Diabetes mellitus is caused by the abnormality of carbohydrate metabolism which is linked to low blood insulin level or insensitivity of target organs to insulin. Despite considerable progress in the treatment of diabetes by oral hypoglycemic agents, search for newer drugs continues because the existing synthetic drugs have several limitations. The herbal drugs with antidiabetic activity are yet to be commercially formulated as modern medicines, even though they have been acclaimed for their therapeutic properties in the traditional systems of medicine. Type 2 diabetes usually occurs in obese individuals and is associated with hypertension and dyslipidemia [5]. Digestion of starch by key gastrointestinal enzymes can be retarded and this can benefit the diabetic patient. Release of glucose from food sources is the main factor affecting postprandial hyperglycemia. α-*amylase* and α-*glucosidase* are the enzymes involved in the digestive process of polysaccharides and carbohydrates. The enzyme inhibitors such as acarbose, miglitol and voglibose are known to reduce postprandial hyperglycemia primarily by interfering with the carbohydrate-digesting enzymes and delaying glucose absorption [6].

Marsdenia brunoniana Wight& Arn. is a plant of the genus *Marsdenia* belongs to Asclepiadaceae family. *M. brunoniana* is one such rare medicinal twining shrub found in Tamilnadu and Karnataka states of India [7]. It has long been used by tribes and native medical practitioners to treat various chronic disorders including diabetes. Literature review revealed that no phytochemical and pharmacological studies have been carried out in this plant. Previous work performed in our laboratory revealed that the plant extracts having sting antioxidant and free radical scavenging activity. In continuation of our earlier study, the present work is aimed to evaluate the alpha *glucosidase* and alpha *amylase* inhibitory activity of various solvent extracts of leaves of *Marsdenia brunoniana* using *in vitro* assay systems.

MATERIALS AND METHODS

Chemicals and Reagents

α-*glucosidase*, α-*amylase*, 4-nitrophenyl α-D-glucopyranoside were obtained from Sigma Aldrich Chemical Co St. Louis M.O., USA. 3,5-dinitro salicyclic acid (DNS), methanol, dipotassium hydrogen phosphate (KH_2PO_4), potassium dihydrogen phosphate (KH_2PO_4), sodium chloride were procured from HiMedia Laboratories, Mumbai, India. Acarbose was obtained from Bayer Pharmaceuticals Pvt. Ltd., Mumbai, India. All other chemicals procured were of analytical grade.

Collection and Extraction

The leaves of *M. brunoniana* was collected from Sirumalai Hills, near Dindugal, Tamilnadu in the month of January 2012 and the authenticity of the plant was confirmed from Botanical survey of India, Coimbatore. The shade dried coarse powder of *M.brunoniana* (1.5 kg) was extracted with various solvents by increasing order of polarity viz. Hexane, chloroform, ethyl acetate and ethanol by using soxhlet extractor for 72 h. After completion of extraction, each extract was filtered, concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50 °C). The residues were then stored in vacuum dessicator. The residue obtained after extraction with ethanol was extracted with water by cold maceration process for 72 h. The extractive values and nature of the extracts of *M. brunoniana* were tabulated.

Preliminary phytochemical screening

Prepared plant extracts of *M. brunoniana* were analyzed for the presence of various phytochemical constituents employing standard procedures. Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

In vitro antidiabetic activity

Enzyme inhibitory activity of *Marsdenia brunoniana*

α- glucosidase inhibitory activity

α-glucosidase was pre-mixed with various extracts of *M. Brunoniana* at different concentration (1000 –15.625 μg/ml) and 3 mM p-nitrophenyl α-D -glucopyranoside as a substrate in potassium phosphate buffer was added to the mixture to start the reaction. The reaction was incubated at 37^{o}C for 30 min and stopped by addition of 2 ml of 0.1 M Na_2CO_3. *Alpha glucosidase* activity was determined by measuring release of p-nitrophenyl α-D-glucopyranoside at 420 nm [8]. Inhibition rates were calculated as percentage controls using the formula:

$$\% \text{ inhibition} = [(A420_{Control} - A420_{Test})/A420_{Control}] \text{ X } 100$$

IC_{50} values were determined from dose-response curves of percentage inhibition versus extract concentration and compared with the IC_{50} of acarbose determined under similar conditions.

α- amylase inhibitory activity

α-amylase was pre-mixed with different solvent extracts of *M. brunoniana* at various concentrations (1000 – 15.625 μg/ml) and starch as a substrate was added as a 0.5% starch solution in phosphate buffer to start the reaction. The reaction was carried for 20 minutes and terminated by adding 2 ml of DNS reagent (1% 3,5-dinitrosalysalic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was then heated for 15 min at 100^{o}C and *alpha amylase* activity was

determined by measuring absorbance at 540 nm [9]. Inhibition rates were calculated as percentage controls using the formula:

$$\% \text{ inhibition} = [(A540_{Control} - A540_{Test})/A540_{Control}] \text{ X } 100$$

IC_{50} values were determined from dose-response curve of percentage inhibition versus *M. brunoniana* concentration and compared with the IC_{50} of the synthetic inhibitor of *alpha amylase* (acarbose) determined under similar conditions.

RESULTS

Preliminary Qualitative Analysis

Colour, nature and extractive yields of prepared extracts of *M. brunoniana* are presented in Table 1.

Ethanol and water extracts showed highest extractive yield when compared to other solvents. Ethyl acetate gives moderate extractive yield. Preliminary qualitative analyses of the leaf extracts of *M. brunoniana* were undergone for the detection of various phytochemical constituents. The plant extract showed the presence of a variety of phytochemicals. Hexane extracts showed the presence of phytosterols whereas chloroform extract contains phytosterols and alkaloids. Ethyl acetate extract showed the presence of phenolic and flavonoid compounds. Ethanol extract showed the presence of majority of the phytochemicals present in the plant. Aqueous extract contains more polar compounds like carbohydrates, proteins, tannins and glycosides. Terpenoids, saponins and gums and mucilages were found to be absent in all the prepared extracts. The preliminary qualitative analysis report data is displayed in Table 2.

α- glucosidase inhibitory Activity

The various extracts of *M. brunoniana* are subjected to the study of inhibition of *α- glucosidase* enzyme. Ethanol and ethyl acetate extracts showed maximum activity with the lowest IC_{50} value. The chlorofrm and water extracts have shown a moderate activity in the inhibition of *α- glucosidase*. Acarbose at a concentration of 0.15 to 10µg/ml was employed and served as reference standard drug for *α-glucosidase* inhibitory activity. The IC_{50} values of all the extracts of *M. brunoniana* denote that it has intense activity in the inhibition of enyme *glucosidase*. The IC_{50} value of acarbose against *alpha glucosidase* was found to be 0.57µg/ml (Table3).

α- amylase inhibitory Activity

The various extracts of *Marsdenia brunoniana* were analyzed for *α- amylase* inhibitory activity. The maximum effect was found on the extracts of ethanol and ethyl acetate with lowest IC_{50} values. The IC_{50} values of chloroform and water extracts showed moderate activity. All the tested extracts showed better enzyme inhibitory activity when compared to acarbose, whose IC_{50} values was found to be 265.27 µg/ml(Table 3).

Table 1. Color, nature and extractive yields of various extracts of *Marsdenia brunoniana*

Name of the Extract	Colour	Nature	Yield (%)
Hexane	Dark green	Sticky semisolid	2.6
Chloroform	Dark greenish yellow	Solid	1.5
Ethyl acetate	Greenish yellow	Solid	6.3
Ethanol	Yellowish brown	Solid	8.6
water	Brown	Solid	10.4

Table 2. Preliminary Phytochemical Studies of various extracts of *Marsdenia brunoniana*

Phytochemical constituents	Name of the Extract				
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
Carbohydrates	+	+	+	+	+
Phytosterols	+	-	-	+	-
Alkaloids	-	+	-	-	-
Glycosides	-	-	-	+	+
Terpenoids	-	-	-	-	-
Proteins & aminoacids	-	-	-	+	+
Saponins	-	-	-	-	-
Tannins	-	-	-	+	+
Phenolic compounds	-	-	+	+	+
Flavonoids	-	-	+	+	+
Fixed oils & Fats	+	-	-	-	-
Gums & Mucilages	-	-	-	-	-

(+) Presence (-) Absence

Table 3. α- *glucosidase* and α- *amylase* inhibitory activity of various extracts of *Marsdenia brunoniana*

Extracts/Standard	IC_{50} Value (µg/ml)*	
	α-glucosidase	***α -amylase***
Hexane	>1000	>1000
Chloroform	153.23 ± 2.11	138.9 ± 3.96
Ethyl acetate	99.63 ± 2.93	100.42 ± 3.43
Ethanol	85.28 ± 4.86	87.48 ± 5.34
Water	120.77 ± 4.1	122 ± 2.63
Acarbose	0.5627 ± 0.053	265.27 ± 12.51

*Average of three determinations; Data are expressed as mean ± SEM

DISCUSSION

Diabetes mellitus is possibly the world's largest growing metabolic disease and as the knowledge on the heterogeneity of this disorder is advanced, the need for more appropriate therapy increases. Medicinal plant extracts and herbal formulations have recently been reviewed and have gained importance for the control of diabetes mellitus. They are being used directly or indirectly for the preparation of many modern drugs [10]. Although, the plant *M. brunoniana* have been used by the tribal people, it have not gained much importance as medicines and one of the reasons being lack of specific standards being prescribed for herbal medicines and supportive animal or clinical trials . In the present study, we investigated the leaves of the traditional medicinal plant, *M. brunoniana* for its possible significance in control of diabetes by carbohydrate enzymes inhibitory activity. Medicinal plants have wide range of phytochemical constituents ranging from both non-polar to polar. Thus, the plant materials were extracted to ensure complete extraction of all non-polar and polar phytochemical components and thereby all the constituents are exposed to the screening studies. The nature has provided abundant plant wealth for all the living creatures, which possess medicinal virtues. Therefore, there is a necessity to explore their uses and to ascertain their therapeutic properties. Hence, the present study aims to open new avenues for the improvement of medicinal uses of *M. brunoniana*.

The treatment goal of diabetes patients is to maintain near normal levels of glycemic control, in both the fasting and post-prandial states. Many natural resources have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine. *α-amylase* catalyses the hydrolysis of α-1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and *α-glucosidase* further breaks down the disaccharides into simpler sugars, readily available for the intestinal absorption. The inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes. Therefore, effective and nontoxic inhibitors of *α-amylase* and *α-glucosidase* have long been sought [11].

Experimental animal studies [12] and clinical studies also have shown that inhibitors of both *α-amylase* and *α-glucosidase* can suppress the production and absorption of glucose from the small intestine. Furthermore, some inhibitors of *α–amylase* and *α–glucosidase* such as phaseolamin, acarbose and vogiblose are currently used to suppress postprandial glucose levels in diabetic patients [13]. Plants are known to produce a large variety of *glucosidase* inhibitors that provide protection against insects and microbial pathogens [14, 15]. Therefore, the prepared plant extracts of *M. brunoniana* were analyzed for *α-amylase* and *α-glucosidase* inhibitory activity.

The intestinal digestive enzymes *alpha-glucosidase* and *alpha-amylase* are plays a vital role in the carbohydrate digestion. One antidiabetic therapeutic approach reduces the post prandial glucose level in blood by the inhibition of *alpha-glucosidase* and *alpha-amylase* enzymes. These can be an important strategy in management of blood glucose [16]. Recent advances in understanding the activity of intestinal enzymes (*α-amylase* and *α-glucosidase* both are important in carbohydrate digestion and glucose absorption) have lead to the development of newer pharmacological agents. A high postprandial blood glucose response is associated with micro- and macro-vascular complications in diabetes and is more strongly associated with the risk for cardiovascular diseases than are fasting blood glucose [17].

α-Glucosidase enzymes in the intestinal lumen and in the brush border membrane play main roles in carbohydrate digestion to degrade starch and oligosaccharides to monosaccharides before they can be absorbed. It was proposed that suppression of the activity of such digestive enzymes would delay the degradation of starch and oligosaccharides, which would in turn cause a decrease in the absorption of glucose and consequently the reduction of postprandial blood glucose level elevation [18]. *Alpha-glucosidase* inhibitor retards the digestion of carbohydrates and slows down the absorption. Acarbose and miglitol are competitive inhibitor of *α-glucosidases* and reduces absorption of starch and disaccharides [19]. Hence one of the therapeutic approaches for reducing postprandial blood glucose levels in patient with diabetes mellitus is to

prevent absorption of carbohydrate after food intake. Inhibition of these enzymes reduced the high postprandial blood glucose peaks in diabetes. *α- amylase* inhibitors act as an anti-nutrient that obstructs the digestion and absorption of carbohydrates. Acarbose is complex oligosaccharides that delay the digestion of carbohydrates. It inhibits the action of pancreatic *amylase* in breakdown of starch. Synthetic inhibitor causes side effect such as abdominal pain, diarrhoea and soft faeces in the colon [17].

The *in vitro α- glucosidase* and *α-amylase* inhibitory study results indicate that *M. brunoniana* possess significant *in vitro* antidiabetic activity. The mechanism by which *M. brunoniana* exerted action may be due to its action on carbohydrate binding sites of α-1,4-glucosidic linkage in starch and other related polysaccharides have also been targets for the suppression of postprandial hyperglycemia. This enzyme is responsible in hydrolyzing dietary starch into maltose which then breaks down to glucose prior to absorption. Since *α-amylase* play an important role in starch breakdown in human beings and animals, the presence of such inhibitors in food stuffs may be responsible for impaired starch digestion [20-21]. Thus, *α-amylase* inhibitor may be of value as novel therapeutic agents.

Phytochemical screening of *M. brunoniana* suggested the presence of alkaloids, phenols, flavonoids and terpenoids. Taking in to consideration the results of other similar *in vitro* studies, which have attributed the *α-glucosidase* inhibitory activity of some plant material extracts to the presence of flavonoids, polyphenols as well as their glycoside derivatives, it is reasonable to suggest that the *α-glucosidase* inhibitory effect of *M. brunoniana* observed in the current study could also be due to the presence of flavonoids and/or other phenolic compounds.

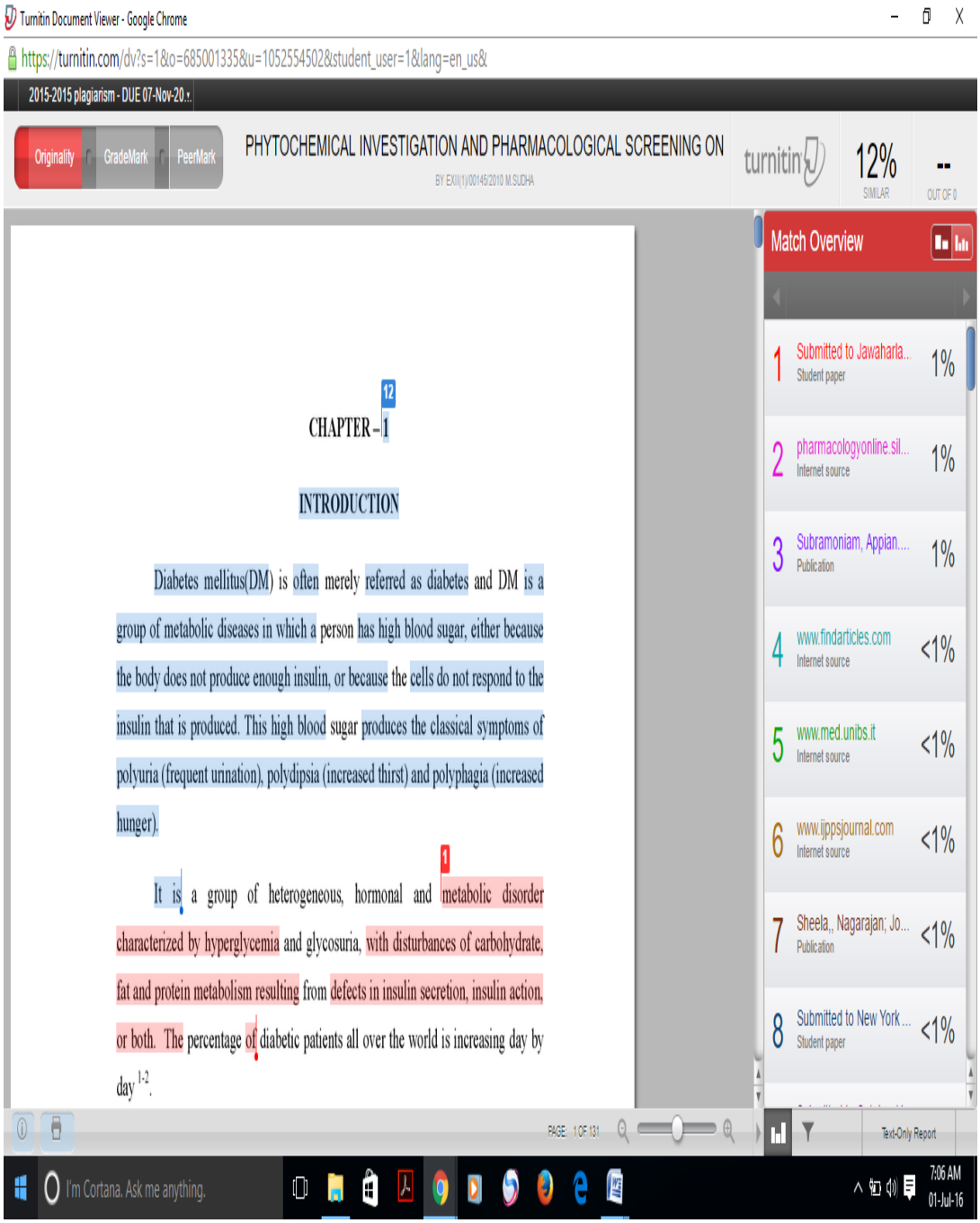
CONCLUSION

In conclusion, our findings showed that *M. brunoniana* have the potential to be explored further to identify the anti diabetic compounds in this plant. The above conducted *in vitro* studies depict an appreciable increase in the glucose uptake by the plant extracts. It was observed that, the plant extracts inhibited *alpha glucosidase* and *alpha amylase* enzyme thereby helps in the inhibition of the formation of glycated end products. We can therefore conclude from this study that, the presence of the phytochemicals in this plant might be the reason for these inhibitions and that the plants may essentially contain herbal bioactive compounds which require further structural elucidation and characterization methodologies to identify the bioactive constituents. Further, ex vivo and in vivo investigations should be done for confirming the anti diabetic activity of this plant. The plant extracts understudy can serve as therapeutic agents and can be used as potential sources of novel bioactive compounds for treating Diabetes mellitus.

REFERENCES

1. Malviya N, Jain S, Malviya SAPNA. Antidiabetic potential of medicinal plants. *Acta Pol Pharm.*, *67*(2), 2010, 113-118.
2. Mamun-or-Rashid ANM, Hossain MS, Naim Hassan B, Kumar Dash M, Sapon A, Sen MK. A review on medicinal plants with antidiabetic activity. *J Pharmacog Phytochem.*, *3*(4), 2014, 149-159.
3. Arvind K, Pradeepa R, Deepa R, Mohan V. Diabetes & coronary artery disease. *Indian J Med Res.*, *116*, 2002, 163.
4. Mukesh R, Namita P. Medicinal plants with antidiabetic potential-A Review. *American-Eurasian J Agric Environ Sci.*, *13*(1), 2013, 81-94.
5. Arumugam G, Manjula P, Paari N. A review: Anti diabetic medicinal plants used for diabetes mellitus. *J Acute Dis.*, *2*(3), 2013, 196-200.
6. Rang HP, Dale MM, Ritter JM, More PK. Pharmacology 5th ed., Chruchill Livingstone, New Delhi, pp.385.
7. Natarajan D, Britto S.J, Balaguru B, Nagamurugan N, Soosairaj S, Arockiasamy DI. Identification of conservation priority sites using remote sensing and GIS: A case study from Chitteri hills, Eastern Ghats, Tamil Nadu. *Curr sci.*, *86*(9), 2004, 1316-1323.
8. Sanap SP, Ghosh S, Jabgunde AM, Pinjari RV, Gejji SP, Singh, Dhavale DD. Synthesis, computational study and glycosidase inhibitory activity of polyhydroxylated conidine alkaloids—a bicyclic iminosugar. *Org Biomol Chem.*, *8*(14), 2010, 3307-3315.
9. Kim YM, Jeong YK, Wang MH, Lee WY, Rhee HI. Inhibitory effect of pine extract on α-*glucosidase* activity and postprandial hyperglycemia. *Nutrition.*, *21*(6), 2005, 756-761.
10. Odhav B, Kandasamy T, Khumalo N, Baijnath H. Screening of African traditional vegetables for their alpha-*amylase* inhibitory effect. *J Med Plants Res.*, 4, 2010, 1502-1507.
11. Bhandari MR, Jong-Anurakkun N, Hong G, Kawabata J. α-*Glucosidase* and α-*amylase* inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata*, Haw.). *Food Chem.*, *106*(1), 2008, 247-252.
12. Hara Y, Honda M. The inhibition of alpha *amylase* by tea polyphenols, *Agric Biol Chem.*, 54, 1990, 1939-1945.
13. Sanap SP, Ghosh S, Jabgunde AM, Pinjari RV, Gejji SP, Singh S, Chopade BA , Dhavale DD. Synthesis, computational study and glycosidase inhibitory activity of polyhydroxylated conidine alkaloids – a bicyclic iminosugar. *Org Biomol Chem.*, 8, 2010, 3307-3315.
14. Ryan CA. Proteinase inhibitor gene families: strategies for transformation to improve plant defense herbivores. *Bio Essays.*, 10, 1989, 20-24.

15. Lu S, Deng P, Liu X, Luo J, Han R, Gu X, Liang S, Wang X, Li F, Lozanov V, Patthy A, Pongor S. Solution structure of a-*amylase* inhibitor of the crop plant amaranth. *J Biol Chem*., 274, 1999, 20473-20478.
16. Manikandan R, Vijaya Anand A, and Durai Muthumani. *In vitro* anti-diabetic activity of methanolic extract of *Psidium guajava* leaves. *Int J Curr Microbiol App Sci.,* 2(2), 2013, 15-19.
17. Narkhede MB, Ajimire PV, Wagh AE, Mohan M, Shivashanmugam AT. In vitro antidiabetic activity of *Caesalpina digyna* (R.) methanol root extract. *Asian J Plant Sci Res.*, *1*(2), 2011, 101-106.
18. Marshal JJ. Hypothesized that negatively charged residues of pilaic acid from membrane. *Am Chem Soc Symposium Series,* 15, 1975, 244-266.
19. Jaffe WG, Lette CLV. Heat labile growth inhibiting factors in beans (*Phaseolus vulgaris*). *J Nutr.*, 94, 1968, 203-210.
20. Jung M, Park M, Lee HC, Kang YH, Kang ES, Kim SK. Antidiabetic agents from medicinal plants. *Curr Med Chem*., 13, 2006, 1203–1218.
21. Andrade-Cetto A, Cardenas R, Ramirez-Reyes B. Hypoglycemic effect of *Cecropia peltata* L. on N5-STZ type 2 diabetic rats. *Pharmacologyonline*, 3, 2007, 203-210.


Turnitin Document Viewer - Google Chrome
https://turnitin.com/dv?s=1&o=685001335&u=1052554502&student_user=1&lang=en_us&
2015-2015 plagiarism - DUE 07-Nov-20.r...
Originality
GradeMark
PeerMark
PHYTOCHEMICAL INVESTIGATION AND PHARMACOLOGICAL SCREENING ON
BY EXII(1)/0014S/2010 M.SUDHA
turnitin
12%
SIMILAR
--
OUT OF 0
CHAPTER-1
INTRODUCTION
Diabetes mellitus(DM) is often merely referred as diabetes and DM is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because the cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger).
It is a group of heterogeneous, hormonal and metabolic disorder characterized by hyperglycemia and glycosuria, with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The percentage of diabetic patients all over the world is increasing day by day [1-2].
Match Overview
1 Submitted to Jawaharla... Student paper 1%
2 pharmacologyonline.sil... Internet source 1%
3 Subramoniam, Appian.... Publication 1%
4 www.findarticles.com Internet source <1%
5 www.med.unibs.it Internet source <1%
6 www.ijppsjournal.com Internet source <1%
7 Sheela,, Nagarajan; Jo... Publication <1%
8 Submitted to New York ... Student paper <1%
PAGE: 1 OF 131
Text-Only Report
I'm Cortana. Ask me anything.
7:06 AM
01-Jul-16

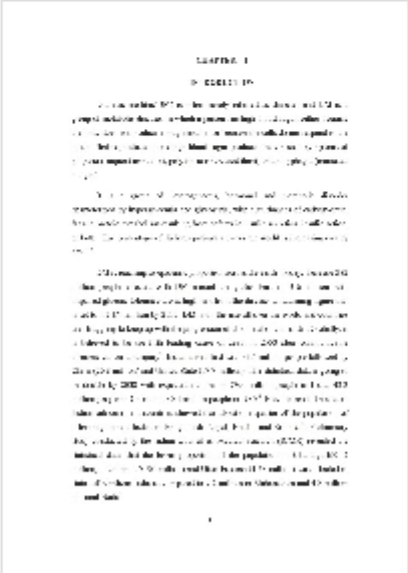
turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	Exii(1)/00145/2010 M.SUDHA
Assignment title:	2015-2015 plagiarism
Submission title:	PHYTOCHEMICAL INVESTIGATION..
File name:	Thesis_as_on_29.06.2016.doc
File size:	2M
Page count:	131
Word count:	26,998
Character count:	146,796
Submission date:	29-Jun-2016 09:34PM
Submission ID:	685001335



CHATPER – 11

BIBLIOGRAPHY

1. American Diabetes Association. Standards of medical care in diabetes. Diabetes Care. 2007; 30: 4–41.

2. Malviya N, Jain S and Malviya S. Antidiabetic potential of medicinal plants. ActaPoloniae Pharmaceutica. 2010; 67: 113–118.

3. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27(5): 1047-1053.

4. Kaveeshwar SA, Cornwall J. The current state of diabetes mellitus in India. The Australasian Medical Journal. 2014; 7(1): 45-48.

5. Anjana RM, Pradeepa R, Deepa M,Datta M, Sudha v,Unnikrishnan R,Bhansali A, Joshi SR, Yajnik CS, Dhandhania VK, Nath LM, Das AK, Rao PV, Madhu SV, Shukla DK, Kaur T, Priya M, Nirmala E, Parvathi SJ, Subhashini S, Subhashini R, Ali MK, Mohan V. Prevalence of diabetes and prediabetes (impaired fasting glucose and/or impaired glucose tolerance) in urban and rural India: phase I results of the Indian Council of Medical Research-INdiaDIABetes (ICMR-INDIAB) study. Diabetologia. 2011; 54(12): 3022-3027.

6. Xu H, Dembski M, Yang Q, Moriarty A, Tayber O, Chan H, Kapeller R and Tartanglia LA. Dual specificity mitogen-activated protein (MAP) kinase phospatase-4 plays a potential role in insulin resistance. J BioI Chem. 2003; 278: 30187-30192.

7. Storlien LH, Kriketos AD, Calvert GD, Haur LA and Jenkins AB. Fatty acid, triglycerides and syndromes of insulin resistance. Prostaglandins, Leukotrienes and Essential fatty acids. 1997; 57: 379-385.

8. American Diabetes Association. Report of the Expert Committee on the diagnosis and classification of diabetes mellitus, Diabet Care. 2005; 25: S5-S20.

9. Current Medical Diagnosis and Treatment, 44 Ed, Lange Medical Books. McGraw-Hill, Medical Division. 2005; 1157-1201.

10. National Diabetes Data Group. Diabetes in America. 2nd ed. Bethesda, Md. National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, NIH. 2005, publication no. 95-1468.

11. Sheriff, S., Fischer, J.E., Balasubramaniam, A. Amylin inhibits insulin stimulated glucose uptake in C2C12 muscle cell line through a cholera-toxin sensitive mechanism. Biochimicaet Biophysica Acta. 1992; 1136: 219-222.

12. Dewitt, D.E., Irl, B.H. Outpatient Insulin Therapy in Type I and Type II Diabetes Mellitus: Scientific Review. Journal of American Medical Association. 2003; 289(17): 2254-2264.

13. Bevan, P. Insulin signalling. Journal of Cell Science. 2001; 114: 1429-1430.

14. Herman WH, Engelgau MM, Zhang Y and Brown MB. Use of GHb (HbAlC) to screen for undiagnosed diabetes in the U.S. population. Diabet Care.2000; 23: 1207-1208.

15. Dey L, Zhang L and Yuan CS. Anti-diabetic and anti-obese effects of ginseng berry extract: comparison between intraperitoneal and oral administration. Am J Clin Med. 2002; 30: 645-647.

16. Luna B and Feinglos MN. Oral agents in management of type 2 diabetes mellitus. Am Fam Physician. 2001;63: 1747-1756.

17. Sy, G.Y., Cisse, A., Nongonierma, R.B., Sarr, M., Mbodj, N.A., Faye, B. Hypoglycaemic and antidiabetic activity of acetonic extract of Vernoniacolorata leaves in normoglycaemic and alloxan-induced diabetic rats. Journal of Ethnopharmacol. 2005; 98: 171-175.

18. Zhang B.B, Moller D.E. New approaches in the treatment of type 2 diabetes. Current Opinion in Chemical Biolog. 2000; 4: 461- 467.

19. Ahamad, M., Khan, M.A., Qureshi, R.A. Ethanobotanical study of some cultivated plants of Chhuch region (district of Attock). Journal of Hamdard Medicus. 2003; 6: 15-19.

20. Sinha A Formica C, Tsalamandris C, Panagiotopoulos S, Hendrich E, Deluise M, Seemam E and Jerums G. Effects of insulin on body composition in patients with insulin-dependent and non-insulin-dependent diabetes. Diabet Med. 1996; 13: 40-6.

21. Bailey CJ and Day C. Traditional plant medicines as treatments for diabetes. Diabet Care.1989; 12: 553-564.

22. Matsumoto N, Ishigaki F, Ishigaki A, Iwashina H and Hara Y. Reduction of blood glucose levels by tea catechins. Biosci Biotech Biochem.1993; 57: 525-527.

23. Maga JA. Simple phenol and phenolic compounds in food flavor. CRC Crit Rev Food SciNutr.1978; 10: 323-372.

24. Welsch CA, Lechane PA and Wessennan BP. Dietary phenolic compounds: inhibition of Na+ -dependent D-glucose uptake in rat intestinal brush border membrane vesicles. J Nutr.1989; 119: 1968-1704.

25. Joffe DJ. Effect of extended release Gymnema in Type 2 diabetes in children and adolescents (Consensus Statement), Diabet Care. 2000; 23: 381-89.

26. The wealth of India – Vol. VI: L-M. Council for Scientific and Industrial Research (CSIR), New Delhi, India, 1998, 305-306.

27. Kirtikar KR and Basu BD. Indian Medicinal Plants, Vol III, 2nd Edn., International book publisher, Dehradun. 2008,1627-1628.

28. Kottaimuthu R. Ethnobotany of the Valaiyans of Karandamalai, Dindigul District, Tamil Nadu, India. Ethnobotanical Leaflets. 2008; 12: 195-203.

29. R. Kottaimuthu, R. Ganesan, R. Kumuthakalavalli and N. Manikandan. On the rediscovery of marsdeniatirunelvelica (asclepiadaceae) - a rare and little known endemic species from Western Ghats. Bio Diversity. Elixir Bio Diver.2011; 38: 4243-4244.

30. Jiang S, Qiu L, Li Y, Li L, Wang X, Liu Z, Guo Y, Wang H. Effects of Marsdenia tenacissima polysaccharide on the immune regulation and tumor growth in H22 tumor-bearing mice. Carbohydr Polym. 2016; 137: 52-58.

31. Wang Q, Cao J, Wang P, Ge M. Antitumor Effect of C21 Steroidal Glycosides on Adenoid Cystic Carcinoma Cell Line SACC83. Clin Lab. 2015; 61(10): 1553-1560.

32. MohdNasuha NA, Choo YM. A new flavone from Malaysia Borneo Marsdenia tinctoria. Nat Prod Res. 2015; 30: 1-5.

33. Sikdar S, Mukherjee A, Khuda-Bukhsh AR. Anti-lung cancer potential of pure esteric-glycoside condurangogenin A against non small-cell lung cancer cells in vitro via p21/p53 mediated cell cycle modulation and DNA damage-induced apoptosis. Pharmacogn Mag. Suppl. 2015; 1: S73-85.

34. Pang X, Kang LP, Yu HS, Zhao Y, Han LF, Zhang J, Xiong CQ, Zhang LX, Yu LY, Ma BP. New polyoxypregnane glycosides from the roots of Marsdenia tenacissima. Steroids. 2015; 93: 68-76.

35. Gupta V, Kumar A, Khare A, Khare NK. Pregnane and pregnanetetraglycoside from Marsdeniaroylei. Nat Prod Res. 2011; 25(10): 959-973.

36. Gupta V, Kumar A, Khare A, Khare NK. Two pregnaneoligoglycosides from Marsdeniaroylei. Nat Prod Res. 2009; 23(14): 1342-1354.

37. Sethi A, Paswan S, Srivastava S, Khare NK, Bhatia A, Kumar A, Bhatia G, Khan MM, Khanna AK, Saxena JK. A new pregnane glycoside from Marsdeniaroylei as potential antioxidant and antidyslipidemic agents. J Asian Nat Prod Res. 2008; 10(11-12): 1023-1028.

38. Shivanisaini and Sunil Sharma. Antidiabetic effect of helianthus annuus l., seeds ethanolic extract in streptozotocin- nicotinamide induced type 2 diabetes mellitus.International Journal of Pharmacy and Pharmaceutical Sciences. 2013; 5(2): 382-387.

39. Sunday Oyedemi, graemebradley,and anthonyafolayan. antidiabetic activities of aqueous stem bark extract of strychnoshenningsiigilg in streptozotocin-nicotinamide type 2 diabetic rats. Iran J Pharm Res. 2012; 11(1): 221–228.

40. RakeshBarik, Sanjay Jain, Deep Qwatra, Amit Joshi, GirrajSharanTripathi, and Ravi Goyal. Antidiabetic activity of aqueous root extract of Ichnocarpusfrutescens in streptozotocin-nicotinamide induced type-II diabetes in rats. Indian J Pharmacol. 2008; 40(1): 19–22.

41. Jayaprasad B, Sharavanan PS, Sivaraj R. Antidiabetic effect of Chloroxylonswietenia bark extracts on streptozotocin induced diabetic rats. Beni-Suef University Journal of Basic and Applied Sciences. 2016; 5(1): 61-69.

42. Subramaniam Ramachandran, Aiyalu Rajasekaran, and Natarajan Adhirajan In Vivo and In Vitro Antidiabetic Activity of Terminalia paniculata Bark: An Evaluation of Possible Phytoconstituents and Mechanisms for Blood Glucose Control in Diabetes ,Hindawi Publishing Corporation, ISRN Pharmacology Article ID 484675, Vol. 2013, 1-10.

43. Pierre W, Gildas AJ, Ulrich MC, Modeste WN, Benoît NT, Albert K. Hypoglycemic and hypolipidemic effects of Bersamaengleriana leaves in nicotinamide/streptozotocin-induced type 2 diabetic rats. BMC complementary and alternative medicine. 2012; 12(1): 1.

44. Sharma U, Sahu RK, Roy A, Golwala DK. In vivo Antidiabetic Antioxidant Potential of Stephaniahernandifolia in Streptozotocin-Induced-Diabetic Rats. Journal of Young Pharmacists. 2010; 2(3): 255-260.

45. Madhavi M, Kiran S, Ramesh D. Evaluation of antidiabetic, antihyperlipidemic and antioxidant activities of Acacia leucophloea in streptozotocin-nicotinamide induced type II diabetic in rats. Global Journal of Pharmacology. 2014; 8(1): 64-72.

46. Bisht R, Bhattacharya S. Effect of various extracts of Desmodiumgangeticum on streptozotocin-nicotinamide induced type-2 diabetes. Asian Journal of Plant Science and Research. 2013; 3(3): 28-34.

47. Rahim AA, Mohamad J, Alias Z. Antidiabetic Activity of Aqueous Extract of Leptospermum flavescens in Alloxan Induced Diabetic Rats. SainsMalaysiana. 2014; 43(9): 1295-1304.

48. Verma A, Dewangan P, Kesharwani D, Kela SP. Hypoglycemic and hypolipidemic activity of scopoletin (Caumarin derivative) in streptozotocin induced diabetic rats. Int. J. Pharm. Sci. 2013; 22(1): 79-83.

49. Pradeep Kumar Bhateja and Randhir Singh, "Antidiabetic Activity of Acacia tortilis (Forsk.) Hayne ssp. raddiana Polysaccharide on Streptozotocin-Nicotinamide Induced Diabetic Rats," BioMed Research International, vol. 2014, Article ID 572013, 2014. doi:10.1155/2014/572013, 1-9

50. Tripathi YB. Insulin secreting and alpha-glucosidase inhibitory activity of hexane extract of Annonasquamosa Linn. in streptozotocin (STZ) induced diabetic rats. Indian journal of experimental biology. 2014; 52(6): 623-629.

51. Ramachandran S, Naveen KR, Rajinikanth B, Akbar M, Rajasekaran A. Antidiabetic, antihyperlipidemic and in vivo antioxidant potential of aqueous extract of Anogeissuslatifolia bark in type 2 diabetic rats. Asian Pacific Journal of Tropical Disease. 2012; 2: S596-602.

52. Sabitha V, Ramachandran S, Naveen KR, Panneerselvam K. Investigation of in vivo antioxidant property of Abelmoschusesculentus (L) moench. fruit seed and peel powders in streptozotocin-induced diabetic rats. Journal of Ayurveda and integrative medicine. 2012; 3(4): 188.

53. Liu C, Song J, Teng M, Zheng X, Li X, Tian Y, Pan M, Li Y, Lee RJ, Wang D, Di Wang PD, Diabetic Sprague Dawley Rats, Antidiabetic and Antinephritic Activities of Aqueous Extract of Cordyceps militaris Frui Body in Diet-Streptozotocin-Induced Diabetic Sprague Dawley Rats, Hindawi Publishing Corporation, Vol.2016,1-12

54. Ramachandran S, Rajasekaran A, Kumar KM. Antidiabetic, antihyperlipidemic and antioxidant potential of methanol extract of Tectonagrandis flowers in streptozotocin induced diabetic rats. Asian Pacific journal of tropical medicine. 2011; 4(8): 624-631.

55. Ahmed OM, Moneim AA, Yazid IA, Mahmoud AM. Antihyperglycemic, antihyperlipidemic and antioxidant effects and the probable mechanisms of action of Rutagraveolens infusion and rutin in nicotinamide-streptozotocin-induced diabetic rats. DiabetologiaCroatica. 2010; 39(1): 15-35.

56. Pari L, Saravanan R. Antidiabetic effect of diasulin, a herbal drug, on blood glucose, plasma insulin and hepatic enzymes of glucose metabolism in hyperglycaemic rats. Diabetes, obesity and metabolism. 2004; 6(4): 286-292.

57. Akter R, Hasan SR, Hossain MM, Ahmed T, Majumder MM, Akter M. In vitro Evaluation of Antioxidant Potential of Leaves of Opuntiadillenii Haw. Stamford Journal of Pharmaceutical Sciences. 2009; 2(1): 22-26.

58. Subramanian R, Asmawi MZ, Sadikun A. In vitro alpha-glucosidase and alpha-amylase enzyme inhibitory effects of Andrographispaniculata extract and andrographolide. ActaBiochim Pol. 2008; 55(2): 391-398.

59. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes. 1991; 40(4): 405-412.

60. K.M. Mathew. Illustrations on the Flora of the Tamil Nadu Carnatic. Asclepiadaceae. The Rapinat herbarium, St.Joseph's college, Tiruchirapalli. 1982; 2: 450.

61. K.M.Mathew. The Flora of the TamilNadu carnatic.The Rapinat herbarium, St.Joseph's college, Tiruchirapalli. 1983; 3: 952-953.

62. A.P.Jagtap and N.P. Singh. Fascicles of Flora of India. Fascicle 24. Botanical Survey of India.124-125.

63. Harborne JB. Methods of plant analysis. In Phytochemical Methods, Chapman and Hall, London. 1973, 1-32.

64. Wagner H, Bladt S and Zgainski EM. Plant Drug Analysis, Springer – Verlag, Berlin, 1984, 298-334.

65. Kokate CK. Practical Pharmacognosy 4th Edition, VallabhPrakashan, Delhi, 1994, 107-111.

66. Nenadis N, Wang L, Tsimidou M and Zhang H. Estimation of scavenging activity of phenolic compounds using the ABTS assay. J Agric Food Chem. 2004; 52: 4669–4674.

67. Re R, Pellegrini M, Proteggente A, Pannala A, Yang M and Rice EC. Antioxidant activity applying an improved ABTS radical cationdecolorisation assay. Free Radic. Biol. Med. 1999; 26: 1231–1237.

68. Huong NTT, Malsumato K, Kasai R, Yamasaki K and Watanabeth. In vitro antioxidant activity of Vietnamese ginseng saponin and its components. Biol Pharm Bull. 1998; 21: 978–981.

69. Elizabeth K and Rao MNA. Oxygen radical scavenging activity of curcumin. Intl J Pharm. 1990; 58: 237.

70. Jayaprakasha GK, JaganmohanRao L and Sakariah KK. Antioxidant activities of flavidin in different in vitro model systems. Bioorg Med Chem. 2004; 12: 5141–5146.

71. Oyaizu M. Studies on products of browning reaction: antioxidative activity of products of browning reaction prepared from glucosamine. Jpn J Nutr. 1986; 44: 307-315.

72. Fejes S, Blazovics A, Lugasi A, Lemberkovics E, Petri G and Kery A. In vitro antioxidant activity of Anthriscuscerefolium L. (Hoffm.) extracts. J. Ethnopharmacol. 2000; 69: 259-265.

73. Sanap SP, Ghosh S, Jabgunde AM, Pinjari RV, GejjiSP, Singh S, Chopade BA, Dhavale DD. Synthesis, computational study and glycosidase inhibitory activity of polyhydroxylatedconidine alkaloids – a bicyclic iminosugar. Org Biomol Chem. 2010; 8: 3307-3315.

74. Kim YM,Jeong YK, Wang MH, Lee WY, Rhee HI. Inhibitory effects of pine bark extract on alpha-glucosidase activity and postprandial hyperglycemia. Nutrition. 2005; 21: 756-761.

75. Lee JC, Kim HR, Kim J and Jang YS. Antioxidant property of an ethanol extract of the stem of Opuntiaficus-indica var. saboten. Journal of Agricultural and Food Chemistry. 2002; 50(22): 6490–6496.

76. Vijayan P, Vinod Kumar S, Dhanaraj SA, Mukherjee PK, Suresh B. *In vitro* cytotoxicity and antitumor properties of Hypericummysorense and Hypericumpatulum. Phytotherapy Research. 2003; 17: 952-956.

77. Yang C, Watson RT, Elmendorf JS, Sacks DB and Pessin JE. Calmodulin antagonists inhibit insulin-stimulated GLUT4 (glucose transporter 4) translocation by preventing the formation of phosphatidylinositol 3,4,5-trisphosphate in 3T3L1 adipocytes. Mol. Endocrinol. 2000; 14: 317–326.

78. Indian Pharmacopoeia, Chemical Tests and Assays, The Controller of Publications, Govt. of India, New Delhi, 1996, A34-A89.

79. Kumaran A and Karunakaran J. In vitro antioxidant activities of methanol Extracts of five Phyllanthus species from India. LWT. 2006; 40: 344–352.

80. Schanderl SH. In: Method in Food Analysis. Academic Press New York, 1970, 709.

81. Hsu C. Antioxidant activity of extract from Polygonumaviculare L. Biol. Res. 2006; 39: 281-288.

82. Sadasivam S and Manickam A. Biochemical Methods. 2nd Edition, New Age International (P) Ltd., New Delhi, 1991, 184-185.

83. The Organization of Economic Co-operation and Development. The OECD guideline for Testing of Chemical: 423 Acute Oral Toxicity, OECD, Paris, 2001, 1-14.

84. The Organization of Economic Co-operation and Development. The OECD guideline for Testing of Chemical: 407 Repeated Dose 28-days Oral Toxicity Study in Rodents, OECD, Paris, 2008, 1-13.

85. Masiello P, Broca C, Gross R, Roye M, Manteghetti M and Hillaire-Buys D. Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes. 1998; 47: 224-229.

86. Pari L and Srinivasan S. Antihyperglycemic effect of diosmin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-nicotinamide-induced diabetic rats. Biomed Pharmacother. 2010; 64: 477-481.

87. Pradeep K, Mohan CV, Gobianand K and Karthikeyan S. Silymarin modulates the oxidant-antioxidant imbalance during diethylnitrosamine induced oxidative stress in rats. Eur J Pharmacol. 2007; 560: 110-116.

88. Ramachandran A, Das AK, Joshi SR,Yajnik CS, Shah S, and Prasanna Kumar KM. Current Status of Diabetes in India and Need for Novel Therapeutic Agents. J Assoc Physician India. 2010; 58: 7-9.

89. Devasagayam TPA and Tarachand U. Decreased lipid peroxidation in the rat kidney during gestation. BiochemBiophys Res Commun. 1987; 145: 134-38.

90. Sinha AK (1972). Colorimetric assay of catalase. Anal Biochem.1972; 47: 389-394.

91. Marklund S, Marklund G. Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem. 1974; 47: 469-474.

92. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG and Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science. 1973; 179: 588-590.

93. Habig WH, Pabst MJ and Jakoby WB. Glutathione S- transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem. 1974; 249: 7130-7139.

94. Kumar EK, Janardhanan DK. Antidiabeticactivity of alcoholicstemextract of Nerviliaplicata in streptozotocin-nicotinamideinducedtype2diabeticrats. J Ethnopharmacol. 2011; 133: 480-483.

95. Balakumar P, Chakkarwar AV, Kumar V, Jain A and Reddy J. Experimental models for nephropathy.JRenin Angiotensin Aldosterone Syst. 2008; 9: 189-195.

96. Bieke FS, De vriese DS, Voorde VJ, Rasch R, Lameire HN and Flyvbjerg A. Long-term renal changes in the gota-kakizaki rat a model of lean type 2 diabetes. Nephrol Dial Transplant. 2004; 19: 1092-1097.

97. Alberti KG, Zimmet P, Shaw J. IDF Epidemiology Task Force Consensus Group. The metabolic syndrome-a new worldwide definition. Lancet 2005; 366: 1059-1062.

98. Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care. 1996; 19: 257-267.

99. Gallou G, Rueland A, Campion L, Maugendre D, Le Moullec N, Legras B, Allanic H, Cloarec L. Increase in Thiobarbituric acid reactive substances and

vascular complications in type 2 diabetes mellitus. DiabeteMetab. 1994; 20: 258-264.

100. Maxwell SR, Thomason H, Sandler D, Leguen C, Baxter MA, Thorpe GH, Jones AF & Barnett AH . Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. Eur J Clin Invest. 1997; 27: 484–490.

101. McLennan SV, Heffernan S, Wright L, Rae C, Fisher E, Yue DK, Turtle JR. Changes in hepatic glutathione metabolism in diabetes. Diabetes. 1991; 40(3): 344-348.

102. Christine H. Foyer and Graham Noctor. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context Plant. Cell and Environment. 2005; 28: 1056–1071.

103. Bnouham M, Ziyyat A, Mekhfi H, Tahri A, Legssyer A. Medicinal plants with potential antidiabetic activity - A review of ten years of herbal medicine research (1990-2000). Int. J. Diabetes Metab. 2006; 14: 1-25.

104. Moller DE. New drug targets for type 2 diabetes and the metabolic syndrome. Nature. 2001; 414(6865): 821-827.

105. Halliwell B and Gutteridge JMC. Free radicals in biology and medicine, 4th Edition, Oxford UK, 2007.

106. Valko M, Izakovic M, Mazur M, Rhodes CJ and Tesler J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem. 2004; 266: 37-56.

107. Poli G, Leonarduzzi G, Biasi F and Chiarpotro E. Oxidative stress and cell signaling. Curr Med Chem. 2004; 11: 1163-1182.

108. Rajkapoor B, Burkan ZE and Senthilkumar R. Oxidants and Human diseases: Role of antioxidant medicinal plants- A Review. Pharmacologyonline. 2010; 1: 1117-1131.

109. Sancehz-Moreno C. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci Tech Intl. 2002; 8: 121-37.

110. Soare JR, Dinis TCP, Cunha AP and Almeida LM. Antioxidant activity of some extracts of Thymus zygis. Free Radic Res.1997; 26: 469-78.

111. Deighton N, Brennan R, Finn C and Davis HV. Antioxidant properties of domesticated and wild Rubusspecies. J Sci Food Agric. 2000; 80: 1307-1313.

112. Halliwell B. Reactive oxygen species in living systems: Source, Biochemistry and role in human diseases. Am J Med. 1991; 91: 14-22.

113. Nakayama T. Suppression of hydroperoxide induced cytotoxicity by polyphenols. Cancer Res. 1994; 54: 1991s-1993s.

114. Sakanaka S, Tachibana Y and Okada Y. Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (Ka-Kiniha-Cha). Food Chem. 2005; 89: 569-575.

115. Dorman HJD, Peltoketo A, Hiltunen R and Tikkanen MJ. Characterization of the antioxidant properties of deodorized aqueous extracts from selected Lamiaceae herbs. Food Chem. 2003; 35: 255-262.

116. Gordon MH. The mechanisms of antioxidant action in vitro. In BJF Hudson (Ed.) Food antioxidants. Elsevier Applied Science, London, 1990, 1-18.

117. Holasova M, Fiedlerova V, Smrcinova H, Orsak M, Lachman J and Vavreinova S. Buck wheat – the source of antioxidant activity in functional foods. Food Res Intl. 2002; 35: 207- 211.

118. Gheldof N and Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. J Agric Food Chem. 2002; 50: 3050-3055.

119. Osawa T. Novel natural antioxidants for utilization in food and biological systems. In: Uritani I, Garcia VV, Mendoza EM (eds.) Postharvest biochemistry of plant food materials in the tropics. Japan Scientific Societies Press, Tokyo, Japan, 1994, 241-251.

120. Haslam E. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. J Nat Prod. 1996; 59(2): 205-215.

121. Gutteridge JM. Biological origin of free radicals, and mechanisms of antioxidant protection. ChemBiol Interact. 1994; 91(2-3): 133-140.

122. Bailey CJ. Potential new treatments for type 2 diabetes. Trends in pharmacolsci. 2000; 21: 259-65.

123. Sanap SP, Ghosh S, Jabgunde AM, Pinjari RV, Gejji SP, Singh S, Chopade BA and Dhavale DD. Synthesis, computational study and glycosidase inhibitory activity of polyhydroxylatedconidine alkaloids – a bicyclic iminosugar. Org BiomolChem. 2010; 8: 3307-3315.

124. Cheng AYY andFantus IG. Oral antihyperglycemic therapy for type 2 diabetes mellitus. Canadian Med Assoc Journal. 2005; 172: 213-226.

125. Matsui T, Tanaka T, Tamura S, Toshima A, Miyata Y and Tanaka K. Alpha glucosidase inhibitory profiles of catechins and theaflavins. J Agric Food Chem. 2007; 55: 99-105.

126. Bhandari MR, Jong-Anurakkun N, Hong G and Kwabata J. Alpha glucosidase and alpha amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (Bergenia ciliate, Haw.). Food Chem. 2008; 106: 247-252.

127. Smith C, Marks AD and Lieberman M. Marks's Basic Medical Biochemistry: A Clinical Approach, 2nd Edition, Lipincott Williams & Wilkins, Baltimore, Maryland 21201-2436 USA, 2005.

128. Hara Y and Honda M. The inhibition of alpha amylase by tea polyphenols. Agric Biol Chem. 1990; 54: 1939-1945.

129. Ryan CA. Proteinase inhibitor gene families: Strategies for transformation to improve plant defense herbivores. BioEssays. 1989, 20-24.

130. Lu S, Deng P, Liu X, Luo J, Han R, Gu X, Liang S, Wang X, Li F, Lozanov V, Patthy A and Pongor S. Solution structure of a-amylase inhibitor of the crop plant amaranth. J Biol Chem. 1999; 274: 20473-20478.

131. Latner A. Carbohydrate metabolism: Abnormalities of post absorptive blood sugar level. In Clinical Biochemistry, 2nd Ed., WB Saunders, Philadelphia, USA. 1958, 48.

132. Bollen M and Stalmans T. The antiglycogenolytic action of 1-deoxynojirimycin results from a specific inhibition of the α-1,6-glucosidase activity of the debranching enzyme. Eur J Biochem, 1989; 181: 775-780.

133. Yoshikuni Y. Inhibition of intestinal α-glucosidase activity and postprandial hyperglycemia by moranoline and its N-alkyl derivatives. AgricBiolChem. 1988; 52: 121-28.

134. Marshal JJ. Hypothesized that negatively charged residues of pilaic acid from membrane. Am ChemSoc Symposium Series. 1975; 15: 244-266.

135. Jaffe WG and Lette CLV. Heat labile growth inhibiting factors in beans (Phaseolus vulgaris). J Nutr. 1968; 94: 203-210.

136. Yoon SH and Robyt JF. Study of the inhibition of four alpha amylase by acarbose and its 4IV-alpha- maltododecaosyl analogues. Carbohydr Res. 2003; 338: 1969-1980.

137. Subramanian R, Asmawi MZ and Sadikun A. In vitro α-glucosidase and α-amylase enzyme inhibitory effects of Andrographispaniculata extract and andrographolide. Acta Biochimica Polonica, 2008; 55: 391-98.

138. Pari L andSatheesh MA. Effects of pterostilbene on hepatic key enzymes of glucose metabolism in streptozotocin and nicotinamide induced diabetic rats. Life Sci. 2006; 79: 641-645.

139. Jung M, Park M, Lee HC, Kang YH, Kang ES and Kim SK. Antidiabetic agents from medicinal plants. Curr Med Chem. 2006; 13: 1203–1218.

140. Andrade-Cetto A, Cardenas R and Ramirez-Reyes B. Hypoglycemic effect of Cecropiapeltata L. on N5-STZ type 2 diabetic rats. Pharmacology online, 2007; 3: 203-210.

141. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000; 9;408 (6809): 239-247.

142. Singh BN, Singh BR, Singh RL, Prakash D, Dhakarey R, Upadhyay G, Singh HB. Oxidative DNA damage protective activity, antioxidant and anti-

quorum sensing potentials of Moringaoleifera. Food ChemToxicol. 2009; 47(6): 1109-1116.

143. Eldar-Finkelman H, Krebs EG. Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. ProcNatlAcadSci U S A. 1997; 2;94(18): 9660-9664.

144. Juleen R. Zierath, Harriet Wallberg-Henriksson. From Receptor to Effector: Insulin Signal Transduction in Skeletal Muscle from Type II Diabetic Patients. 2002; 967: 120-134.

145. Ministry of Health and Welfare, Canada health protection branch. The testing of chemicals for carcinogenecity, mutagenicity and teratogenecity. 1977.

146. Perry JS. In: The ovarian cycle of mammals. University Review Biology, Vol 13, Increnne JE (ed), Oliver and Boyd, Edinburgh, 1971.

147. Lee J, Yee ST, Kim JJ, Choi MS, Kwon EY, Seo KI and Lee MK. Ursolic acid ameliorates thymic atrophy and hyperglycemia in streptozotocin-nicotinamide-induced diabetic mice. ChemBiol Interact. 2010; 188: 635-642.

148. Roden M and Bernroider E. Hepatic glucose metabolism in humans–its role in health and disease. Best Pract Res ClinEndocrinolMetab. 2003; 17: 365–383.

149. Weir GC, Clore ET, Zmachinski CJ and Bonner-Weir S. Islet secretion in a new experimental model for non-insulin-dependent diabetes. Diabetes. 1981; 30: 590-595.

150. Masiello P, Broca C, Gross R, Roye M, Manteghetti M and Hillaire-Buys D. Experimental NIDDM: development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes.1998; 47: 224-229.

151. Dixit N, Baboota S, Kohli K, Ahmad S, Ali J. Silymarin: A review of Pharmalogical aspects and bioavilability enhancement approches. Indian J Pharamacol. 2007; 39: 172-179.

152. Swanston-Fiatt SK, Day C, Bailey CJ and Flatt PR. Traditional plant treatments for diabetes: studies in normal and streptozotocin diabetic mice. Diabetologia. 1990; 33: 462-464.

153. G Rajiv Gandhi and P Sasikumar. Antidiabetic effect of MerremiaemarginataBurm. F. in streptozotocin induced diabetic rats. Asian Pac.J.Trop.Biomed. 2012; 2(4): 281-286.

154. Chen B, Bestetti G, Day RM and Turner AP. The synthesis and screening of a combinatorial peptide library for affinity ligands for glycosylated haemoglobin. Biosens Bioelectron. 1998; 13: 779-785.

155. Chandalia HB and Krishnaswamy PR. Glycated hemoglobin. Current sci. 2002; 83: 522-532.

156. Esterbauer H, Schaur RJ and Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free RadicBiol Med. 1991; 11: 81-128.

157. Shaker E, Mahmoud H and Manaa S. Silymarin, the antioxidant component and Silybummarianum extracts prevent liver damage. Food Chem Toxicol, 2010; 48: 803-806.

158. Marie A, Felix O, Errol Y and Helen N. Hepatic function enzymes and lipidperoxidation streptozotocin-induced diabetic rats fed bitter yam steroidal sapogenin extract. Diabetologia Croatica, 2003; 32: 17-23.

159. Wang F, Li M, Cheng L, Zhang T, Hu J and Cao M. Intervention with cilostazol attenuates renal inflammation in streptozotocin-induced diabetic rats. Life Sci. 2008; 83: 828-835.

160. Yousef MW, Omar HA, Ghanayem MN, Wahed MA and Morsy DM. Effect of some calcium channel blockers in experimentally induced diabetic nephropathy in rats. Int J Diabetes Metab. 2005; 14: 39-49.

161. Kiran G, Nandini CD, Ramesh HP and Salimath PV. Progression of early phase diabetic nephropathy in streptozotocin-induced diabetic rats evaluation of various kidney related parameters. Indian J Exp Biol. 2012; 50: 133-140.

162. Noriega CR, Avila O, Gutierrez E, Guerrero MC, Gerciglia R and Rojo C. Hypolipidemic activity of Eryngiumcarlinae on streptozotocin induced diabetic rats. Biochem Res Int. 2012; 10: 60-65.

163. Ramesh B, Viswanathan P and Pugalendi KV. Protective effect of umbelliferone on membranous fatty acid composition in streptozotocin-induced diabetic rats. Eur J Pharmacol. 2007; 56: 231-239.

164. Patel S, Shan SR and Goyal KR. Antihyperglycemic antihyperlipidemic and antioxidant effects a dihar a poly herbal ayurvedic formulation in streptozotocin induced diabetic rats. Indian J Exp Biol. 2009; 47: 564-570.

165. Siu YP, Leung KT, Tong MK and Kwan TH. Use of allopurinol in slowing the progression of renal disease through its ability to lower serum uric acid level. Am J Kidney Dis. 2006; 47: 51-59.

166. Grover JK, Rathi SS and Vats V. Antihyperglycemic effect of Eugenia jambalona and Tinospora cordifoliain experimental diabetes and their effects on key metabolic enzymes involved in carbohydrate metabolism. J. Ethnopharmacol. 2000; 73: 461-470.

167. Ramkumar KM, Ponmanickam P, Velayuthaprabhu S, Archunan G and Rajaguru P. Protective effect of Gymne mamontanum against renal damage in experimental diabetic rats. Food ChemToxicol. 2009; 47: 2516-2525.