PHARMACOLOGICAL STUDIES ON NOVEL ANTI-DIABETIC BIOACTIVE CONSTITUENTS OF SOME ETHNO MEDICINAL PLANTS OF MIZORAM

THESIS

SUBMITTED TO THE
MIZORAM UNIVERSITY, AIZAWL

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
HORTICULTURE, AROMATIC AND MEDICINAL PLANTS

By

Mrs. Binnu Singh
(Reg No. MZU/Ph.D/395 of 02/06/2011)

DEPARTMENT OF HORTICULTURE, AROMATIC
AND MEDICINAL PLANTS
MIZORAM UNIVERSITY
AIZAWL-796004
2016

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CERTIFICATE

This is to cetify that the thesis entitled, **'Pharmacological studies on novel anti-diabetic bioactive constituents of some ethno medicinal plants of Mizoram,'** submitted by Mrs. Binnu Singh, for the degree of **Doctor of Philosophy** in the Mizoram University, Aizawl, Mizoram, embodies the record of original investigations carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D degree. This work has not been submitted for any in any other university.

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DECLARATION BY THE CANDIDATE

Mizoram University

July, 2016

I, Binnu Singh, hereby declare that the subject matter of the thesis entitled "**Pharmacological studies on novel anti-diabetic bioactive constituents of some ethno medicinal plants of Mizoram**'' is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge, to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/ Institutes.

This is being submitted to the Mizoram University for the Degree of Doctor of Philosophy in Horticulture, Aromatic and Medicinal Plants.

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DEDICATED TO

MY PARENTS AND MY SON

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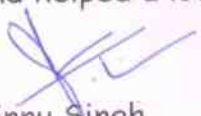

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CHAPTER 1
INTRODUCTION

CHAPTER 1: INTRODUCTION

Diabetes mellitus is an age old disease of worldwide significance and increasing prevalence. It is a metabolic disorder characterized by significantly raised blood glucose and is a major public health problem because it affects a significant minority of all populations. It has been noted in almost all ancient cultures with a tradition of written language such as Vedic, Chinese, Arabic, and Mediterranean cultures. Sushruta in Ayurveda has mentioned about the sweetness of diabetic urine. The first recorded description of diabetes mellitus dates back to around 1500 by the Ebers papyrus in Egypt (Soumyanath, 2006). In the first century A.D, the Greek physician Aeretaeus coined the word diabetes. Willis, in the 17th century observed that a diabetic's urine was sweet as if imbued with honey or sugar. Dobson in 1755 demonstrated the presence of sugar in the urine of diabetics (Wadkar *et al.,* 2008).

Diabetes mellitus is a carbohydrate metabolism disorder of endocrine system, characterized by high concentration of glucose in the blood. Glucose concentrations in normal individuals are regulated by hormonal factors. Any changes in glucose metabolism regulation results in diabetes, which is usually due to a deficiency of insulin release from the pancreas and/or a reduced response to insulin (Soumyanath, 2006). It occurs due to an absolute or relative deficiency of insulin secretion, action, or both (Ponnusamy *et al.,* 2011). It is characterized by the elevated glucose in plasma and ketoacidosis. Additional symptoms of diabetes mellitus include excessive thirst, glucosuria, polyuria, lipemia and hunger (Sachan *et al.,* 2009).

This disease is characterized by deranged secretions and effects of insulin and glucagons, extensive disturbances of carbohydrates, proteins and lipid metabolism, thickening of capillary basement membrane throughout the body leading to microangiopathy and macroangiopathy and long term complications will affect eye, kidney, nervous system and circulatory system. Not only does diabetes kills, but also causes of kidney failure, neuropathy, adult blindness, heart attack and strokes (Dhasarathan and Theriappan, 2011).

If left untreated, due to acute metabolic decompensation producing dehydration and acidosis, diabetes mellitus may lead to death. Over a period of years, even with treatment, elevated blood glucose levels result in damage to a number of organs, including the eyes, kidneys, nervous system, and blood vessels occurs (Soumyanath, 2006).

Diabetes basically can be categorized in to two types, i.e. diabetes insipidus and diabetes mellitus. Diabetes insipidus is a condition where the urine is normal, of low specific

gravity and excessive by the deficiency of ADH. It is caused due to tumor in posterior pituitary but may be idiopathic (Dhasarathan and Theriappan, 2011). There are two types of diabetes mellitus, one is insulin dependent diabetes mellitus and the other is insulin independent diabetes mellitus.

Type 1 diabetes is a condition due to absolute insulin deficiency secondary to autoimmune destruction of the insulin-containing β - cells of the pancreas gland. A number of lines of evidence suggest a genetic predisposition to developing type 1 diabetes. This type of diabetes represents 5 to 10% of all cases of diabetes.

Type 2 diabetes is a condition due to relative insulin deficiency and/or impaired biological response to insulin ("insulin resistance"). This is a state in which more than normal amounts of insulin are needed to produce a normal metabolic response to insulin. Insulin resistance is asymptomatic and may be found in subject's years before they develop type 2 diabetes. Type 2 diabetes is a more heterogeneous disease compared to type 1 diabetes. Type 2 diabetes accounts for 85% of all cases. The most common associations with insulin resistance are obesity and lack of physical fitness.

Other forms of diabetes may be secondary to other metabolic disorders, such as the endocrine disorder acromegaly in which excessive production of growth hormone inhibits the action of insulin or due to pancreatic problems (e.g., after surgical removal of pancreas) (Soumyanath, 2006).

Till date no drug is available or discovered which can cure diabetes (Dhasarathan and Theriappan, 2011).The underlying goal of all diabetes treatment and management is to maintain an adequate blood glucose concentration (Wadkar *et al.*, 2008). In critical and necessary situation, intake of hypoglycemic drugs solve the complications related with diabetes, but are not curative and continuous use of the synthetic anti-diabetic drugs causes side effects and toxicity Therefore, the need for discovering more effective and safe oral hypoglycaemic gents is ongoing (Zhou *et al* ., 2009).

The global prevalence of diabetes is rapidly rising at an alarming rate By 2030 the prevalence of diabetes for all age-groups worldwide is estimated to be around 4.4% compared to 2.8% in 2000. A projected rise from 171 million in 2000 to 366 million people with diabetes in 2030 is estimated (Mohan *et al.*, 2007).

In developing countries, the urban population is projected to double. There had been an important demographic change to diabetes prevalence across the world from a mild disorder of the elderly to a major cause of morbidity and mortality affecting the youth and middle aged people. Life expectancy may be halved by this disease. India is

endangered with a even more worst situation because of sedentary life style and food habit (Dhasarathan and Theriappan, 2011). The disorder affects more than 100 million people worldwide and by 2030 it is predicted to reach 366 million (Ponnusamy *et al.*, 2011).

Diabetes is a serious globally prevailing problem (Paul *et al.*, 2012). The term diabetes is derived from a Greek work "*diabanein*" which means to pass through, which is in reference to the excessive urine produced as a symptom of this disease (Shivashankar and Mani, 2011). It is a chronic metabolic characterized by elevated blood glucose levels which occurs when the body cannot use insulin effectively or cannot secrete enough insulin or both (Samsha, 2013; Bastaki S, 2005, IDF Diabetes Atlas, 2013). Insulin is required by body cells to absorb glucose (Samsha, 2013).

Insulin is the most potent anabolic hormone known (Saltiel and Kahn, 2001). Insulin secretion from the pancreas reduces glucose output by the liver, enhances glucose uptake by skeletal muscle, and suppresses fatty acid release from fat tissue (Stumvoll *et al.*, 2005). It increases the expression or activity of enzymes that catalyse glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyse degradation. It promotes the synthesis and storage of carbohydrates, lipids and proteins and inhibits their degradation and release into the circulation while stimulating the uptake of glucose, amino acids and fatty acids into cells (Saltiel and Kahn, 2001). The regulation of metabolism by insulin is depicted in **Figure - 1.1**.

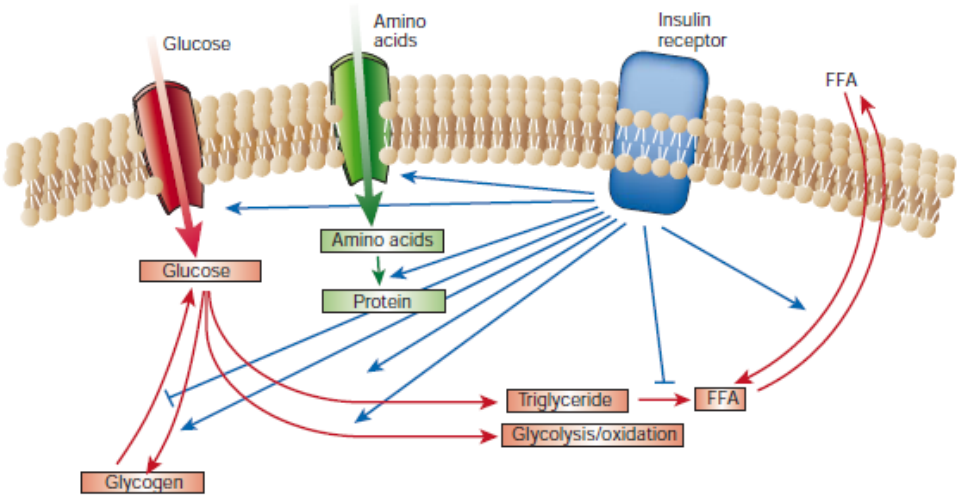


Figure - 1.1: The Regulation of metabolism by insulin
(Source: Saltiel and Kahn, 2001)

Insulin deficiency can cause disturbances of carbohydrate, fat and protein metabolism thereby leading to conditions such as chronic hyperglycemia, glycosuria, polyuria,

polyphagia, polydypsia, ketonaemia and negative nitrogen balance (Paul *et al.*, 2012; Bastaki, 2005). The details of the same is explained in detail in **Figure – 1.2**.

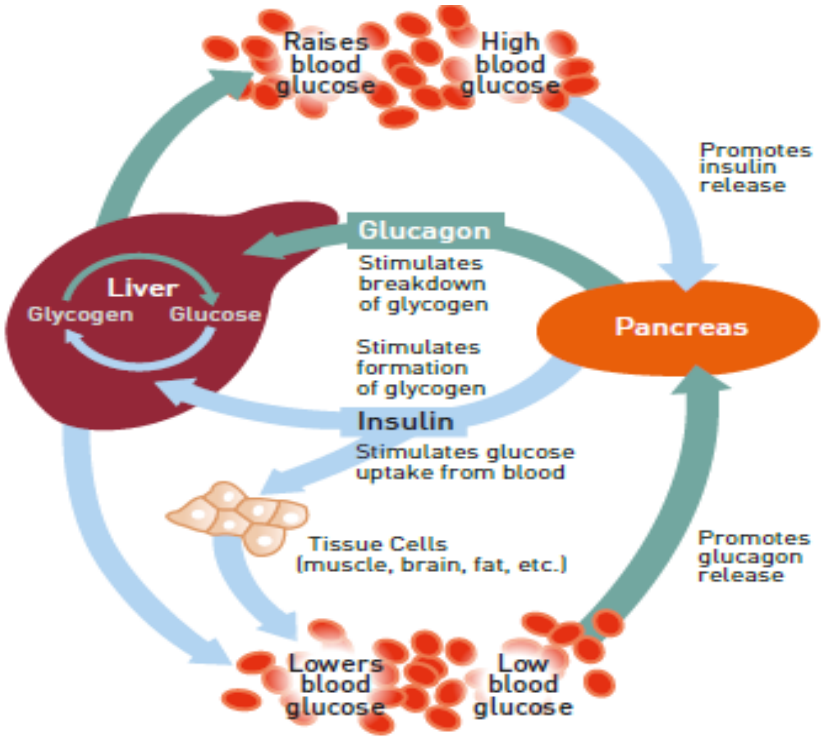


Figure – 1.2: Insulin Production and Action
(**Source:** IDF Diabetes Atlas, 2013)

Criteria for screening and diagnosis of Diabetes

In 1965, the World Health Organization (WHO) put forward the diagnostic criteria and the classification of diabetes and then by the National Diabetes Data Group (NDDG) in 1979. This was followed by recommendations by the WHO in 1980 and 1985 (Bastaki, 2005). The updated recommendations are published both by the American Diabetes Association (ADA) and by the WHO. Both groups agreed on the recommendations and criteria (Bastaki, 2005). These recommendations and updates are provided as **Table 1.1** and **Table 1.2**.

Table -1.1: Summary of WHO diagnostic criteria for diabetes and intermediate hyperglycaemia: At a glance

	1965	1980	1995	1999
Normal **Fasting glucose** **2-h glucose**	 Not specified < 6.1 mmol/l	Not defined	Not defined	 < 6.1 mmol/l Not specified but < 7.8 mmol/l implied
Diabetes **Fasting glucose** **2-h glucose**	Not specified < 7.2 mmol/l	< 8.0 mmol/l And / or ≥ 11.1 mmol/l	< 7.0 mmol/l or ≥ 11.1 mmol/l	< 7.0 mmol/l or ≥ 11.1 mmol/l
IGT **Fasting glucose** **2-h glucose**	Referred to as boderline state 6.1 to 7.1 mmol/l	< 8.0 mmol/l And ≥ 8.0 and < 11.0 mmol/l	< 7.0 mmol/l And ≥ 7.8 and < 11.1 mmol/l	< 7.0 mmol/l And ≥ 7.8 and < 11.1 mmol/l
IFG **Fasting glucose** **2-h glucose**	Not defined	Not defined	Not defined	≥ 6.1 and < 7.0 mmol/l And < 7.8 mmol/l (if measured

(Source: WHO Publication, 2006)

Table – 1.2: Comparison of 1999 WHO and 2003 ADA diagnostic criteria

	WHO 1999	ADA 2003
Diabetes **Fasting glucose** **2-h glucose***	≥ 7.0 mmol/l or ≥ 11.1 mmol/l	≥ 7.0 mmol/l or ≥ 11.1 mmol/l
IGT **Fasting glucose** **2-h glucose**	≥ 7.0 mmol/l or ≥ 7.8 mmol/l and < 11.1 mmol/l	Not required ≥ 7.8 mmol/l and < 11.1 mmol/l
IFG **Fasting glucose** **2-h glucose**	6.1 and 6.9 mmol/l and (if measured) (measurement recommended)	5.6 and 6.9 mmol/l Measurement not recommended (but if measured should be < 11.1 mmol/l)

(Source: WHO Publication, 2006)
*Venous plasma glucose 2-h after ingestion of 75 g oral glucose load

Diabetes may be diagnosed based on A1C criteria or plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) as depicted in **Table 3.3**. The same tests are used to both screening for and diagnose diabetes (Diabetes Care, 2015).

Table – 1.3: Criteria for the diagnosis of diabetes

Test	Results	Interpretation
HbA1c	6.5% or higher	Diabetes
	5.7% - 6.4%	Impaired glucose tolerance
	Lower than 5.7%	Normal

Fasting* Plasma Glucose	126 mg/dL or higher	Diabetes
	140 -199 mg/dL	Impaired glucose tolerance
	Lower than 140 mg/dL	Normal
Random Plasma glucose	200 mg/dL or higher	Diabetes
	100 – 125 mg/dL	Impaired glucose tolerance
	Lower than 100 mg/dL	Normal

(Source: Diabetes Care 2015)
*Fasting is defined as no calorie intake for atleast 8 h

Prevalence

Currently around 382 million people worldwide or 8.3% of adult population have diabetes. This estimate is expected to jump to 592 million, or one adult in 10 by 2035, if these trend continues. Approximately 65,000 children worldwide develop type 1 diabetes on an annual basis. In 2013, the death toll of people aged between 20 and 79 years who died from diabetes is approximately 5.1 million which is 8.4% of global all-cause mortality in this age group. In countries such as China, India, USA, and the Russian Federation, the highest number of deaths occurred due to diabetes. **Figure – 1.3** depicts the Top 10 countries of number of people with diabetes (IDF Diabetes Atlas, 2013).

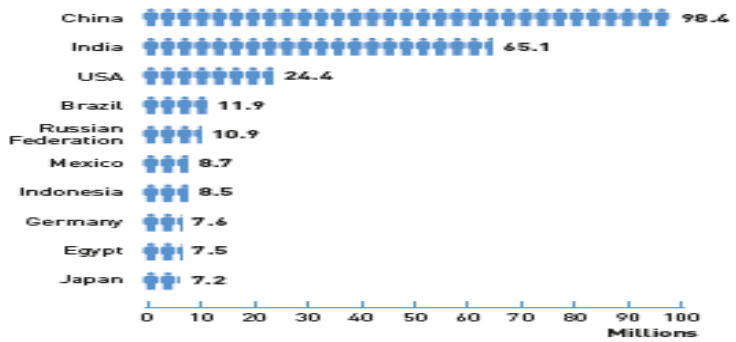


Figure – 1.3: Top 10 countries / territories of number of people with diabetes (20-79 years)
(Source: IDF Diabetes Atlas, 2013)

Types of Diabetes

Diabetes is generally characterized as existing in four major clinical classes:

1. Type 1 - Insulin-dependent diabetes mellitus [IDDM]
2. Type 2 - Noninsulin-dependent diabetes [NIDDM]
3. Gestational diabetes mellitus [GDM]
4. Other specific types of diabetes

Type 1 - Insulin-dependent) diabetes mellitus [IDDM]

It is a T cell mediated (CD4+ and CD8+) catabolic disorder characterized by conditions:

- Very low or absence of circulating insulin
- Elevated plasma glucagon and
- Failure of pancreatic beta cells to respond to all insulin-secretory stimuli (Paul *et al.,* 2012)

This type of diabetes occurs in patients who have little or no endogenous insulin secretory capacity (Bastaki S, 2005). The pancreas shows lymphocytic infiltration and destruction of insulin-secreting cells of the islets of Langerhans (Paul *et al.,* 2012). The pathophysiology of type 1 diabetes and its causes are presented in **Figure – 1.4** and **Figure - 1.5** respectively.

Type 1 diabetes occurs due to an autoimmune process which results in immunological destruction of pancreatic ß cells, thereby leading to insulin deficiency (Bastaki S, 2005), (IDF Diabetes Atlas, 2013). Autoimmune process of ß-cell destruction is due to the presence of islet cell antibody (ICA), anti-glutamic acid decarboxylate (anti-GAD), IA-2 or insulin antibodies. Autoimmune diseases such as Grave's disease, Hashimoto's thyroiditis and Addison's disease may be associated with type 1 diabetes mellitus. For survival, these patients will require insulin therapy (Bastaki, 2005).

The symptoms in both types of diabetes are similar but vary in intensity. Symptoms in type 1 diabetes are more rapid and more typical (Bastaki, 2005). The signs and symptoms of type 1 diabetes include frequent urination, extreme hunger, unusual thirst, unusual weight loss, extreme fatigue, cramps, constipation, blurred vision, candidiasis and irritability (Samsha, 2013, Bastaki, 2005). Patients with long standing type 1 diabetes are susceptible to macrovascular disease (coronary artery, heart, and peripheral vascular diseases) and microvascular complications (Bastaki, 2005).

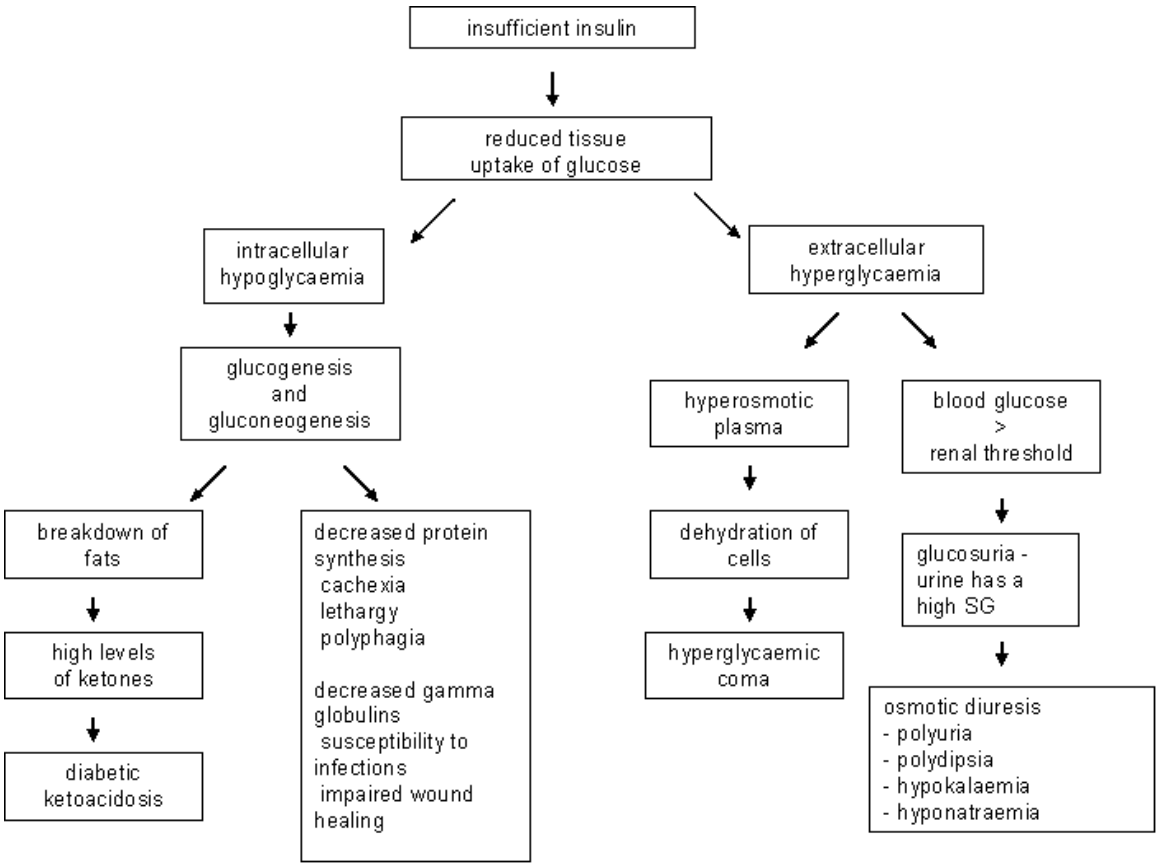


Figure – 1.4: Pathophysiology of Type 1 Diabetes (Paul *et al.*, 2012)

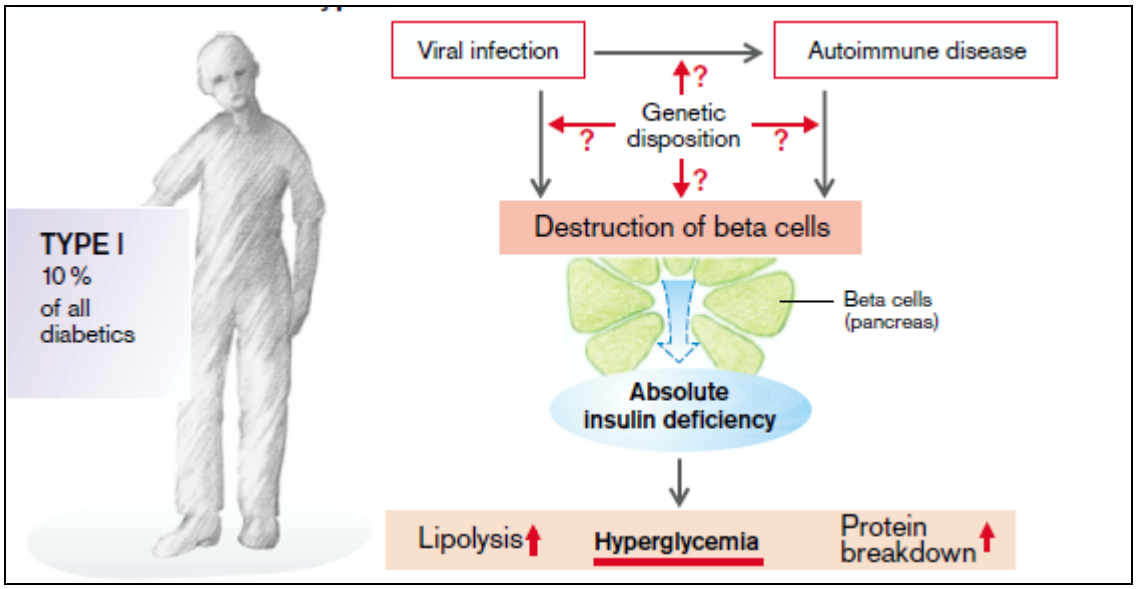


Figure - 1.5: Causes of Diabetes Mellitus - Type I
(Source: Silbernagl and Lang, 2000)

Type 2 - Noninsulin-dependent diabetes [NIDDM]

Type 2 Diabetes is characterized by progressive insulin secretory defect and insulin resistance (Paul *et al.*, 2012), (Bastaki, 2005). Both insulin secretion and insulin action are affected in type 2 diabetes. Decreased insulin secretion reduces insulin signalling in its target tissues and insulin resistance pathways affect the action of insulin in each of the major target tissues. This leads to increased circulating fatty acids and the hyperglycaemia of diabetes. The raised concentrations of glucose and fatty acids in the bloodstream in turn will feed back to worsen both insulin secretion and insulin resistance (Stumvoll *et al.*, 2005). The various factors shown that contribute to the pathogenesis of type 2 diabetes are (Ozougwu *et al.*, 2013):

- Obesity/overweight (especially excess visceral adiposity)
- Excess glucorticoids (Cushing's syndrome or steroid therapy)
- Excess growth hormone (acromegaly)
- Polycystic ovary disease
- Lipodystrophy (acquired or genetic, associated with lipid accumulation in liver)
- Autoantibodies to the insulin receptor
- Mutations of insulin receptor
- Mutations of the peroxisome proliferators' activator receptor γ (PPAR γ)
- Mutations that cause genetic obesity (e.g., melanocortin receptor mutations)
- Hemochromatosis (a hereditary disease that causes tissue iron accumulation)

Insulin secretory defect and insulin resistance leading to chronic hyperglycaemia and multiple disturbances in carbohydrate, protein and fat metabolism including (General practice management of Type 2 diabetes, 2014-2015):

- β islet cell dysfunction
- α cell dysfunction with elevated glucagon levels
- hepatic gluconeogenesis
- insulin resistance with elevated glucose production
- muscle cell insulin resistance with decreased glucose uptake
- kidney adaptation with altered gluconeogenesis
- increased glucose reabsorption via increased sodium glucose transporter protein activity
- diminished incretin hormonal production or incretin resistance
- maladaptive cerebral hormonal responses to insulin and appetite increased lipolysis with elevated free fatty acids (General practice management of Type 2 diabetes, 2014-2015).

The pathophysiology of type 2 diabetes and its causes are presented in **Figure 1.6** and **Figure 1.7** respectively.

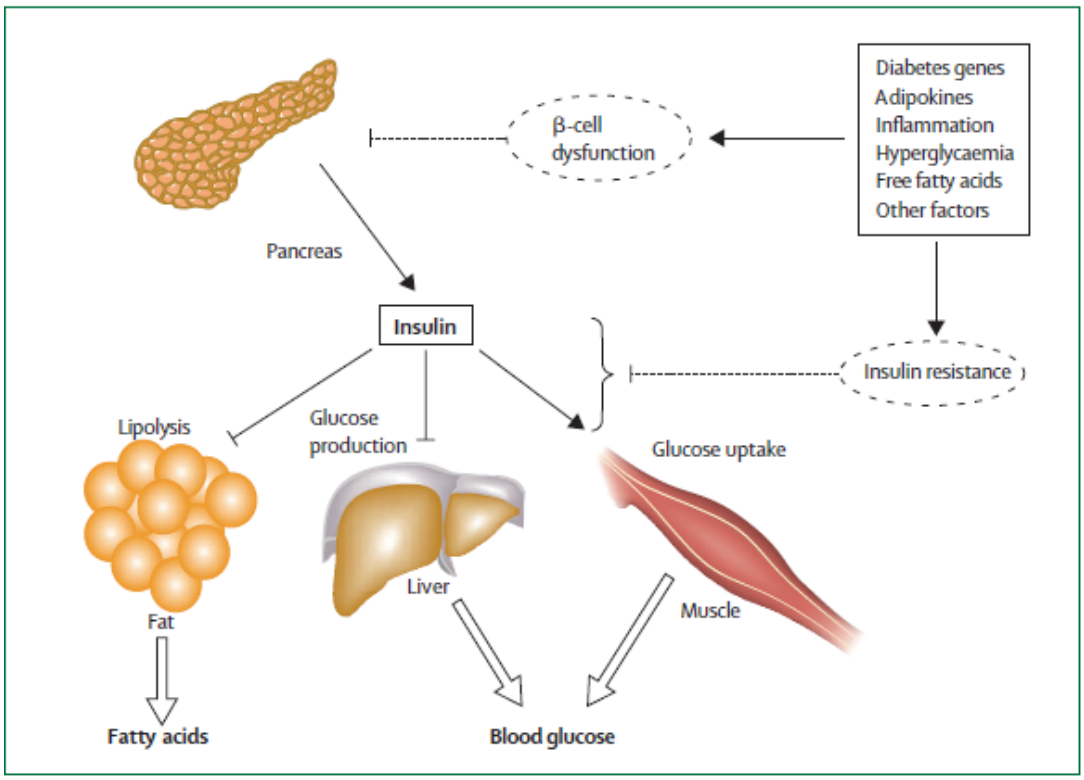


Figure - 1.6: Pathophysiology of hyperglycaemia and increased circulating fatty acids in type 2 diabetes
(Source: Stumvoll *et al.*, 2005)

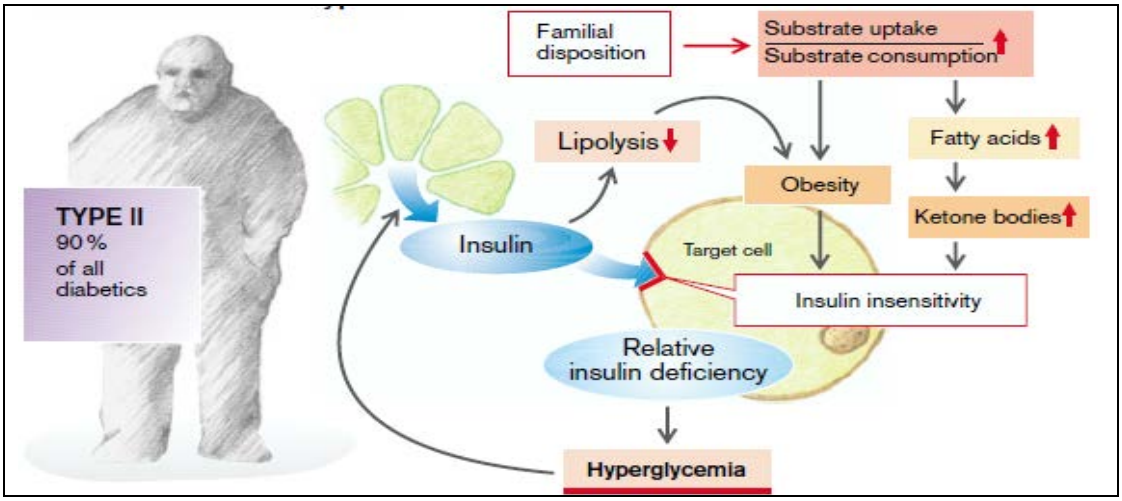


Figure – 1.7: Causes of Diabetes Mellitus - Type II
(Source: Silbernagl and Lang, 2000)

Type 2 diabetes is a serious global health problem and constitutes about 85% to 95% of all diabetes. It has evolved due to various factors such as rapid cultural and social changes, ageing populations, increasing urbanization, dietary changes, reduced physical activity, and unhealthy lifestyle (IDF Diabetes Atlas, 2013). Many people suffering from

type 2 diabetes exhibit no symptoms of the disease or the symptoms can be very mild and most cases are diagnosed due to complications or incidentally (Bastaki S, 2005). These symptoms include any of the type 1 diabetes symptoms, blurred vision, tingling or numbness in the hands or feet, are slow healing of cuts and bruises, recurring skin, gum, or bladder infections and frequent infections (Samsha, 2013). The type of diabetes is usually controlled through dietary therapy, exercise and hypoglycemic agents (Bastaki S, 2005).

Patients with type 2 diabetes commonly associated with raised blood pressure, a disturbance of blood lipid levels and a tendency to develop thrombosis. They are susceptible to a high risk of large vessel atherosclerosis commonly associated with hypertension, hyperlipidemia and obesity (Bastaki S, 2005), (National Institute for Health and Care Excellence, 2014).

It carries increased cardiovascular risk such as coronary artery disease (leading to heart attacks, angina); peripheral artery disease (leg claudication, gangrene); and carotid artery disease (strokes, dementia). Microvascular complications of diabetes include eye damage (blindness), kidney damage (sometimes requiring dialysis or transplantation) and nerve damage (resulting in amputation, painful symptoms, erectile dysfunction, and other problems) (National Institute for Health and Care Excellence, 2014).

Gestational diabetes mellitus (GDM)

The onset or recognition of this form of diabetes is due to development of insulin resistance later in the pregnancy (IDF Diabetes Atlas, 2013), (General practice management of Type 2 diabetes, 2014-2015) usually during second or third trimester (Bastaki S, 2005). It occurs in about 4% of all pregnancies (Bastaki S, 2005). This form of diabetes generally resolves once the pregnancy ends (IDF Diabetes Atlas, 2013). Patients with GDM an increased risk of developing diabetes, usually type 2 diabetes (Bastaki S, 2005). Also babies born to mothers who have hyperglycaemia in pregnancy have increased risk of developing type 2 diabetes later in life (IDF Diabetes Atlas, 2013).

Other specific types of diabetes

Other types of diabetes may occur due to the following reasons (Bastaki S, 2005), (IDF Diabetes Atlas, 2013), (American Diabetes Association, 2010):

- Genetic defects of the pancreatic ß cell or in insulin action pathways (insulin receptor mutations or post-receptor defects)

- Disease of the exocrine pancreas (e.g., Pancreatitis, pancreatic reaction, cystic fibrosis, Trauma/ pancreatectomy, Neoplasia, Hemochromatosis, Fibrocalculous pancreatopathy)
- Drug - or chemical-induced causes (e.g. in the treatment of HIV/AIDS or after organ transplantation). Certain drugs like glucocorticoids, pentamidine, niacin, Vacor, Nicotinic acid, Thyroid hormone, Diazoxide, Thiazides, Dilantin and a-interferon may also lead to diabetes mellitus.
- Endocrinopathies producing insulin counter regulatory hormones excess (e.g., Acromegaly, Cushing's syndrome, Glucagonoma, Pheochromocytoma, Hyperthyroidism, Somatostatinoma, Aldosteronoma) (Bastaki S, 2005) (American Diabetes Association, 2010)
- Infections such as congenital rubella and Cytomegalovirus may also lead to diabetes mellitus (American Diabetes Association, 2010).
- Uncommon forms of immune-mediated diabetes such as "Stiff-man" syndrome and Anti-insulin receptor antibodies may also lead to diabetes mellitus (American Diabetes Association, 2010)
- Other genetic syndromes sometimes associated with diabetes are Down syndrome, Klinefelter syndrome, Turner syndrome, Wolfram syndrome, Friedreich ataxia, Huntington chorea, Laurence-Moon-Biedl syndrome, Myotonic dystrophy, Porphyria, Prader-Willi syndrome (American Diabetes Association, 2010).

Maturity-onset diabetes of the young (MODY) is a familial form of Non-Insulin Dependent diabetes mellitus with autosomal-dominant inheritance. It generally develops in childhood, adolescence or young adulthood, and is characterized by primarily insulin-secretion defects. MODY is not a single entity, but involves genetic, metabolic, and clinical heterogeneity (Bastaki, 2005). All the above type of diabetes is diagrammatically explained in **Figure 1.8**.

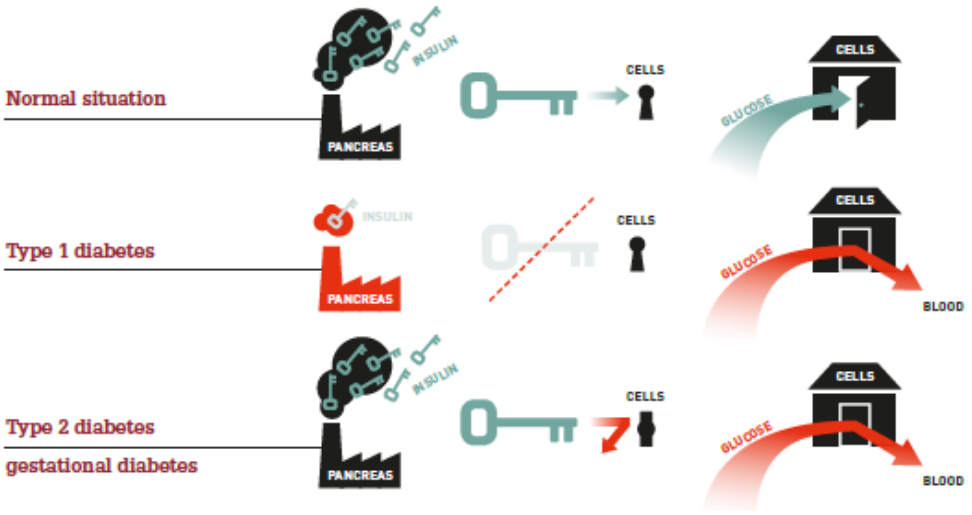


Figure – 1.8: Pathophysiology of diabetes
(Source: IDF Diabetes Atlas, 2013)

The characteristics of different types of diabetes are summarized in the **Table 1.4** below:

Table - 1.4: Characteristics of different types of diabetes

Diabetes type	Age of onset	Etiology	Clinical	Insulin requirement
Type 1 Diabetes	Childhood but can be of any age	Unknown etiology - possibly hereditary , autoimmune, or related to viral infections	Lack of insulin production	Immediately
Type 2 Diabetes	Adults usually older than 30 years of age	Unknown etiology - possibly hereditary and/or lifestyle	Ineffective use of insulin	Within 10 years
Gestational	Pregnancy but may develop Type 2	Pregnancy	Hormones interfere with the action of insulin	Case specific but usually resolve with diet, exercise and delivery
Latent autoimmune diabetes of the adult	Adult	One antibody seen in Type 1 – etiology as in Type 1	Slow progression of Type 1	Within 4 years
Insulin resistance Syndrome	Adult	Unknown etiology – possibly hereditary and/or lifestyle	Pre-diabetes or metabolic syndrome	None

(Source: Rogers, 2007)

Associated complications related with diabetes

People suffering from diabetes are at risk of developing a number of disabling and life-threatening health problems such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness (**Figure 1.9**). These complications lead to reduced life expectancy, increasing disability and enormous health costs (IDF Diabetes Atlas, 2013).

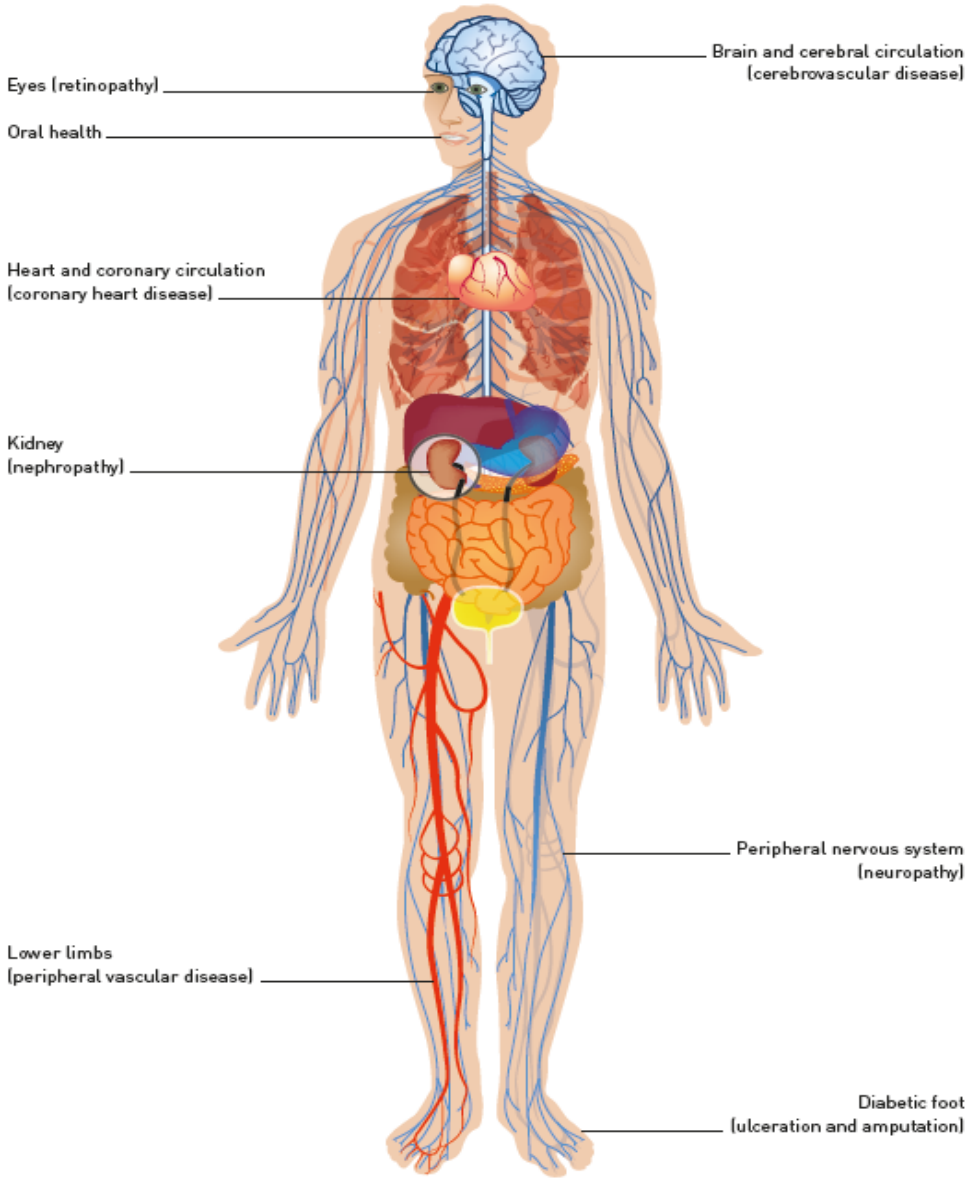


Figure – 1.9: Complications related with diabetes
(Source: IDF Diabetes Atlas, 2013)

Acute effects of insulin deficiency

The absence of insulin in acute insulin deficiency, affects the glucose metabolism resulting in hyperglycemia. The extracellular accumulation of glucose leads to hyperosmolality which in turn leads excess amount of glucose in the kidney thereby leading to glucose excretion in the urine and an osmotic diuresis with renal loss of water (polyuria), Na+, and K+, dehydration, and thirst.

No hypokalemia occurs despite the renal loss of K^+, because the cells give up K^+ as a result of reduced activity of Na^+-K^+-2 Cl^- co-transport and of Na^+-K^+- ATPase. The negative K^+ balance is disguised by the extracellular K^+ concentration. In such a situation, administration of insulin causes a life-threatening hypokalemia. Hypovolemia and impairment of the circulation occurs because of dehydration caused due to diuresis. This results in release of aldosterone, epinephrine and glucocorticoids. Aldosterone increases the K^+ deficiency, while the release of epinephrine and glucocorticoids exacerbates the catabolism. The renal excretion of glucose diminishes due to reduced renal blood flow and thus encourages the hyperglycemia.

Further phosphate (Pi) and magnesium are lost and excreted by the kidney. When there is insulin deficiency, proteins are broken down to amino acids in muscles and other tissues leading to muscular weakness. Lipolysis leads to release of fatty acids into blood (hyperlipidacidemia). Accumulation of acetoacetic acid and β-hydroxybutyric acid produced by the liver from the fatty acids leads to acidosis, thereby forcing the patient to breathe deeply (Kussmaul breathing). Some of the acids are broken down to acetone (ketone bodies). Also, triglycerides are formed in the liver from fatty acids and incorporated into VLDL thereby developing fatty liver. The breakdown of proteins and fat and polyuria results in weight loss. The abnormal metabolism, electrolyte disorders and the changes in cell volume can impair neuronal function and cause hyperosmolar or ketoacidotic coma.

Hyperglycemia and hyperosmolarity are the main effects of relative insulin deficiency. Increased proteolysis and lipolysis (ketoacidosis) are added to these effects in case of absolute insulin deficiency (Silbernagl and Lang, 2000).

The acute effects of diabetes mellitus are depicted in **Figure 1.10**.

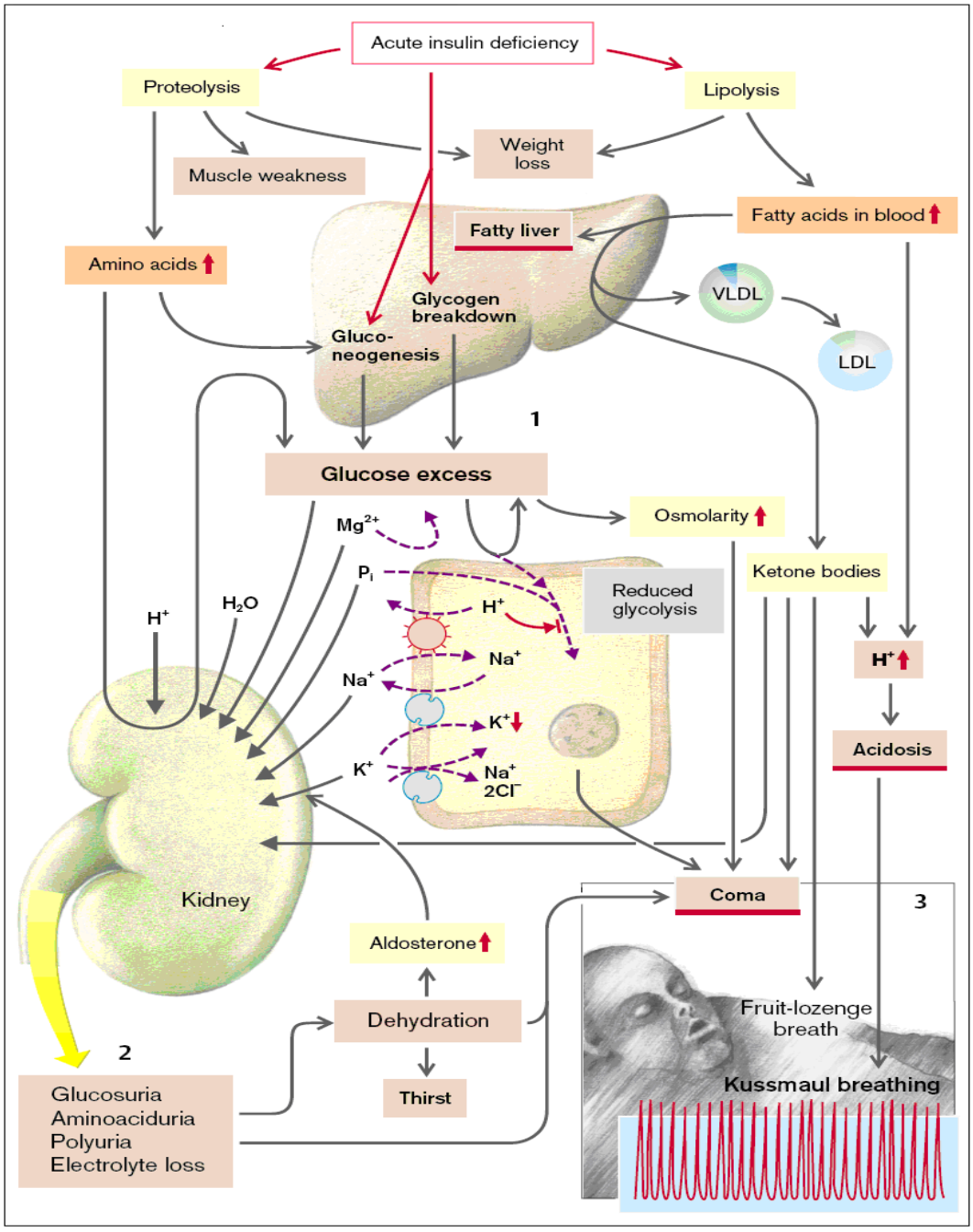


Figure - 1.10: Acute effects of Diabetes Mellitus
(Source: Silbernagl and Lang, 2000)

Late complications of prolonged hyperglycemia (Silbernagl and Lang, 2000).

Inadequately treated relative or absolute insulin deficiency in the course of years or decades will lead to metabolic abnormalities and will cause extensive irreversible changes in the body. Hyperglycemia plays a central role in this.

Glucose is reduced to sorbitol in cells that contain the enzyme aldosereductase. Hexahydric alcohol cannot pass through the cell membrane, as a result of which its cellular concentration increases and the cell swells. Sorbitol accumulation in the lens of the eye leads to water being incorporated and impairing lenticular transparency (cataract). Sorbitol accumulation in the Schwann cells and neurons reduces nerve conduction (polyneuropathy), affects the autonomic nervous system, reflexes, and sensory functions.

As a result of extracellular hyperosmolarity, cells that do not take up glucose in sufficient amounts will shrink. Shrunk lymphocytes have impaired functions such as the formation of superoxide's, which are important for immune defense, thus diabetics are more prone to infection of the skin (boils) or kidney (pyelonephritis). These infections, in turn, increase the demand for insulin, because they lead to an increased release of insulin-antagonistic hormones. Hyperglycemia promotes the formation of sugar containing plasma proteins such as fibrinogen, haptoglobin, α_2 -macroglobulin as well as clotting factors V–VIII. Clotting tendency and blood viscosity may increase and raise the risk of thrombosis.

By binding of glucose to free amino-groups of proteins and a subsequent irreversible Amadori reaction, advanced glycation end products (AGEs) are formed. They occur in increasing amounts in the elderly. A protein network can be formed through the formation of pentosin. AGEs promote the deposition of collagen in the basement membranes of the blood vessels by binding to respective receptors of the cell membrane.

The formation of connective tissue is in part stimulated via transforming growth factor β (TGF- β). The collagen fibers can be changed by glycosylation. Both these changes produce thickening of the basement membranes with reduced permeability and luminal narrowing (microangiopathy). It causes changes in the retina, which are results of microangiopathies that ultimately may lead to blindness (retinopathy). In the kidney glomerulosclerosis (Kimmelstiel– Wilson) develops, which can result in proteinuria, reduced glomerular filtration rate due to a loss of glomeruli, hypertension, and renal failure. Due to the high amino acid concentration in plasma, hyper-filtration occurs in the remaining intact glomeruli resulting in its damage as well. A rise of VLDL in blood and the raised clotting tendency of the blood together with hypertension promotes the development of a microangiopathy that can further damage the kidneys and cause myocardial infarction, cerebral infarction, and peripheral vascular disease.

Lastly, glucose can react with hemoglobin (HbA) to form HbA_{1c}. Increased concentration of HbA_{1c} in blood indicates hyperglycemia. HbA_{1c} has a higher oxygen affinity than HbA and thus releases oxygen in the periphery less readily. The persisting insulin deficiency further leads to a reduction in the erythrocytic concentration of 2, 3-bisphosphoglycerate (BPG), which, as allosteric regulator of hemoglobin, reduces its oxygen affinity. The BPG deficiency also results in an increased oxygen affinity of HbA.

The late complications of diabetes mellitus are depicted in **Figure 1.11.**

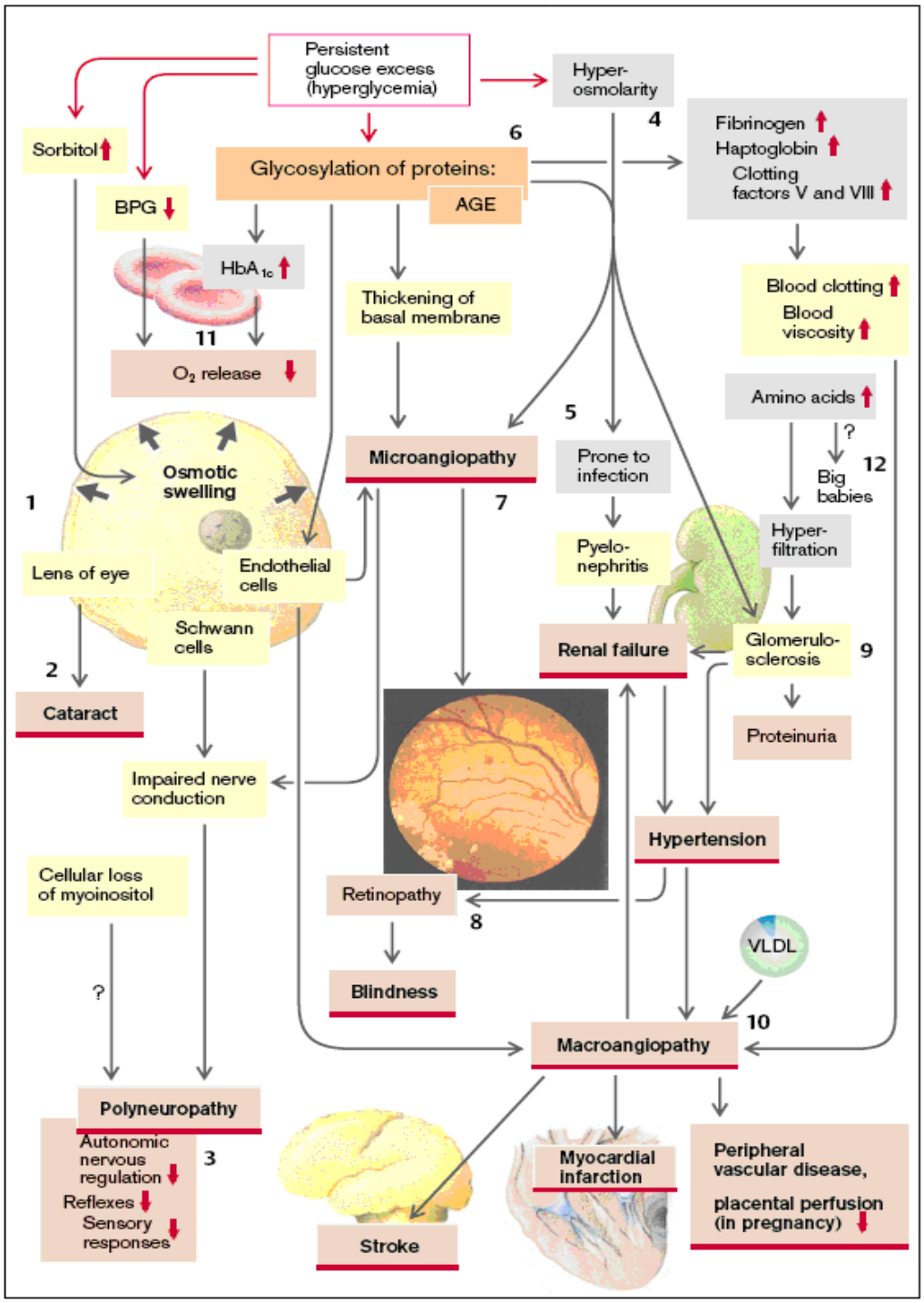


Figure - 1.11: Late complications of Diabetes Mellitus
(Source: Silbernagl and Lang, 2000)

Screening of Type 1 and Type 2 Diabetes

Early Warning Signs for Type 1 and Type 2 Diabetes includes symptoms such as increased urination, increased thirst, increased appetite or unexplained weight loss. In case one or more of the symptoms is present, a blood glucose level should be checked (Stang and Story, 2005).

(a) Screening for Type 1 Diabetes
No screening recommendations for the diagnosis of type 1 diabetes in adolescents have been established.

(b) Screening for Type 2 Diabetes
The screening recommendations listed in **Table 1.5** are from the American Diabetes Association (Stang and Story, 2005).

Table – 1.5: Testing for Type 2 Diabetes in children and Adolescents

Criteria*	Overweight (BMI $\geq 85^{th}$ percentile for age and gender, weight for height $\geq 85^{th}$ percentile or weight $\geq$ 120% of ideal for height), plus any two of the following risk factors: • Family history of type 2 diabetes in first- or second degree relative • Race/ethnicity (American Indian, African American, Hispanic, Asian/Pacific Islander) • Signs of insulin resistance or conditions associated with insulin resistance (acanthosis nigricans, hypertension, dyslipidemia, polycystic ovary syndrome)
Age of initiation	Age 10 years or at onset of puberty if puberty occurs at a younger age.
Frequency	Every 2 years
Test	Fasting plasma glucose is the preferred method for screening

*Clinical judgment should be used to test for diabetes in high-risk patients who do not meet these criteria.
(Source: Stang and Story, 2005)

Differentiation is based on age, rate of clinical onset, body weight, family history and urinary ketones. Once a diagnosis is made it is important to determine the type of diabetes. Usually there is clear clinical evidence and differentiation is easy. Few features which clearly differentiate type 1 and 2 diabetes in young people is depicted in **Table 1.6** (National Institute for Health and Care Excellence, 2014).

Table – 1.6: Features to differentiate type 1 and 2 diabetes in young people (National Institute for Health and Care Excellence, 2014)

	Type 1 diabetes	**Type 2 diabetes**
Onset	Acute - symptomatic	Slow – often asymptomatic
Clinical picture	Weight loss Polyuria Polydipsia	Obese Strong family history of type 2 diabetes 'Ethnicity – high prevalence population Acanthosis nigricans PCOS
Ketosis	Almost always present	Usually absent
C-peptide	Low/ absent	Normal/ elevated
Antibodies	ICA positive Anti-GAD positive ICA 512 positive	ICA negative Anti-GAD negative ICA 512 negative
Therapy	Insulin variability	Lifestyle, OHA or insulin
Associated auto-immune disease	Yes	No

PCOS –Polycystic ovarian syndrome; ICA – Islet cell antibodies; Anti GAD: Glutamic acid decarboxylase antibodies; OHA: Oral hypoglycemic agent

Prevention

Early treatment and prevention of diabetes is clearly of pivotal importance in reducing the population burden. Diabetes can be delayed or prevented in individuals at high risk adequate diet and exercise programme, Lifestyle modification and intervention with medications. Medications are associated with unwanted side- effects (Stumvoll *et al.,* 2005).

Type 1 diabetes is an autoimmune destruction of the beta cells, and currently there is no treatment to prevent it. Prevention of type 2 diabetes requires identifying children, teens and adolescents at risk and providing them appropriate knowledge and support to help reduce risk factors. 40-80% of teens diagnosed with type 2 diabetes are overweight and the incidence of overweight is increasing. Positive lifestyle modifications through healthy food choices, increased physical activity and achievement/maintenance of a healthy weight are few preliminary options to prevent Type 2 diabetes. A 5-10% decrease in body weight and 30 minutes/day of moderate physical resulted in a 58% reduction in diabetes (Stang and Story, 2005).

Table 1.7 states the percentage of preventable complications of diabetes mellitus (Rogers, 2007).

Table - 1.7: Prevention Potential for Diabetes Complications

Potential complications	Percentage of preventable complications
Kidney failure	50% (with better control of blood pressure and blood glucose levels)
Blindness	Upto 90% (with proper screening and care)
Amputation	Upto 85% (with implementation of foot care program that include regular examinations and patient education)
Death due to heart disease or stroke	Upto 30% (with improved control of blood pressure, blood glucose and lipid level)
Heart disease and stroke	Upto 50% (with improved control of blood pressure and cholesterol and lipid level)
Nerve disease	40% (with a 1% reduction in hemoglobinA1c test)

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. Herbal remedies, in many regions of the world, continue to be more accessible and affordable than conventional drugs. The herbal products today symbolise safety in contrast to the synthetics that are regarded as unsafe to human and environment. In the traditional treatment of diabetes plant materials have played an important role. They represent the first line of treatment for diabetes patient. Diversity and similarity can be found in the use of plants across the world. Each region of the world has its *Materia medica* of antidiabetic remedies based on the local flora.

The North East region of India is well known for its traditional medical treatments. The traditional system of cure and information of the healing properties of the available plants in these regions has been passed on from one generation to the other, and by large has remained undocumented. A consolidated account and analysis of the total use pattern with regard to ailment and traditional treatments by different communities is not available. Therefore, in the present study an attempt has been made to find out the bioactive compounds of some important ethno-medicinal plant(s) of Mizoram that can be used as an effective pharmaceutical agent for the cure of diabetes mellitus in human beings.

The aim of the study was to investigate the following objectives:

1. To collect and screen some common anti-diabetic ethno medicinal plants of Mizoram
2. To select and identify two potential plant(s) for detailed investigation
3. To extract and perform pharmacognostic and phytochemical evaluation of the selected plant/plant parts
4. To qualitatively identify the phytoconstituents present in the selected plants/plant parts by means of standard phytochemical screening tests.
5. To investigate pharmacological activity of the selected plants through a battery of tests which includes *in vitro* antioxidant studies, *in vitro* α-glucosidase activity, *in vitro* α-amylase activity, *in vitro* effect on glucose uptake in L6 cell line, *in vitro* study on GLUT-4 gene expression in L-6 cell line, *in vivo* acute oral toxicity study, *in vivo* anti-diabetic activity against STZ induced diabetic rat model.

CHAPTER 2
REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

2.1 Management of diabetes (Type 2 Diabetes Practical Targets and Treatments, 2005)

A. Diet and nutrition

With proper diet and nutrition, effective management of type 2 diabetes as well as associated cardiovascular risk factors such as hypertension, dyslipidemia and obesity can be achieved.

Nutrition forms an integral part of the management of diabetes. The major goals of nutritional management in diabetes management are to:

- Achieve and maintain optimal blood glucose levels
- Reduce cardiovascular risk factors, including dyslipidemia and hypertension
- Provide a balanced, nutritional diet.
- Weight control

B. Physical activity

Physical activity plays an important role in the management of type 2 diabetes. People with diabetes who undertake regular physical activity have demonstrated substantially lower mortality rates. Physical activity improves insulin sensitivity, thus improving glycaemic control, and may help with weight reduction.

C. Pharmacological treatment of hyperglycaemia

The pharmacological treatment of hyperglycaemia are aimed to target two key metabolic abnormalities in type 2 diabetes i.e., insulin resistance and impaired insulin secretion. Each hypoglycaemic agent targets one of these abnormalities, and combination therapy is often required to address both components.

Hypoglycaemic agent which directly stimulates insulin secretion includes Sulphonylureas and glinides. Thiazolidinedione's and metformin improve insulin sensitivity while $\propto$-glucosidase inhibitors slow down carbohydrate absorption, hence reducing the need for post-prandial insulin secretion.

C.1. Metformin:

Metformin is recommended as first line therapy in the obese and overweight diabetics as well as in non-obese patients.

C.2. Sulphonylureas:

Sulphonylureas stimulate insulin secretion by the beta cells, and lowers HbA1c. These are usually used as second or third line agents.

C.3. Thiazolidinedione's

The thiazolidinedione's improve insulin sensitivity, by improving cellular response to insulin action; however, they do not enhance insulin production. They decrease HbA1c and do not cause hypoglycaemia. The most common side effect is weight gain which usually results from increased subcutaneous fat rather than accumulation of visceral fat. Fluid retention may also occur, and in those patients with pre-existing heart disease, cardiac failure may be precipitated. Thiazolidinedione's should not be initiated in patients with active liver disease or transaminase levels above 2.5 times the upper limit of normal.

E.g. Rosiglitazone and pioglitazone

C.4. ∝–glucosidase inhibitors

∝-glucosidase inhibitors improve overall glycaemic control by slowing down carbohydrate absorption from the jejunum, thereby decreasing postprandial blood glucose and, to a lesser degree, fasting glucose. They have a weight-neutral or weight reducing effect and are used as first-line therapy in association with diet, or in combination with Sulphonylureas, metformin and insulin. E.g., acarbose, miglitol and voglibose

C.5. Glinides

These are a new generation of sulphonylurea-like agents and are now available in several countries. The compounds stimulate early insulin secretion and reduce post-prandial hyperglycaemia. It may be used as monotherapy or in combination therapy with biguanides or thiazolidinedione's and has to be taken with each meal.

E.g., nateglinide and repaglinide

C.6. Insulin

When adequate glycaemic control can no longer be achieved with oral agents alone, insulin is used. Type 2 diabetes is a progressive disorder and with time, loss of beta cell function may cause inadequate glycaemic control despite maximum oral therapy. In such a scenario, insulin is often needed to achieve good glycaemic control, and is generally considered for all patients on maximum oral therapy with HbA1c > 6.5%.

When unintentional weight loss occurs at any time during the course of diabetes, including at the time of diagnosis, early treatment with insulin should be strongly considered. Insulin should usually be used in combination with oral agents, as they limit the weight gain, and reduce the hypoglycaemia associated with insulin therapy. In lean symptomatic patients, if there is uncertainty about the diagnosis of diabetes type, insulin should be considered as first-line therapy.

Figure – 2.1 depicts the pharmacological treatment of hyperglycaemia agents according to site of action.

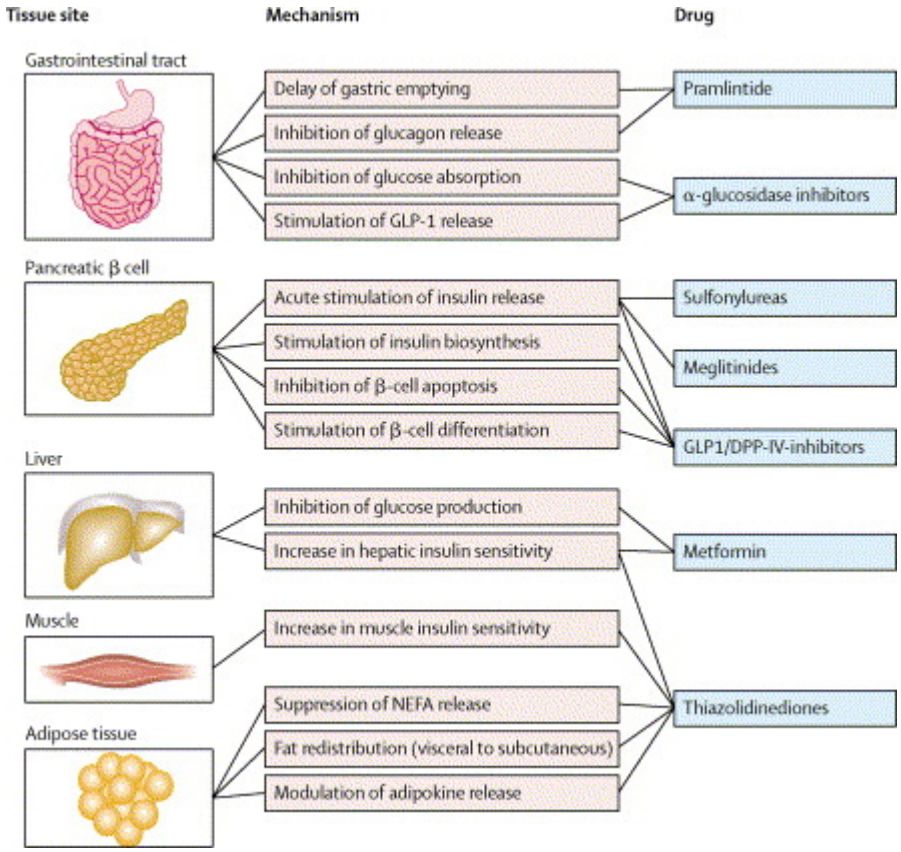


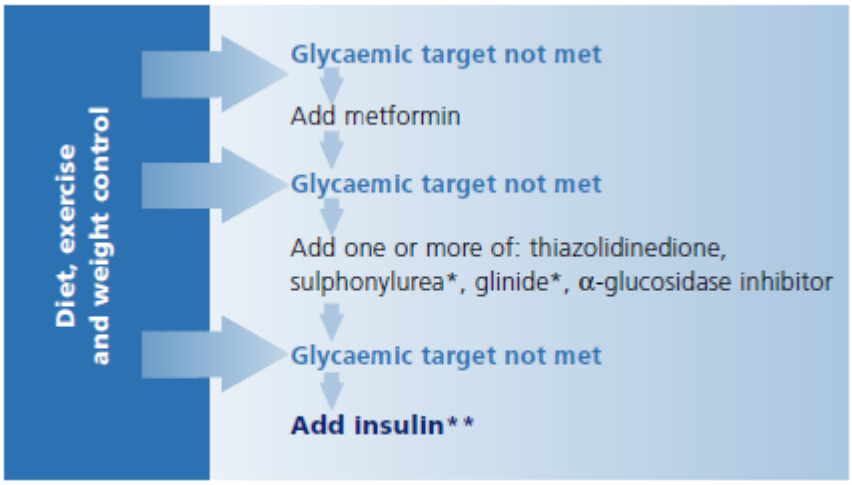
Figure – 2.1: Pharmacological treatment of hyperglycaemia according to site of action
(Source: Stumvoll *et al.*, 2005)

2.2 Combination oral therapy

Combination therapy capitalises on the complimentary modes of action of the different drug classes. Metformin, Sulphonylureas (or glinides), thiazolidinedione's, and ∝-glucosidase inhibitors may be used in various combinations with each other or with insulin when treatment targets are not achieved. Fixed-dose combination tablets are now available, and may be a convenient way of administering oral combinations.

The management algorithm for overweight patients with type 2 diabetes mellitus and management algorithm for normal weight patients with type 2 diabetes mellitus is displayed in **Table 2.1** and **Table 2.2**.

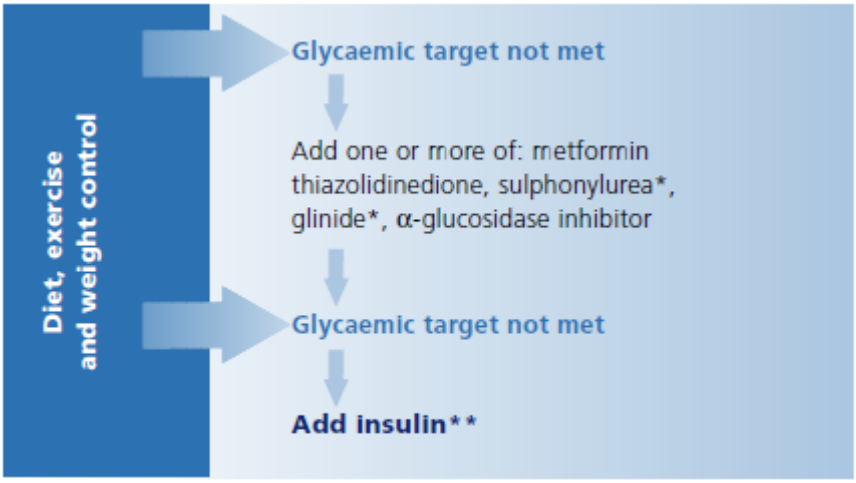
Table – 2.1: Management algorithm for overweight patients with type 2 diabetes mellitus



* Sulphonylureas and glinides should not be combined with each other.

** In some countries, combination of insulin with thiazolidinediones is not approved.

Table – 2.2: Management algorithm for normal weight patients with type 2 diabetes mellitus



* Sulphonylureas and glinides should not be combined with each other.

** In some countries, combination of insulin with thiazolidinediones is not approved.

The potential sequences of anti-hyperglycemic therapy for patients with type 2 diabetes is depicted in **Figure – 2.2**, with the usual transition moving vertically from top to bottom (although horizontal movement within therapy stages is also possible, depending on the circumstances).

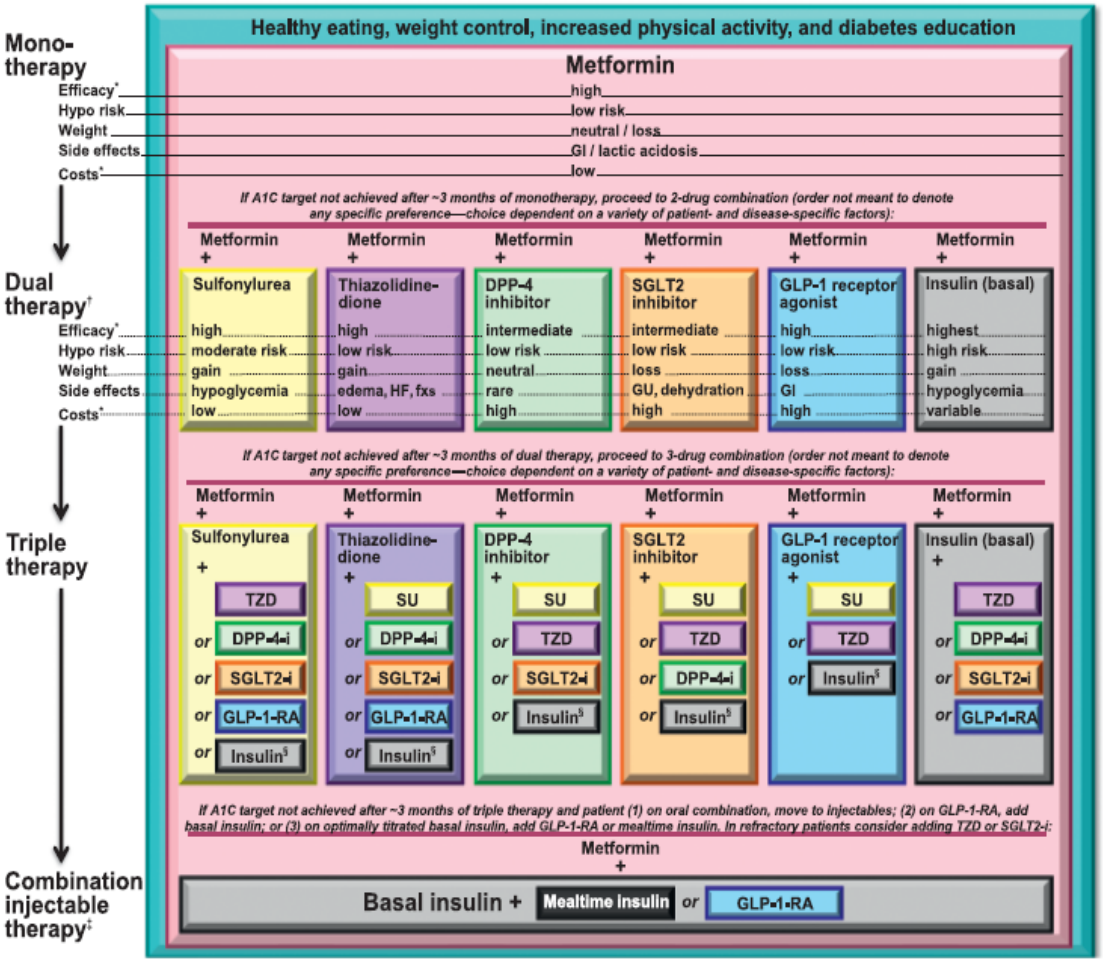


Figure – 2.2: General recommendations for anti-hyperglycemic therapy in type 2 diabetes (Source: Diabetes Care, 2015)

DPP-4-i: DPP-4 inhibitor; Fxs: fractures; GI, gastrointestinal; GLP-1-RA, GLP-1 receptor agonist; GU, genitourinary HF, heart failure; Hypo, hypoglycemia; SGLT2-i, SGLT2 inhibitor; SU, sulfonylurea; TZD, thiazolidinedione

2.3 Newer Approaches in The Treatment of Diabetes Mellitus

- **Peroxisome Proliferator Activated Receptors (PPARs):** PPARs are transducer proteios belonging to nuclear receptor super family. Three major types of PPARs, encoded by separate genes are PPARa, PPARpto and PPARy'. Peroxisome - proliferator activated receptor gamma (PPARy) is a transcription factor activated by thiazolidinedione's (TZDs).

  PPAR ∝/βDual Agonist: These agents ameliorate the hyperglycemia and hyperlipidemia associated with Type 2 diabetes. e.g. tesaglitazar (AZ-242), ragaglitazar and muraglitazar.

- **Glucagon like Peptide-1 (GLP-1) Hormone:** It is the incretin hormone acting via GLP-1 receptor (a G-protein coupled receptor). This hormone stimulates insulin secretion and biosynthesis and inhibits glucagon release leading to reduced hepatic glucose output, when blood glucose levels are high. It also slows gastric emptying and reduces appetite thereby serving as an 'ileal brake".

- **β3 - Adrenoreceptor Agonist:** These agents showed marked selectivity for stimulation of lipolysis thereby stimulating oxygen and energy consumption in skeletal muscle and adipose tissue. Currently these compounds are under trial.

- **Liver Selective Glucocorticoid Antagonists:** These agents antagonize the action of insulin, thereby inhibiting glucose disposal and promoting hepatic glucose production and output.

- **Dipeptidyl Peptidase IV Inhibitors:** These agents stabilize endogenous GLP-1 and induce insulin secretion in a glucose-dependent manner.

- **Protein Tyrosine Phosphatase-1b (PTP-1b):** PTP-lB, belongs to non transmembrane class of enzymes expressed in nearly all tissues. PTP-lB acts as negative regulator of insulin signaling. It causes dephosphorylation of insulin receptor thereby causing negative regulation of insulin signaling.

- **Glycogen synthase kinase (GSK-3):** The key enzyme involved in glycogen metabolism has an ability to phosphorylate and inhibit glycogen synthase (GS), deactivation of GS and decrease its affinity to allostetic activation by glucose-6-phosphate.

- **DiaPep277:** It is a synthetic peptide which is known to modulates the immune response that leads to autoimmune diabetes by diminishing or blocking the immunological destruction of beta cells. Benefit is expected in newly diagnosed adults with Type 1 diabetes, newly diagnosed child or adolescent with Type 1 diabetes.

- **Gene therapy:** Gene therapy is highlighted as a most hopeful technology of the 21st century. Its major goal in Diabetes Mellitus (DM) is to maintain euglycemia in face of wide variations in dietary intake. The risk-benefit ratio of gene therapy in DM is better than that of lifelong injections of insulin, and islet transplantation, which faces the problems of donor shortage and rejection.

- **Recombinant Human Glutamic Acid -rhGAD65:** Type I diabetes results from autoimmune destruction of islet β cells. Recently, DNA vaccination encoding GAD65, insulin or HSP70 has shown to be effective in preventing Type I diabetes in the NOD mice.

- **Otelixizumab:** Otelixizumab is one of the several investigational monoclonal antibodies that target CD3, a T lymphocyte receptor involved in normal cell signaling. The drug works by blocking the function of effector T -cells, which mistakenly attack and destroy insulin producing beta cells while stimulating regulatory T cells, which are understood to protect against effector T-cell damage, thus preserving the beta cells' normal ability to make insulin.

- **Stem cell therapy:** Type 1 diabetes is caused by the body's own immune system attacking its pancreatic islet beta cells and requires daily injections of insulin to regulate the patient's blood glucose levels. A new method uses stem cells from cord blood to re-educate a diabetic's own T cells and consequently restart pancreatic function reducing the need for insulin.

Newer approaches are directed towards improving currently available anti-diabetic drugs and finding the new compounds. Few of these in future may not only control symptoms and modify the natural course of diabetes, but also potentially prevent or cure the disease (Patel *et al.*, 2013).

2.4 Ethnomedicines Vs Diabetes

Mankind has a long history of use of herbal medicines. For centuries plants have been used to treat human diseases. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments and for treating and preventing diabetes (Mitra A, 2007). The Rig Veda and Ayurveda (4500-1600 BC) reveal that ancient Indians had a rich knowledge of the use of medicinal plants (Chakravarty and Kalita, 2011).

Plants are important sources of medicines. As estimated by WHO (2001), 80% of world population rely on medicinal plants for their primary health care needs. Of the 3,50,000 plant species known so far, about 35,000 to 70,000 plants are used worldwide for medicinal purposes. Less than about 0.5% of these have been investigated for their phytochemical and pharmacological potential (Murugesan *et al*., 2011).

The plant kingdom represents an enormous reservoir of putative lead compounds to be discovered for various diseases. Medicinal plants are the best source of drugs and therefore plants should be investigated to understand better about their properties, safety and efficacy. At least 25% of the prescription drugs approved contain bioactive compounds derived from or modeled after plant natural products (Murugesan *et al*., 2011). WHO (1980) has also recommended the evaluation of the effectiveness of plants in conditions where there are no safe modern drugs (Chakravarty and Kalita, 2011).

Across India, ethnomedicines are widely used. The traditional folklore healthcare system has a long history and is very deeply rooted in rural and tribal populations. Practice of ethnomedicine is an important vehicle for understanding indigenous societies and their relationships with nature. Scientific knowledge varies from region to region. Traditional healers employ methods based on the ecological, socio-cultural and religious background of their people to provide health care (Rai and Lalramnghinglova, 2011). India occupies the topmost position in the use of herbal drugs since ancient, utilizing nearly 600 plant species in different formulations. Majorities of people in India depend on crude drugs for the treatment of various diseases. This is evidenced from well-documented indigenous system of medicines, Ayurveda and Unani. The Materia Medica of these systems contains a rich heritage of indigenous herbal drugs (Chakravarty and Kalita, 2011).

India is one of the richest countries in the world in relation to genetic resources of medicinal and aromatic plants with 11% of the total known plants having medicinal properties. India is estimated to be having over 45,000 plant species representing about 7% of the world's flora; however, it covers only 2% of the earth's surface. India has medicinal plants distributed in different geographical and environmental conditions. It includes elements of three biodiversity hotspots – the Himalayas, the Western Ghats, and

Indo-Burma. These are highly endangered Eco regions. A large part of the NE India is botanically under-explored or even unexplored (Rai and Lalramnghinglova, 2011).

The knowledge of the system of diabetes mellitus is known to Indians since prehistoric ages. Its earliest reference dates back to 1000 BC in the Ayurvedic literature. Diabetes mellitus was known as 'Asrava' during Vedic era (6000 BC). Its detailed description is available in Charak Samhita, Sushruta Samhita and Vagbhatta. Asthanga Haridaya (600 AD) is the first medical treatise in which clear definition of madhumeha/diabetes mellitus is provided by mentioning glycosuria (madhviv mehati-honey like urine). Ayurvedic physician Sushruta (about 400 BC) and Charak were also aware of this disease in Ayurveda (Chakravarty and Kalita, 2011).

Herbal medicines playa a major role in controlling diabetes and have been used since long for the treatment of diabetes. They are accepted as an alternative therapy for diabetic treatment (Chakravarty and Kalita, 2011), (Shivashankar and Mani, 2011). Medicinal plants used in the treatment of diabetes mellitus are perceived to have lesser side effect compared to synthetic therapeutic agents known to be associated with many side effects (Mbaka *et al*., 2010).

Several plants mentioned in the indigenous Indian system of medicine were used for the cure of diabetes. About 800 plants in the Indian subcontinent are known to possess anti-diabetic potential (Chakravarty and Kalita, 2011).

Few anti diabetic medicinal plant found in India and found to be effective in diabetes and their complications are listed in **Table 2.3** below (Chakravarty and Kalita, 2012; Shivashankar and Mani, 2011; Grover *et al*., 2002):

Table – 2.3: Potential **Anti diabetic medicinal plant reported from India**

S.No.	Name of plant	Family
1.	*Annona squamosa* Linn	Annonaceae
2.	*Argyreia speciosa* (Linn. f.)	Convulaceae
3.	*Andrographis paniculata* (Burm.f.)	Acanthaceae
4.	*Aegle marmelos* (L.) Corrêa	Rutaceae
5.	*Azardirachta indica* A.Juss.,	Meliaceae
6.	*Acacia catechu* (Willd.)	Leguminosae
7.	*Aerva lanata* (L.) Juss.	Amarantaceae
8.	*Allium cepa* Linn	Amaryllidaceae
9.	*Allium sativum* Linn	Amaryllidaceae
10.	*Aloe vera* (L.) Burm.f.	Xanthorrhoeaceae
11.	*Alpinia calcarata* Roxb.,	Zingiberaceae
12.	*Benincasa hispida* (Thunb)	Cucurbitaceae
13.	*Barleria prionitis* Linn	Acanthaceae
14.	*Crateva nurvula* (Lour.)	Capparidaceae

15.	*Cocculus hirsutus* DC.	Menispermaceae
16.	*Capsicum annum* Linn	Solanaceae
17.	*Cedrus deodara* Roxb	Coniferae
18.	*Coccinia indica* W&A	Cucurbitaceae
19.	*Cassia auriculata* (L.) Roxb.	Caesalpiniaceae
20.	*Cassia glauca* Linn	Caesalpinaceae
21.	*Capparis sepiaria* Linn	Capparidaceae
22.	*Cajanus cajan* Adans.	Fabaceae
23.	*Coccinia indica* (L.) Voigt	Cucurbitaceae
24.	*Caesalpinia bonducella* (L.) Roxb.	Caesalpiniaceae
25.	*Emblica officinalis* S Gaertn	Phyllanthaceae
26.	*Eugenia jambolana* Lam	Myrtaceae
27.	*Ficus bengalenesis* Linn	Moraceae
28.	*Ficus gibosa* BI	Moraceae
29.	*Ficus glomerata* Roxb	Moraceae
30.	*Gymnema sylvestre* R.Br	Asclepiadaceae
31.	*Helicteres isora* Linn	Sterculiaceae
32.	*Holostemma annulare* K.Schum	Asclepiadaceae
33.	*Holostemma* ada Kodien	Asclepiadaceae
34.	*Helicteres isora* Linn	Sterculiaceae
35.	*Hemidesmus indicus* (L.) R.Br.	Apocynaceae
36.	*Jatropha curcas* Linn	Euphorbiaceae
37.	*Mimosa pudica* Linn	Fabaceae
38.	*Momordica charanti* Linn	Cucurbitaceae
39.	*Ocimum sanctum* Linn	Lamiaceae
40.	*Plumbago rosea* Linn	Plumbaginaceae
41.	*Pterocarpus marsupium* Roxburgh	Fabaceae
42.	*Rubia cordifolia* Linn	Rubiaceae
43.	*Rosa canina* Linn	Rosaceae
44.	*Salacia fruticosa* Linn	Celastraceae
45.	*Salacia oblonga* Wall	Hippocrateaceae
46.	*Saraca indica* Linn	Leguminosae
47.	*Stroblanthus hyneanus* Nees	Acanthaceae
48.	*Swertia chirayita* Linn	Gentianaceae
49.	*Syzigium cumini* (L.) Skeels.	Myrtaceae
50.	*Trigonella foenum graecum* Linn	Fabaceae
51.	*Trichosanthes dioica* Roxb.	Cucurbitaceae
52.	*Tinospora cordifolia* Miers	Menispermaceae
53.	*Tragia involucrate* Linn	Euphorbiaceae
54.	*Tribulus terrestris* Linn	Zygophyllaceae
55.	*Vinca rosea* (L.) G.Don	Apocynaceae

The medicinal values of various plants extracts have been studied by many scientists in the field of diabetic research and a few of them have been experimentally evaluated and active principles of few plants have also been isolated (Chakravarty and Kalita, 2011). Few of these plants and its active compound are listed in **Table 2.4**.

Table – 2.4: **Potential antidiabetic plants and their active compounds**

Plant	Used part	Active compounds	References
Gallega officinalis	leaves, seeds	galegine	Whitters, 2001; Goldstein and Wieland, 2008; Lemus et al., 1999
Syzygium cumini	seeds, leaves, flower	mycaminose	Trojan-Rodrigues et al., 2011; Kumar et al., 2008
Bauhinia forficata	leaves, flowers, bark	kaempferol-3-neohesperidoside (insulin mimetic)	Trojan-Rodrigues et al., 2011; Cazarolli et al., 2009
Bidens pilosa L.	whole plant	polyacetylenic glucosides	Trojan-Rodrigues et al., 2011; Hsu et al., 2009
Swertia punicea	whole plant	methylswertianin, bellidifolin	Tian *et al.,* 2010
Capparis moon	fruits	gallotannins (chebulinic acid derivatives)	Kanaujia *et al.*, 2010
Artemisia dracunculus L.	whole plant	davidigenin, sakuranetin, 2',4'-dihydroxy-4-ethoxydihydrochalcone, 4,5-di-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 6-demethoxycapillarisin	Wang et al., 2011; Ribnicky *et al.*, 2009; Eisenman *et al.*, 2011; Logendra *et al.*, 2006
Salacia reticulata	root, stem	Salacinol, kotalanol, de-O-sulfated salacinol, de-O-sulfated kotalanol, ponkolanol, salaprinol	Muraoka *et al.*, 2008
Morus alba	leaves	(2S)-euchrenone, chalcomoracin, moracin C, moracin D, moracin N, (2R)/(2S)-euchrenone, moracin N , quercetin, norartocarpetin, several flavanes	Yang *et al.*, 2012
*Morus alb*a	leaves	quercetin 3-(6-malonylglucoside), rutin (quercetin 3-rutinoside), isoquercitrin (quercetin 3-glucoside)	Hansawasdi and Kawabata, 2006; Katsube *et al.*, 2006
Ocimum sanctum	leaves	polyphenols, caffeic acid, p-coumaric acid	Wongsa *et al.*, 2012
Acacia pennata	shoot tips	polyphenols, caffeic acid	Wongsa *et al.*, 2012
Solanum xanthocarpum	fruit	polyphenols, caffeic acid	Wongsa *et al.*, 2012
Macaranga tanarius	seeds	ellagitannins (mallotinic acid, corilagin, chebulagic acid, macatannins A and B)	Puteri and Kawabata, 2010
Salacia oblonga, Salacia chinensis	root, stem, leaves	salacinol, kotalanol, mangiferin	Muraoka *et al.*, 2010; Giron *et al.*, 2009
Eleutherine americana	bulb	eleutherinoside A	Ieyama *et al.*, 2011
Aquilaria sinensis	leaves	mangiferin, iriflophenone 2-*O*-α-L-rhamnopyranoside, iriflophenone 3-*C*-β-D-glucoside, iriflophenone 3,5-*C*-β-D-diglucopyranoside	Feng *et al.*, 2011

Plant	Used part	Active compounds	References
Panax japonicus	root	polyacetylenes, phenolic compounds, one sesquiterpenoid, one sterol glucoside	Chan *et al.*, 2010
Curcuma longa	rhizome	curcumin, demethoxycurcumin, bisdemethoxycurcumin, ar-turmerone	Kuroda *et al.*, 2005
Rhododendron tomentosum Picea mariana	fruit	querceti n	Nistor Baldea *et al.*, 2010
Aronia melanocarpa	fruit	anthocyanins	Kulling and Rawel, 2008; Valcheva- Kuzmanova *et al.*, 2007
Stevia rebaudiana	leaves	alkaloid s, flavonoi ds	Kujur *et al.*, 2010
Nigella sativa	seeds	gallic acid, (–)-p-hydroxybenzoic acid, chlorogenic acid, vanillic acid, p-coumaric, ferulic acid, trans-2- hydroxycinnamic acid, trans-cinnamic acid, epicatechin, (+)-catechin, quercetin, apigenin, amentoflavone, flavone	Bourgou *et al.*, 2008; Meddah *et al.*, 2009
Phaseolus vulgaris L.	seeds	alkaloids, flavonoids, fiber, proteins, tannins, terpenoids, saponins, quercetin, anthocyanin, catechin	Ocho-Anin Atchibri *et al.*, 2010
Marrubium vulgare	aerial part	flavonoi ds	Elberry *et al.*, 2011
Ruta graveolens	leaves	rutin	Ahmed *et al.*, 2010
Carissa carandas	fruit	gallic acid, flavonoids	Itankar *et al.*, 2011
Pinus pinaster	bark	polyphenols: proanthocyanidins, catechin, epicatechin	El-Zein *et al.*, 2011; Bedekar *et al.*, 2010; Liu *et al.*, 2004
Piper retrofractum	fruits	piperidine alkaloids: piperine, pipernonaline,dehydropiperno naline	Kim et al., 2011

(Source: Coman *et al.*, 2012)

Clinically useful drugs, of vital importance in treating various types of diseases including metabolic disorder, have been discovered through screening of natural source of plant origin. Many kinds of natural products, such as terpenoids, alkaloids, flavonoids, phenolics, and some others, have shown antidiabetic potential. Particularly, schulzeines A, B, and C, radicamines A and B, 2,5-imino-1,2,5-trideoxy-L-glucitol, beta-homofuconojirimycin, myrciacitrin IV, dehydrotrametenolic acid, corosolic acid (Glucosol), 4 - (alpha-rhamnopyranosyl) ellagic acid, and 1,2,3,4,6-pentagalloylglucose have shown significant antidiabetic activities (Jung *et al.*, 2006).

Large classes of compounds are available from many plant sources. Natural products such as plant extracts, phytochemicals, and microbial metabolites are currently studied for their potential uses in the treatment and prevention of diabetes mellitus. A number of plant extracts and natural biomolecules have shown very promising effects indicating that the dietary intake of phytochemicals could be a promising strategy for diabetes prevention (Coman *et al.,* 2012).

Polyphenolic compounds, especially flavonoids have been studied a lot with regard to their antidiabetic properties. Flavonoids are of plant origin and are known for their antioxidant, anti-inflammatory, and anti-carcinogenic properties. Dietary intake of flavonoids may be an important alternative diabetes treatments and for the reduction of the risk of the disease. Therapies based on phytochemicals therefore constitute a novel pharmacological approach for treatment or an approach that would reinforce existing treatments. Different classes of plant compounds with anti-hyperglycemic activity, together with few examples from each class are summarized as follows (**Table 2.5**):

Table - 2.8: **Classes of reported antidiabetic compounds having antidiabetic efficacy and their details**

Classes of compounds		Compounds
Flavonoids	Anthocyanidins	cyanidin, delphynidin, petunidin, peonidin, pelargonidin, malvidin
	Anthocyanins	Anthocyanidins + sugar residue (glucose, galactose, arabinose, rhamnose, xylose) e.g. cyanidin 3-glucoside, cyanidin 3-galactoside, pelargonidin-3-arabinoside
	Flavones	Flavonols: quercetin, kaempferol, myricetin, isorhamnetin, rutin (quercetin-3-O-rutinoside), kaempferol-3-neohesperidoside, isoquercitrin (quercetin 3-glucoside), apigenin
		Flavanones: sakuranetin
		Flavanonol: taxifolin (dihidroquercetin), dihidrokaempferol
	Isoflavones	genistein
	Flavan-3-ols	catechins (proanthocyanidins), epicatechins
	Isoflavonoids	phytoestrogens
Chalcones		davidigenin, 2',4'-dihydroxy-4-methoxydihydrochalcone
Tannins		gallotannins: 1,3,6-tri-O-galloyl-2-chebuloyl-β-D-glucopyranoside; 1,3,6-tri-O-galloyl-2-chebuloyl ester-β-D-glucopyranoside ellagitannins: mallotinic acid, corilagin, chebulagic acid, macatannins A and B
Xanthones		mangiferin, bellidifolin, swerchirin, methylswertianin
Organic acids		kaurenoic acids, p-coumaric acid, vanillic acid, gallic acid, *p*-hydroxybenzoic acid
Cinamic acid derivatives		caffeic acid, ferulic acid, chlorogenic acid
Sugars		salacinol, kotalanol, ponkolanol, salaprinol

Classes of compounds	Compounds
Curcuminoids	curcumin, demethoxycurcumin, bisdemethoxycurcumin
Alkaloids	conophylline, piperine, pipernonaline, dehydropipernonaline

(Source: Coman *et al.,* 2012)

Compounds such as acarbose, miglitol, and voglibose (**Figure 2.3**) are a few examples of natural compounds of microbial origin that are currently used in the treatment of Type 2 Diabetes Mellitus (Coman *et al.*, 2012).

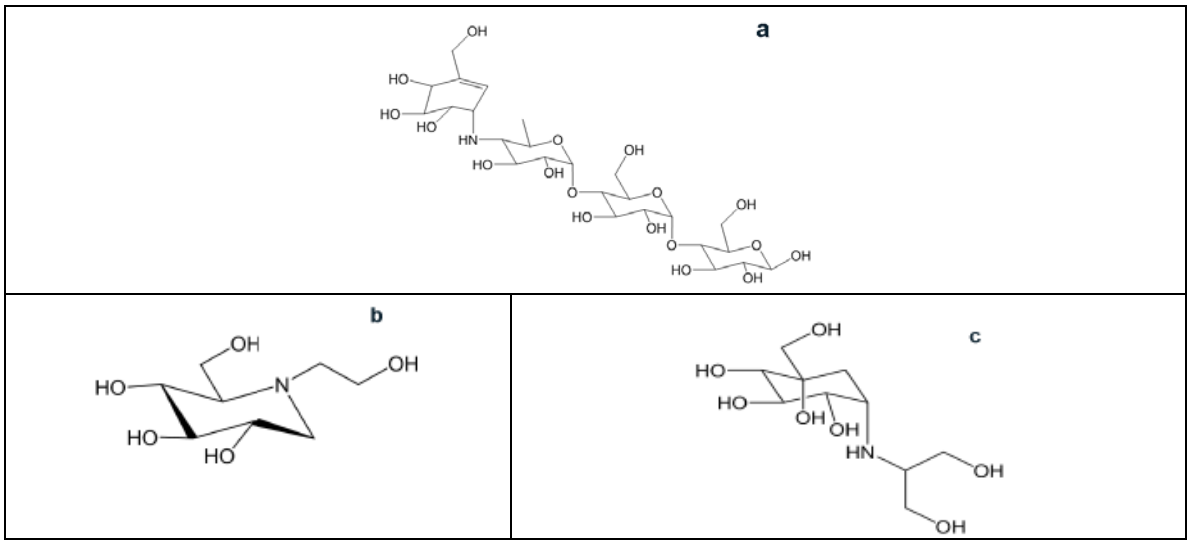


Figure – 2.3: Molecular structures of the (a) acarbose, (b) miglitol, and (c) voglibose (Coman *et al.,* 2012**)**

Acarbose is a pseudotetrasaccharide, a secondary metabolite produced by the bacterium species Actino-planes sp. SE50. Miglitol and voglibose both contain only one sugar ring. Miglitol is synthesised from D-glucose by Glucanobactor oxydans, while voglibose is synthesised from valilamine that is obtained from the fermentation of Streptomyces culture broth (Coman *et al.,* 2012).

The Northeastern (NE) region of India comprises of eight states namely Arunachal Pradesh, Assam, Meghalaya, Manipur, Tripura, Mizoram, Nagaland and Sikkim. This region harbors more than 180 major tribal communities of the total 427 tribal communities found in India (Chakravarty and Kalita, 2012; Rai and Lalramnghinglova, 2010b).

Mizoram state in North East India has served as the location of our studies of ethnomedicines. Over the last ten years, 302 plants from 96 families were recorded as being used by the indigenous Mizo and other tribal communities (Rai and Lalramnghinglova, 2011).

Mizoram is an important state of north east India, sandwiched between Burma and Bangladesh (**Figure – 2.4**). The state is bordered by Chin Hills of Myanmar in the east, Chittagong hills of Bangladesh and Tripura state in the west, Manipur state and Cachar district of Assam in the north and on the south Arakan hill ranges of Myanmar. It covers an area of 21,087 km^2. It is extended between latitude 21° 58' - 24° 45' and 24° 35' N and between 92°15' and 93°29' E longitude and also is a part of the 34 mega-biodiversity hotspots of the world. The altitude ranges from 500 to 2157 m (Rai and Lalramnghinglova, 2011; Rai and Lalramnghinglova, 2010; Kar *et al.*, 2013).

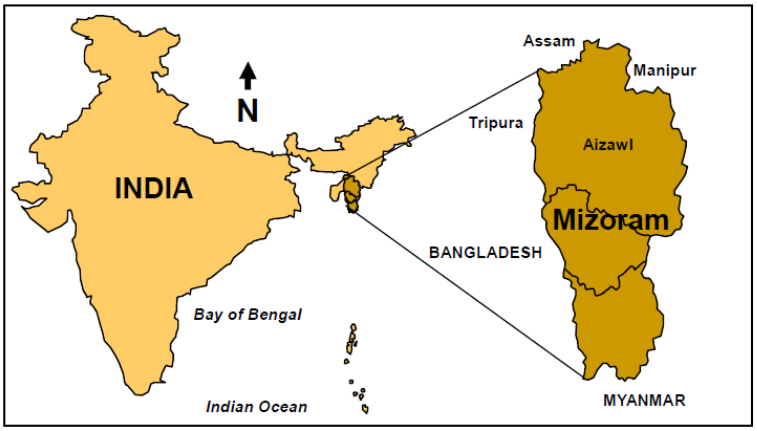


Figure – 2.4: Location of Mizoram

Temperature varies from 18 to 29^{0}C in summer and from 11 to 24^{0}C in winter. Mizoram is under direct influence of monsoon, with heavy rains from May to September and a maximum annual rainfall ranges 2000-3200 mm (Rai and Lalramnghinglova, 2010, Kar *et al.*, 2013).

The soil type of Mizoram varies from sandy loam, clayey loam to clay. These factors have influenced the physiography and climate of the state. Such a diverse topography and climate have favored the occurrence of rich flora and fauna in various forest type (Kar *et al.*, 2013). 90.68% of the geographical area of Mizoram is covered with forest. The total forest area of Mizoram is 15,825 km^2 and grows from 500 to 2,157 m in altitude. These forest harbors 2200 flowering plant species. The vegetation of Mizoram, according to the proposed classification, is tropical evergreen and semi-evergreen forest in the lower altitude hills; sub-tropical to montane sub-tropical in the high hills. However, these forests are exposed to various anthropogenic disturbances, e.g., shifting cultivation, timber logging, extraction of fuel wood by poor/rural tribals, industrialization and in fact urbanization in some parts of Mizoram like Aizawl (Rai and Lalramnghinglova, 2010; Kar *et al.*, 2013).

CHAPTER 3
COLLECTION AND SCREENING OF COMMON ANTI-DIABETIC ETHNO MEDICINAL PLANTS OF MIZORAM

CHAPTER 3: COLLECTION AND SCREENING OF COMMON ANTI-DIABETIC ETHNO MEDICINAL PLANTS OF MIZORAM

Every human society has its own practices concerning health and diseases as well as their management. The Mizo traditional health care practices and disease treatment systems are based on their belief and observation. The medicinal use of plant parts in the management and treatment of diseases has been an age long practice among different tribes (Lalmuanpuii *et al.,* 2013). The present study was proposed to screen and investigate the pharmacological activity of the selected anti-diabetic ethno medicinal plants commonly used among the tribal communities of Mizoram (**Table 3.1**).

Table – 3.1: Anti-diabetic ethnomedicinal plants from Mizoram

Scientific name	Local/Mizo name	Family	Part Used	Mode of Utilization/Uses
Bryphytum sensativum (l.) DC.	Zarero	Oxalidaceae	Leaves	A saline extract of 10 -20 ml from matured leaves is consumed twice daily for two months.
Cassia alata L	Mongrangiangtong	Caesalpiniaceae	Leaves	Decoction of leaves taken orally twice for 6-8 weeks.
Cassia occidentalis L	Hant thenga	Caesalpiniaceae	Bark	50 g bark is used to make infusion and is given orally daily, for 8-10 weeks
Catharanthus roseus Linn.	Kumtluang	Apocynaceae	Leaf, root & stem	Decoction of roots, stem & leaves is useful in diabetes. 20 mL is taken orally once a day for eight to ten weeks.
Cinnamomum tamala T. Nees & Eberm	Lappyrring	Lauraceae	Leaves	The powder is made from dried leaves and 5 g per day is taken orally for 5 – 6 weeks.
Dillenia pentagyna Roxb.	Kaihzawl	Dilleniaceae	Bark	Decoction of the bark is prepared from 100 g bark. This is taken orally once a day for 6-8 weeks taken orally for diabetes.
Eucalyptus globules Labill	Eucalyptus	Myrtaceae	Leaves	Decoction of leaves is used for diabetes.
Ficus bengalensis L.	Hmawng	Moraceae	Bark	Infusion of bark is used in diabetes.
Ficus semicordata Miq	Theipui	Moraceae	Bark	Decoction of the bark is prepared and 20 to 30 ml is taken orally once a day, for 5 to 6 weeks.
Inula cappa DC.	Buarthau	Asteraceae	Leaf	The leaves are crushed with Plantago asiatica and Lobelia angulata and 20 to 30 ml juice is taken orally once a day, for 6 to 8 weeks for diabetes .
Ichnocarpus	Dudhkuri lota	Apocynaceae	Root	The root powder 1 or 2 gm is

Scientific name	Local/Mizo name	Family	Part Used	Mode of Utilization/Uses
frutescens (L.) R.Br.				administered along with milk and also; root decoction is taken orally, daily once, for 4 to 6 weeks.
Mallotus roxburghianus Mull. Arg	Zawngtenawh-lung	Euphorbiaceae	Leaves	The decoction is prepared from leaves and is taken orally ¼ cup (25 ml) twice daily as tea, for 3 to 4 months.
Momordica charantia L.	Tita Kela	Cucurbitaceae	Fruit	2-3 fruits are cooked and consumed, and also 50 ml raw fruit juice is taken orally once a day for 5-6 weeks.
Musa glauca Roxb	Saisu	Musaceae	Seeds	The seeds are powdered and 5 to 10 gm of powder is taken orally twice a day for 6 to 8 weeks
Lepionurus sylvestris Blume	Anpangthuam	Opiliaceae	Leaves	Leaves are boiled and the water is taken ½ cup (50 ml) once a day for 6 to 8 weeks
Picrasma javanica Blume	Thingdamdawi	Simaroubaceae	bark	Decoction is prepared from bark, and two tablespoonful's (15 ml) of decoction are taken orally twice a day for 6 to 8 weeks
Phyllanthus fraternus Webster.	Mitthi sunhlu	Euphorbiaceae	Whole plant	50 ml infusion of plant taken twice daily for diabetes
Thunbergia grandiflora Roxb.	Zawngafian	Acanthaceae	Leaf	Juice of the leaves is used for diabetes
Vitex peduncularis Wall	Thingkhawilu	Lamiaceae	bark	Decoction is prepared from bark and ½ cup (50 ml) is orally taken twice a day, for 2-3 months.

(Rai and Lalramnghinglova, 2010a; Rai and Lalramnghinglova, 2010b)

CHAPTER 4
SELECTION AND IDENTIFICATION OF THE MOST POTENTIAL PLANT(S) FOR DETAILED INVESTIGATION

CHAPTER 4: SELECTION AND IDENTIFICATION OF THE MOST POTENTIAL PLANT(S) FOR DETAILED INVESTIGATION

The plant materials to be investigated were selected on the basis of literature data, ethno botanical and traditional uses of the plants. The traditional usage of the plant is used to prioritize which plants should be extracted and screened for biological activity.

Among the many plants ethno medicinal widely used by Mizo traditional folklore and tribal populations for the management and treatment of diabetes mellitus, the following plants were screened for further selection and investigation. This was also based on the review of literature and information obtained from traditional healers and herbalist. These medicinal plants are:

- *Picrasma javanica* Blume
- *Mallotus roxburghianus* Mull.Arg
- *Lepionurus sylvestris* Blume
- *Ficus bengalenesis* Linnaeus
- *Phyllanthus fraternus* G.L. Webster

Among the above 5 medicinal plants, two were selected for further extraction and evaluation. The plants selected were *M. roxburghianus* and *P. fraternus*.

I. *Mallotus roxburghianus* Mull.Arg (Family - Euphorbiaceae)

Classification
Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Malpighiales
Family: Euphorbiaceae
Genus: Mallotus
Species: roxburghianus Müll.Arg.

M. roxburghianus Muell. (Family - Euphorbiaceae), a deciduous tree, is a traditional plant of north east India found particularly in the tropical evergreen forests and mixed bamboo forests distributed in the region of Chittagong Hill tract of Bangladesh and Myanmar. It is a native plant of Mizoram and is used in the traditional medicine by the tribal people of Mizoram (Rana *et al.*, 2005, Lalhlenmawia *et al.*, 2007). Locally it is Zawngtenawhlung, its Mizo name. It is found widely in Mizoram particularly in the

catchments area of river Khawthlangtuipui between Tlabung and Dinthar and within Dampa Wildlife sanctuary (Pachuau, 1994; Rai and Lalramnghinglova, 2010b).

The plant is a shrub to small tree; young part softly pubescent; bark grey, rough; leaves alternate, caudate-acuminate, distantly serrature, 6-15 x 10-15 cm; base rounded; nerve 5 at the base, intramarginal; tertiaries scalariform; veinlets reticulate; petiole up to 15 cm long; flowers racemes, terminal, as long as leaves; fruits 3-lobed, sub-globose. It sheds its leaf during the month of October to December. New leaf grows from January. It flowers during the month of May to June and bears fruit at August to September (Lalramnghinglova H, 2003).

The pictures of *M. roxburghianus* plant and leaves are provided in **Figure - 4.1** and **Figure 4.2** respectively.

Figure - 12: *M. roxburghianus* plant

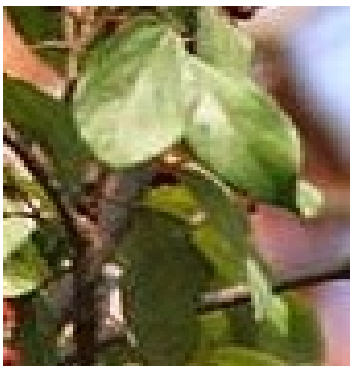


Figure 13: Leaves of *M. roxburghianus*

Traditionally the tribal people of Mizoram people used *M. roxburghianus* plant as follows.

1. For Diabetes: Decoction of leaves is taken orally for diabetes at ¼ cup (25 ml approximately) twice daily.
2. for hepatitis and fever: Young twigs with 4-7 leaves are boiled with chicken and rice and the soup is taken as an effective cure for hepatitis and fever.
3. For hypertension: Infusion of the leaves with the bark of *Alstonia scholaris* is taken orally 2 times a day for hypertension.
4. For the treatment of inflammatory conditions (Lalramnghinglova H, 2003).
5. For jaundice and hepatomegaly - Twigs are boiled and the soup is taken daily/drunk (1 cup (100ml) twice daily) (Rai and Lalramnghinglova, 2010b).

The antioxidant activity of the plant may be responsible for its uses in these (Lalramnghinglova H, 2003).

Compounds isolated and identified from the leaves of *M. roxburghianus* include (Rana *et al.*, 2005).

1. Beta-sitosterol
2. Stigmasterol
3. Betulinic acid
4. 4-hydroxybenzoic acid
5. Beta-sitosterol-beta-D-glucoside
6. 3-(1-C-beta-D-glucopyranosyl)-2,6-dihydroxy-5-methoxybenzoic acid
7. 2,4,8,9,10-pentahydroxy-3,7-dimethoxyanthracene-6-O-beta-D-rhamnopyranoside
8. Bergenin

The chloroform soluble portion of the alcoholic extract of leaf, and compounds 3, 6, 7, and 8 exhibited encouraging antioxidant activities (Rana *et al.*, 2005). The ethanol extract of *M. roxburghianus* has been reported for its anti-oxidant activity (Lalhlenmawia *et al.*, 2007).

II. *Phyllanthus fraternus* (Family - Euphorbiaceae)

Classification

Kingdom: Plantae
Phylum: Tracheophyta
Class: Magnoliopsida
Order: Malpighiales
Family: Euphorbiaceae
Genus: *Phyllanthus*
Species: *fraternus* G.L. Webster

Description of the plant: The leaves of this plant alternate and are pinnately compound. Flowers are unisexual, minute and green. Fruits capsules from rows under side of leaves. It is a small, erect, annual herb (Dicotyledonous) that grows 30–40 cm in height.

Geographical distribution: This pantropical weed and are indigenous to the rainforests of the Amazon and other tropical areas throughout the world, including the Bahamas, southern and north east India and China. It is quite prevalent in the Amazon and other wet rainforests, growing and spreading freely (Matur *et al.,* 2009), (Mehta *et al.,* 2013). It comprise about 750 species in tropical and subtropical region (Mehta *et al.,* 2013). This plant is locally found in Mizoram in Tahsil and Hnahthial area.

Common names: *P. fraternus* is commonly called Bhumyamlaki, Bhoiamli, gulf leaf-flower, Chanca piedra, quebra pedra, stone braker, arranca-pedras, carry-me-seed, hurricane weed, para-parai mi, quinine weed Mache da goyo (Hausa), Gbogbon owun lese (Yoruba) (Matur *et al.,* 2009), (Christian M, 2015). Locally in Mizoram it is called Mitthi-Sunhlu (Kar *et al.,* 2013).

The pictures of *P. fraternus* plant and leaves are provided in **Figure - 4.3** and **Figure - 4.4**.

Figure – 4.3: *P. fraternus* plant

Figure – 4.4: Leaves of *P. fraternus*

P. fraternus is a traditional plant and is used in India as a herbal medicine (Kar *et al.*, 2013). It is widely used as a folklore remedy for the several diseases which includes treatment of blennorrhagia, colic, diabetes, dysentery, fever, flu, tumors, jaundice, vaginitis, dyspepsia, disturbances of kidney and bladder calculi, intestinal infections, hepatitis B virus, vertilago, malaria, diabetes, menorrhagia, sores, chronic dysentry, tubercular ulcers, wound, bruises, scabies, ringworm, dropsical infection, gonorrhoea, genito-urinary disorders, jaundice, indigestion, intermittent fever, anemia, cough, gout,

urinary disease, dermatosis, miscarriage, abdomen tumour, vaginitis, renal disorders and skin eruption (Matur *et al.,* 2009), (Chopade and Sayyad, 2013, Christian, 2015, Singh *et al.,* 2014). In Mizoram, the stem and leaf are taken raw or half cup of its decoction is taken twice a day for diabetic control Rai and Lalramnghinglova, 2010).

This plant is stomachic, carminative, diuretic, digestive, laxative, vermifuge, cooling and astringent (Matur *et al.,* 2009, Christian, 2015). It has anti-dysentric, anti-hepatotoxic and anti-inflammatory, anti-septic, anti-spasmodic, anti-viral, anti-dote to snake bite activities. It leaves are used in scabies, bruises, wound, poultice lessions, swelling, ulcer, spleen and liver disorders and problem of joints (Christian , 2015).

P. fraternus contains medicinally important bioactive compounds and this justifies the use of plant species as traditional medicine for treatment of various diseases (Mehta *et al.,* 2013). Phytochemical screening of the *P. fraternus* plant extract revealed the presence of alkaloids, tannins, saponin, flavonoids, glycosides, resins, phenols and carbohydrates (Matur *et al.,* 2009). However in another study, the phytochemical constitute of the leaf extract of *P. fraternus* were recorded as Alkaloids (Phyllanthin, Hypophyllanthin, Nirphyllin, Phyllnirurin, Phyllanthol, Phyllanthenol, Rhamnopyrenoside, Phyllanthenone, Lintetralin, Astragalin, Cymene, Niranthin, Nirtetralin, Niruriside, Phyllochrysine, 4-Methoxy-Securinine, 4-Methoxy-Nirsecurinine, Limonene, Niruretin, Nirurin, Phyllochrysine), Steroids (β-Sitosterol, Cholesterol), Flavonoids (FG1, FG2, Quercetin, Quercetin heteroside, Quercetol, Quercitrin, 3, 4, 5-Trimethoxy flavonone, 3, 5, 7-Trihydroxy flavonol), Saponins (Triacontanal, Triacontanol) and other compounds (Estradiol, Carilagin, Eellagic acid, Gallic acid, Rutin, Gernanine, Rutinoside, Lupa, Lupeol, Methyl salicylate) (Christian M, 2015).

CHAPTER 5
EXTRACTION FROM THE SELECTED PLANT/PLANT PARTS

CHAPTER 5: EXTRACTION FROM THE SELECTED PLANT/ PLANT PARTS

5.1 Collection and Authentication:

The whole plant or particular parts of plant were collected depending on the part of the plant used traditionally. In this case the leaves of *M. roxburghianus* and whole plant of *P. fraternus* were to be used and evaluated. Fresh plant leaves of *M. roxburghianus* were collected from Siphir and whole plant of *P. fraternus* were collected from Hnahthial. The plants collected were authenticated by a with the available literatures/herbarium at the school of Earth Sciences and natural Resources Management, Mizoram University, Aizawl.

5.2 Drying and Grinding:

The plant materials were washed thoroughly with normal tap water followed by distilled water. Once cleaned, the plant material needs to be dried as soon as possible. Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. The plant materials were then dried on trays at ambient room temperature and in a room with adequate ventilation. Plant materials should be distributed evenly to facilitate homogeneous drying. Protection from direct sunlight is advised to minimize chemical reactions induced by ultraviolet rays. Once dried the plant materials were crushed to powder using grinding machine and grounded into a coarse powder stored in air tight container bottles in room temperature. The dried material should be stored in sealed containers in a dry and cool place. Storage for prolonged periods should be avoided as some constituents may be decomposed.

5.3 Extraction of the dried material and analysis

Extraction of authenticated dried plant material from *M. roxburghianus* (Leaf) and *P. fraternus* (Whole plant) were used to extract the plant secondary metabolites with the help of soxhlet apparatus.

5.4 Chemicals used: Methanol (S.D. Fine Chemicals, Mumbai), Distilled water.

5.5 Principle of operation of Soxhlet apparatus: The solvent vapor, generated by gently heating the reservoir, condenses and is allowed to drip back onto the porous sample cup. The liquid condensate that drips onto the sample performs the extraction which then passes through the container and back into the reservoir. The cycle is repeated continuously and can be sustained as long as needed.

5.6 Method:

The dried plant materials were extracted by maceration extraction process. 300gm of coarse powdered plant material packed in filter paper and were placed in a stoppered apparatus with 80% methanol solvent. The flask was shaken for 10 minutes initially following which it was shaken again 2-3 times at an interval of three hours. The soxhlet extraction was performed for approximately 10 hours. This was then allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter was dissolved. The mixture was then strained. The marc (the damp solid material) was pressed and the combined liquids were clarified by filtration or by decantation after standing. The extracted solvent was evaporated under reduced pressure and dried to get a material of waxy consistency. The extractive value of the extraction was calculated using the following formula:

$$\% \text{ of extraction} = \frac{\text{Weight of dried extract}}{\text{Weight of fresh material}} \times 100$$

5.7 Result and discussion

The % yield value of extraction is illustrated in **Table 5.1**.

Table – 5.1: % yield value of extraction

Plant	Part of plant	weight of sample taken 'a' (g)	weight of extract obtained 'b' (g)	Solvent used	Quantity of solvent (liter)	% Yield value (g)
M roxburghianus	Leaf	300	29.0	Methanol 80%	1.5	9.66
P fraternus	Whole plant	300	31.2	Methanol 80%	1.5	10.4

This dried extract obtained was stored in an airtight container, to avoid any deterioration.

CHAPTER 6
PHARMACOGNOSTIC AND PHYTOCHEMICAL EVALUATION OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 6: PHARMACOGNOSTIC AND PHYTOCHEMICAL EVALUATION OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS

The use of herbs as a source of medicine dates as far back as prehistoric times. Medicinal plants contain phytochemicals that can be used for therapeutic purpose or as precursors for pharmaceutical synthesis. Quality control tests such as identity, purity, content, and other chemical, physical and/or biological properties of herbal products for the purpose of efficacy and safety is essential. World Health Organization (WHO) has prescribed a number of quality control tests that medicinal plant materials should undergo. Preliminary pharmacognostical and phytochemical screening will be useful in the detection of drug adulteration (Seow *et al.,* 2013).

6.1 Aim of the study: Pharmacognosy analysis raw materials from two different species of *M. roxburghianus* (leaf) *and P. fraternus* (whole plant).

6.2 Materials and Methods

6.2.1 Test substance

M. roxburghianus and *P. fraternus* plant were studied.
The raw materials were dried and powdered, the powdered material were analysed for different parameters.

6.2.2 Procedure

Pharmacognostical evaluation of *M. roxburghianus* and *P. fraternus* plant as well as phytochemical evaluation for various phytoconstituents like carbohydrates, glycosides, saponins, alkaloids, flavonoids, phenolics & tannins, phytosterols & triterpenoids, fixed oils and fats etc. were carried out.

I. Pharmacognostic evaluation

1. Determination of Moisture Content or Loss on Drying
2. Determination of Extractive values
    (a) Alcohol soluble extractive
    (b) Water soluble extractive
3. Fluorescence Analysis
4. Determination of Ash Values
    (a) Determination of Total Ash
    (b) Acid Insoluble Ash
    (c) Water Soluble Ash
    (d) Sulphated ash

II. Phytochemical evaluation

1. Detection for carbohydrates
2. Detection of Glycosides
3. Detection of Saponins
4. Detection of Alkaloids
5. Detection of Flavonoids
6. Detection of Phenolics and Tannins
7. Detection of Phytosterols and Triterpenoids
8. Detection of fixed oils and fats

6.2.2.1 Pharmacognostic evaluation

6.2.2.1.1. Determination of moisture content or loss on drying

About 5 g of each raw material were accurately weighed. The air dried material was taken in a previously dried and tarred flat weighting bottle in IR moisture balance and the temperature was adjusted to 105^{o}C and heating was done for 5 minutes. The procedure was repeated for three times for different samples and the loss in weight of the formulation was calculated with respect to the original weight **(**Prashanth *et al.*, 2012**)**.

The LOD was calculated using the following formula:

$$LOD = W1/W2 \times 100$$

where,

W1-weight of raw material after heating
W2- Original weight of the raw material
LOD: Loss on drying

6.2.2.1.2. Determination of Extractive values

(a) Alcohol soluble extractive (ASE)

Macerate 4 g of the air dried drug, coarsely powdered, with 100 mL of 90% ethanol unless otherwise specified in a glass stopper flask for 24 hours. Shake the contents frequently during the first 6 hours & allow it to stand for 18 hours. Thereafter filter rapidly taking precautions against loss of ethanol. Evaporate 25 mL of the filtrate to dryness on a water bath in a tarred flat bottomed petriplate/shallow dish. Dry at 105° for 1hrs in a hot air oven, remove the dish, cool in a desiccator & weigh. Repeat the process

till the concordant weight is obtained calculate the percentage of ethanol-soluble extractive with reference to the air dried drug (Prashanth *et al.,* 2012).

The formula used for calculating percentage of alcohol extractive value is:

$$\% \text{ of alcohol soluble extractive value} = \frac{B\text{-}A \times 4 \times 100}{W}$$

Where,

A= empty wt. of the dish (g)
B= wt. of dish + residue (g)
W= wt. of plant material taken (g).

(b) Water soluble extractive (WSE)

Macerate 4 g of the air dried drug, coarsely powdered, with 100ml of (5%) chloroform water in a glass stopper conical flask for 24hrs, shaking the contents frequently during the first 6 hours. Thereafter filter rapidly by decanting the water extract. Evaporate 25ml of the filtrate to dryness on a water bath in tarred flat bottomed petriplate/shallow dish. Add 2ml of alcohol to the dry residue shake the contents and dry again on water bath. Dry at 105°c for 1hrs in the hot air oven and cool in a desiccator for 30 minutes and weigh. Repeat the process till the concordant weight is obtained. Calculate the % of WSE with reference to the air dried drug (Prashanth *et al.,* 2012).

The formula used for calculating percentage of water extractive value is:

$$\% \text{ of water soluble extractive value} = \frac{B\text{-}A \times 4 \times 100}{W}$$

Where,

A = empty wt. of the dish (g).
B = wt. of dish + residue (g).

6.2.2.1.3. Fluorescence Analysis

The powdered drugs were treated with sodium hydroxide in methanol, sodium hydroxide (NaOH) in water, HCl in water etc. as mentioned in **Table 6.3**. In most of the cases a definite colour variations was observed under ordinary and ultraviolet rays long (360 nm) and short (254 nm) (Chase and Prat, 1949).

6.2.2.1.4. Determination of Ash Values

Ash values are helpful in determining the quality and purity of crude drug, especially in powder form. The determination of ash value is meant for detecting low grade product, exhausted drugs and sandy or earthy matter. It can also be utilized as mean of detecting the chemical constituents by making use of water soluble ash and acid value.

Ash values such as total ash, acid insoluble ash, water soluble ash, acid soluble and sulfated ash were determined. The total ash determination method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological ash", which is the residue of extraneous matter adhering to the plant surface.

a. Determination of Total Ash

About 3 g each of powdered parts were accurately weighed and taken separately in silica crucible, which was previously ignited and weighed. The powder was spread as a fine layer on the bottom of crucible. The powder was incinerated gradually by increasing temperature to make it dull red hot until free from carbon. The crucible was cooled and weighed. The procedure was repeated to get constant weight. The percentage of total ash was calculated with reference to the air dried powder (Indian Pharmacopoeia, 1985).

b. Acid Insoluble Ash

The ash obtained as described above was boiled with 25 mL of 2N HCl for 5 minutes. The insoluble ash was collected on an ash less filter paper and washed with hot water. The insoluble ash was transferred into a crucible, ignited and weighed. The procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air dried drug (Indian Pharmacopoeia, 1985).

$$\text{Acid insoluble ash \%} = \frac{(B - C)}{A} \times 100$$

Where, A = sample weight in g

B = weight of dish + contents after drying (g)

C = weight in g of empty dishes

c. Water Soluble Ash

The ash obtained as described for the total ash, was boiled for 5 minutes with 25 mL of water. The insoluble matter was collected on ash less filter paper and washed with hot water. The insoluble ash was transferred into silica crucible, ignited for 15 minutes. and weighed. The procedure was repeated to get a constant weight. The weight of insoluble matter was subtracted from the weight of total ash. The difference of weight was considered as water soluble ash. The percentage of water soluble ash was calculated with reference to air dried parts respectively (Indian Pharmacopoeia, 1985).

d. Sulphated ash

A silica crucible was heated to red for 10 minutes and was allowed to cool in a desiccator and weighed. A gram of substance was accurately weighed and transferred to the crucible. It was ignited gently at first, until the substance was thoroughly charred. Then the residue was cooled and moistened with 1 mL of concentrated sulfuric acid, heated gently until white fumes are no longer evolved and ignited at $800^{o}C \pm 25^{o}C$ until all black particles have disappeared. The ignition was conducted in a place protected from air currents. The crucible was allowed to cool. A few drops of concentrated sulfuric acid were added and heated. Ignited as before and was allowed to cool and weighed. The operation was repeated until two successive weighing do not differ by more than 0.5 mg (Indian Pharmacopoeia, 1985).

6.2.2.2 Phytochemical evaluation

6.2.2.2.1. Detection for carbohydrates

500 mg of extract was dissolved in 5 mL of distilled water and filtered. The filtrate was used to test the presence of carbohydrates (Rosenthaler L, 1930).

Molisch's test:

Molish reagent: 10 g of alpha napthol was dissolved in 100 ml of 95% methanol to prepare Molish reagent. To the extract, two drops of Molish reagent and few drops of concentrated H_2SO_4 is added, formation of purple-violet ring indicates the presence of carbohydrates.

6.2.2.2.2. Detection of Glycosides:

0.5 g of the extract was hydrolyzed with 20 ml of HCl (0.1 N) and filtered. The filtrate was used to test the presence of Glycosides (Rosenthaler L, 1930; Middeltone H, 1956).

a. Keller-Killiani test: To the extract, few drops of glacial acetic acid and one drop of 5% $FeCl_3$ and concentrated H_2SO_4 was added, formation of reddish brown colour at the

junction of two liquid layers and upper layer turned bluish green indicates the presence of glycosides.

6.2.2.2.3. Detection of Saponins:

Foam test: 1 mL of extract was diluted to make up to 20 mL with distilled water and slowly shake in a graduated cylinder for 15 minutes. One cm layer of foam indicates the presence of saponins (Kokate *et al.*, 2001).

6.2.2.2.4. Detection of Alkaloids

0.5 g of the extract was dissolved in 10 mL of dilute HCL (0.1N) and filtered. The filtrate was used to test the presence of alkaloids (Rosenthaler L, 1930; Peach and Trancey, 1955).

a. Mayer's test:

Mayer's reagent**:** readily available from SD Fine Chemicals, Mumbai.

Filtrate was treated with Meyer's reagent; formation of yellow cream colored precipitate indicates the presence of alkaloids.

b. Dragendrodroff's test:

Dragendroff's reagent:

i) Dissolve 8 g of bismuth subnitrate in 20 ml of nitric acid.

ii) Dissolve 27.2 g of Potassium iodide in 50 ml of distilled water

Mix (i) and (ii) and adjust the volume to 100 ml with distilled water.

Filtrate was treated with Dragendroff's reagent; formation of red colored precipitate indicates the presence of alkaloids.

6.2.2.2.5. Detection of Flavonoids

a. Alkaline reagent test:

To 100 mg 0f extract, few drops of NaOH solution was added in a test tube. Formation of intense yellow color that becomes colorless on addition of few drops of of dilute HCl indicates the presence of Flavonoids (Shellard EJ, 1957).

6.2.2.2.6. Detection of Phenolics and Tannins

100 mg of extract was boiled with 1 ml of distilled water and filtered. The filtrate was used for the following test,

a. Ferric chloride test: To 2 ml of filtrate, 2 ml of 1% ferric chloride solution was added in a test tube. Formation of bluish black color indicates the presence of phenolic nucleus.

b. Test for Tannins: To the extract 0.5 ml NaOH was added, formation of precipitate indicates the presence of tannins (Kokate *et al.,* 2001).

6.2.2.2.7. Detection of Phytosterols and Triterpenoids (Paech and Tracey, 1955; Finar IL, 1959): 0.5 g of extract was treated with 10 ml chloroform and filtered. The filtrate was used to test the presence of Phytosterols and Triterpenoids.

a. Leibermann's test: To 2 ml of filtrate in hot alcohol, few drops of acetic anhydride was added. Formation of brown precipitate indicates the presence of sterols.

b. Leiberman-Bucharat test: To the extract, few drops of acetic acid and concentrated H_2SO_4 were added, deep red ring at the junction of two layers indicates the presence of triterpenes.

c. Salkowaski test: To the extract solution few drops of concentrated Sulphuric acid was added and shaken and allowed to stand, lower layer turns red indicating the presence of sterols.

6.2.2.2.8. Detection of fixed oils and fats

Oily spot test: One drop of extract was placed on filter paper and solvent was allowed to evaporate. An oily stain on filter paper indicates the presence of fixed oil (Rosenthaler L, 1930).

6.3 Results and discussion:

Pharmacognostic and phytochemical evaluation of *M. roxburghianus* (leaf) and *P. fraternus* (whole plant) were performed.

6.3.1 Pharmacognostic evaluation

The pharmacognostic data generated is helpful in determining the quality and purity of a crude drug, especially in powdered form. In this study the parameters used for the evaluation of were moisture content, extractive values by different solvents (alcohol and water), Fluorescence analysis and ash values (total ash, water soluble and acid insoluble ash) (**Table 6.1, Table 6.2, Table 6.3** and **Table 6.4)**.

Table – 6.1: Determination of moisture content

Plant	Plant part used	Moisture content (%)
M. roxburghianus	Leaf	5.6
P. fraternus	Whole plant	9.2

Table – 6.2: Determination of alcohol and water soluble extractive values

Plant	Plant part used	Alcohol soluble extractive values (%)	Water soluble extractive values (%)

M. roxburghianus	Leaf	13.0	18.0
P. fraternus	Whole plant	15.0	21.0

Table – 6.3: Fluorescence analysis

Plant	Test sample + Solvent	Ordinary light	UV short light
M. roxburghianus (Leaf)	Distilled water	Yellow	Green
	Chloroform	Dark yellow	Dark green
	1N NaoH in water	Brown	Dark brown
	1N NaoH in methanol	Yellow	Dark green
	10 % HCL	Light yellow	Light yellow
	10 % H_2SO_4	Light yellow	Light green
P. fraternus (Whole plant)	Distilled water	Brown	Brown
	Chloroform	Dark green	Green
	1N NaoH in water	Dark brown	Dark brown
	1N NaoH in methanol	Green	Yellowish green
	10 % HCL	Brown	Light green
	10 % H_2SO_4	Brown	Light green

Table -6.4: Determination of Ash Values

Plant	Plant part used	Total Ash (%)	Acid insoluble Ash (%)	Water soluble Ash (%)	Sulphated Ash (%)
M.roxburghianus	Leaf	6.0	1.33	1.66	9.5
P.fraternus	Whole plant	6.0	1.0	2.66	8.0

6.3.2 Phytochemical evaluation

The phytochemical study with methanolic extract of *M. roxburghianus* (Leaf) showed the presence of carbohydrates, glycosides, saponins, phenolics, phytosterols and triterpenoids. On the other hand, methanolic extract of *P. fraternus* (whole plant) showed the presence of carbohydrates, glycosides, saponins, alkaloids, tannins, fixed oils and fats. These results are illustrated in **Table 6.5**.

Table – 6.5: Phytochemical evaluation

Detection	Test	*M. roxburghianus* (Leaf) - Methanolic extract	*P. fraternus* (Whole plant) - Methanolic extract
Test for carbohydrates	Molisch's test	(+)	(+)
Test for Glycosides	Keller-Killiani test	(+)	(+)
Test for Saponins	Foam test	(+)	(+)
Test for Alkaloids	Mayer's test	(-)	(+)
	Dragendrodroff's test	(-)	(+)
Test for Flavonoids	Alkaline reagent test	(-)	(-)

Test for Phenolics and Tannins	Ferric chloride test	(+)	(+)
	Test for Tannins	(-)	(-)
Test for Phytosterols and Triterpenoids	Leibermann's test	(+)	(-)
	Leiberman-Bucharat test	(+)	(-)
	Salkowaski test	(-)	(-)
Test for fixed oils and fats	Oily spot test	(-)	(+)

(+) Present, (-) Absent

6.4 Conclusion:

The recent years have witnessed a resurgence of interest in natural medicines world over as people are turning to use medicinal plants and phyto-chemicals in health care. India has one of the oldest cultural traditions of use of its medicinal flora since Vedic period. Ayurveda, Unani, Siddha and other traditional systems of medicine are one of the oldest systems and utilize large number of medicinal plants.

The pharmacognostic evaluation of powdered roots reveals the standard parameters for the quality and purity of herbal drug and also gives information regarding the authenticity of crude drug. Information from the phytochemical may be further used for isolation of various compounds for the treatment of diseases. Many of these compounds have been shown to produce potent hypoglycaemic, antihyperglycaemic, and glucose suppressive activities. These effects might be achieved by facilitating insulin release from beta pancreatic cells, inhibiting glucose absorption in gut, stimulating glycogenesis in liver and/or increasing glucose utilization by the body.

CHAPTER 7
GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS OF *M M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 7: GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS

Gas chromatography-mass spectroscopy (GC-MS) analysis of plant extracts is becoming a very valuable tool to detect the presence of various phytoconstituents before aiming large scale purification and to minimize replicative phytochemical studies. GC-MS is one of the best techniques to identify the constituents of volatile matter, long and branched chain hydrocarbons, alcoholic acids, esters etc. The mass spectrometer analyzes the compounds eluted at different times and percentage of area occurred to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios.

7.1 Aim of the study: To evaluate the chemical constituents in *M. roxburghianus* and *P. fraternus* plant extract by using modern sensitive gas chromatography–mass spectrometry (GC–MS).

7.2 Materials and Methods:

(a) Equipment: Thermo GC – Trace Ultra Version 5.0 gas chromatography system interfaced to Thermo MS DSQ II mass spectro-meter

(b) Column: DB 5 – MS capillary standard non-polar column with dimensions 30 m × 0.25 mm x 0.25 µm

(c) Carrier Gas: Helium gas

(d) Test substance: *M. roxburghianus* and *P. fraternus* plant extract were studied.

(e) Procedure for gas chromatography–mass spectrometry (GC-MS) analysis

The GC-MS analysis of *M. roxburghianus* and *P. fraternus* plant extract was performed using Thermo GC – Trace Ultra Version 5.0 gas chromatography system interfaced to Thermo MS DSQ II mass spectro-meter instrument detector having automatic sampler. For GC/MS detection, an electron ionization system was used. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min, split less flow 1ml/min. The specification of the capillary column used was DB 5 – MS capillary standard non-polar column with dimensions 30 m × 0.25 mm x 0.25 µm. The oven temperature was kept at 70°C and was programmed to reach 260°C at a rate of 6°C/min. The experiment was programmed with total run time of 37.53 min. Injection volume was 1µl.

The total running time was completed within 37.53 min. The chromatogram obtained from gas chromatography was then analyzed in mass spectrometry to get the mass of all fractions. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Interpretation on mass spectrum GC-MS were identified by matching their recorded mass spectra with the data bank mass spectra of GCMS library. The spectrum of the plant extract component was compared with the spectrum of the known components stored in the library. The name, molecular weight and structure of the components of the test materials were ascertained.

7.3 Results and discussion

In the present study, we characterized the phytochemical profile of *M. roxburghianus* and *P. fraternus* plant extract using GC- MS. The gas chromatogram showed that the relative concentration of various compounds getting eluted at different retention times. The relative percentage of the chemical constituents was expressed as percentage by peak area normalization.

The heights of the peak indicate that the relative concentrations of the components present in the plant extract. The mass spectrometer analysis used to identify the nature and structure of the compounds eluted at different retention times. The large fragments into small compounds giving rise to appearance of peaks at different m/z ratios. The mass spectra of plant extract are the fingerprint of phytocompounds of *M. roxburghianus* and *P. fraternus* plant extract which were identified and matched with those of standards available in the data library. Hence, the results of the GC-MS profile of *M. roxburghianus* and *P. fraternus* plant extract showed the presence of many phyto-compounds. The observations recorded that the major constituents of *M. roxburghianus* are presented in **Table 7.1, Figure 7.1** and the major constituents of *P. fraternus* are presented in **Table 7.2**, **Figure 7.2**.

7.3.1 Interpretation of mass spectrum of *M. roxburghianus* plant extract components

The various components present in *M. roxburghianus* plant extract were detected by the GC-MS and which are shown in **Figure - 7.1** and **Tables - 7.1**.

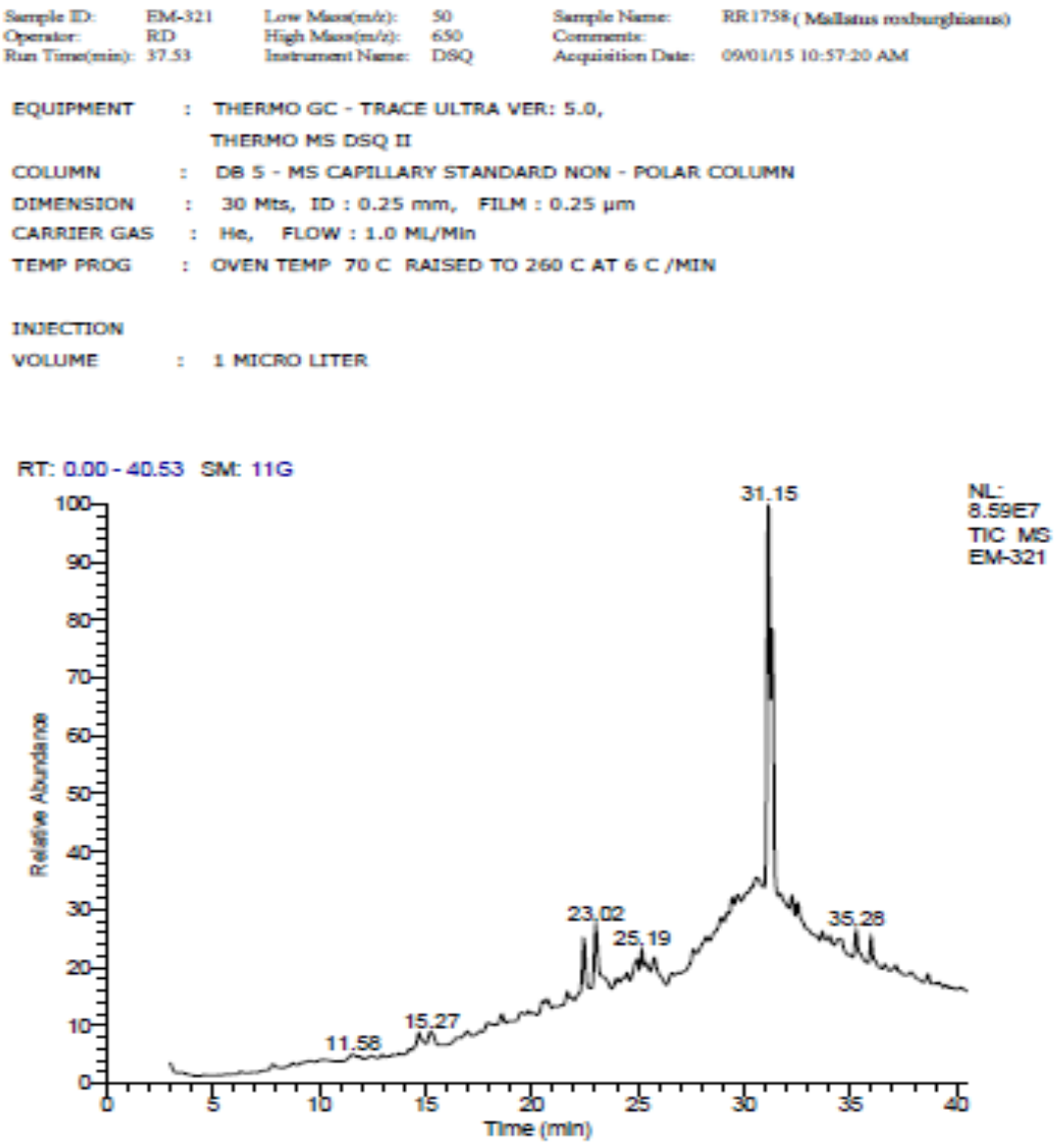
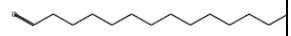
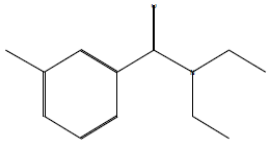
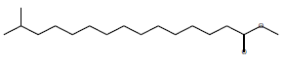
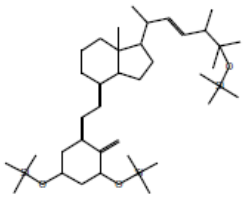
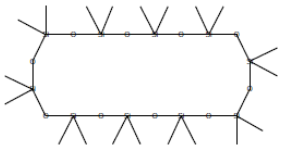
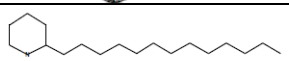
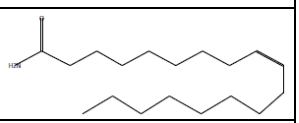
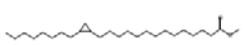
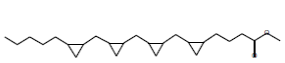
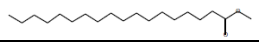
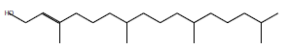
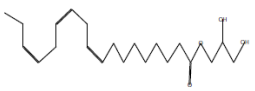
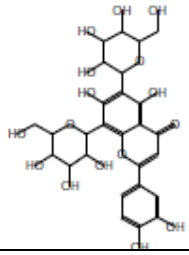
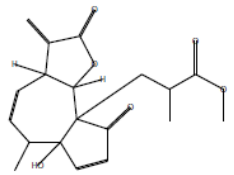
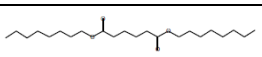
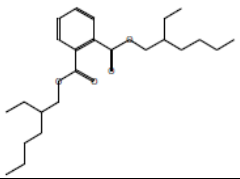
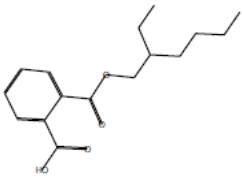
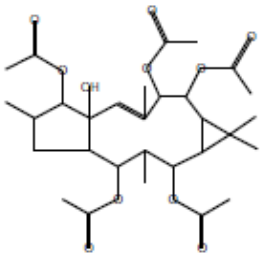
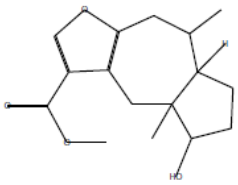
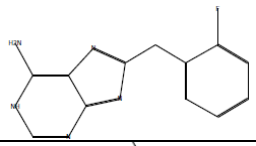
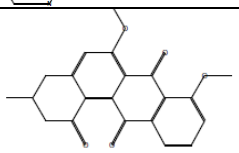


Figure - 7.1: GC-MS chromatogram of *M. roxburghianus* plant extract

Table - 7.1: Phytochemical constituents of *M. roxburghianus* plant extract by GC-MS spectra

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
1	Hexadecanal (CAS)	16.56	$C_{16}H_{32}O$	240 0.99	0.99	
2	Benzamide, N,N-diethyl-3-methyl-	29.11	$C_{12}H_{17}NO$	191	1.56	
3	Pentadecanoic acid, 14-methyl-, methyl ester	12.14	C17H34O2	270	1.66	
4	Tristrimethylsilyl Ether Derivative of 1,25-dihydroxy vitamin D2	21.57	$C_{37}H_{68}O_3Si_3$	644	0.87	
5	Quercetin 7,3',4'-Trimethoxy	22.31	$C_{18}H_{16}O_7$	344	0.87	
6	Cyclodecasiloxane, eicosamethyl-	61.00	$C_{20}H_{60}O_{10}Si_{10}$	740	0.90	
7	Eicosamethylcyclodecasiloxane	61.00	$C_{20}H_{60}O_{10}Si_{10}$	740	0.90	
8	Pyridine, 4-undecyl-	56.22	$C_{16}H_{27}N$	233	0.74	
9	Pyridine, 2-tridecyl-	15.93	$C_{18}H_{31}N$	261	0.74	
10	9-Octadecenamide, (Z)	12.71	$C_{18}H_{35}N_O$	281	0.86	
11	Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester	35.52	$C_{26}H_{50}O_2$	394	2.02	
12	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-	24.90	$C_{25}H_{42}O_2$	374	1.13	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
	pentylcyclopropyl)methyl] cyclopropyl] methyl]cyclopropyl]methyl]-, methyl ester					
13	1-Hexadecanamine	21.29	$C_{16}H_{35}N$	241	4.69	
14	1-Heptadecanamine	74.28	$C_{17}H_{37}N$	255	7.59	
15	Octadecanoic acid, methyl ester	29.06	$C_{19}H_{38}O_2$	298	1.19	
16	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-	53.36	$C_{20}H_{40}O$	296	7.35	
17	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	8.41	$C_{21}H_{36}O_4$	352	4.01	
18	Lucenin 2	29.95	$C_{27}H_{30}O_{16}$	610	1.18	
19	Isochiapin B %2<	16.42	$C_{19}H_{26}O_6$	350	1.47	
20	Silicone Oil	67.25	N/A	0	1.04	NA
21	Hexanedioic acid, dioctyl ester	28.40	$C_{22}H_{42}O_4$	370	1.71	
22	Di-(2-ethylhexyl)phthalate	19.96	$C_{24}H_{38}O_4$	390	44.86	
23	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	16.86	$C_{16}H_{22}O_4$	278	44.86	
24	7aH-	31.47	$C_{30}H_{44}O_{11}$	580	0.74	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
	Cyclopenta[a]cycloprop a[f]cycloundecene-2,4,7 ,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,1 1,11a-dodecahydro- 1,1,3,6,9 -pentamethyl-, 2,4,7,10,11- pentaacetate					
25	Methyl 8,14-Epoxy-15-hydroxy-16-nor-pimarate	64.66	$C_{20}H_{32}O_4$	336	1.53	NA
26	4,4a,5,6,7,7aà,8,9-Octahydro-5á-hydroxy-4aá,8à-dim ethylazuleno[6,5-b]furan-3-carboxylic Acid Methyl Ester	47.94	$C_{16}H_{22}O_4$	278	2.17	
27	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	32.53	$C_{30}H_{50}$	410	1.97	
28	1H-Purin-6-amine, [(2-fluorophenyl)methyl]-(CAS)	36.15	$C_{12}H_{10}FN_5$	243	0.79	
29	dimethoxyrabelomycin	35.40	$C_{21}H_{18}O_5$	350	0.77	

The result pertaining to GC-MS analysis leads to the identification of the number of compounds from the GC fractions of *M. roxburghianus* plant extract and these compounds were identified through mass spectrometry. GC-MS analysis of *M. roxburghianus* plant extract showed the presence of twenty nine bioactive compounds that could contribute towards the medicinal properties to the plant. These constituents are Hexadecanal; Benzamide, N,N-diethyl-3-methyl-; Pentadecanoic acid, 14-methyl-, methyl ester; Tristrimethylsilyl Ether Derivative of 1,25-dihydroxy vitamin D2; Quercetin 7,3',4'-Trimethoxy; Cyclodecasiloxane, eicosamethyl-; Eicosamethylcyclodecasiloxane; Pyridine, 4-undecyl-; Pyridine, 2-tridecyl-; 9-Octadecenamide, (Z); Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester; Cyclopropanebutanoic acid, 2-[[2-[[2-[(2-pentylcyclopropyl)methyl] cyclopropyl] methyl]cyclopropyl]methyl]-, methyl ester; 1-Hexadecanamine; 1-Heptadecanamine;

Octadecanoic acid, methyl ester; 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-; 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- ; Lucenin 2; Isochiapin B; Silicone Oil; Hexanedioic acid, dioctyl ester; Di-(2-ethylhexyl)phthalate; 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester; 7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate; Methyl 8,14-Epoxy-15-hydroxy-16-nor-pimarate ; 4,4a,5,6,7,7aà,8,9-Octahydro-5á-hydroxy-4aá,8à-dimethylazuleno[6,5-b]furan-3-carboxylic Acid Methyl Ester; 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- ; 1H-Purin-6-amine, [(2-fluorophenyl)methyl]- ; and dimethoxyrabelomycin.

Of these 29 constituents, the major chemical components and their % peak area are di-(2-ethylhexyl)phthalate (44.86 %); 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) Ester (44.86 %);1-Heptadecanamine (7.59%); 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (7.35%); 1-Hexadecanamine (4.69%); 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- (4.01%); 4,4a,5,6,7,7aà,8,9-Octahydro-5á-hydroxy-4aá,8à-dim ethylazuleno[6,5-b]furan-3-carboxylic Acid Methyl Ester (2.17%); Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester (2.02%); 2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- (1.97%); Hexanedioic acid, dioctyl ester (1.71%); Benzamide, N,N-diethyl-3-methyl-(1.56%); Pentadecanoic acid, 14-methyl-, methyl ester (1.66%); and Methyl 8,14-Epoxy-15-hydroxy-16-nor-pimarate (1.53 %).

7.3.2 Interpretation of mass spectrum of *P. fraternus* plant extract components

The various components present in *P. fraternus* plant extract were detected by the GC-MS and which are shown in **Figure - 7.2** and **Tables - 7.2**.

Sample ID:	EM-322	Low Mass(m/z):	50	Sample Name:	RR1759 (Phyllanthus fraternus)
Operator:	RD	High Mass(m/z):	650	Comments:	
Run Time(min):	37.51	Instrument Name:	DSQ	Acquisition Date:	09/01/15 11:39:49 AM

EQUIPMENT : THERMO GC - TRACE ULTRA VER: 5.0,
THERMO MS DSQ II
COLUMN : DB 5 - MS CAPILLARY STANDARD NON - POLAR COLUMN
DIMENSION : 30 Mts, ID : 0.25 mm, FILM : 0.25 µm
CARRIER GAS : He, FLOW : 1.0 Ml/Min
TEMP PROG : OVEN TEMP 70 C RAISED TO 260 C AT 6 C /MIN

INJECTION
VOLUME : 1 MICRO LITER

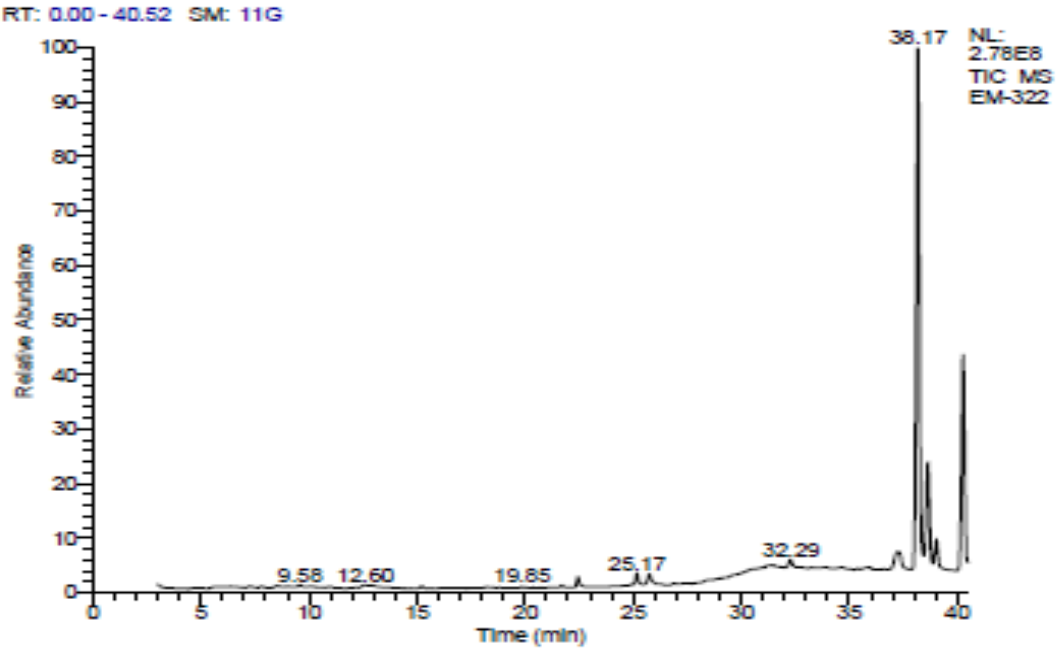
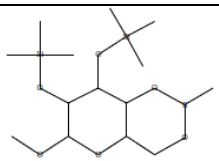
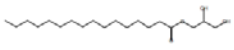
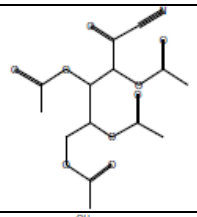
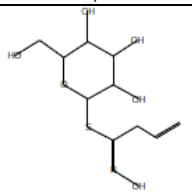
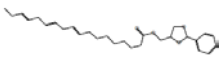
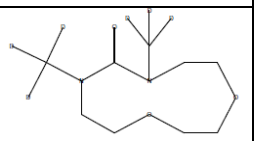
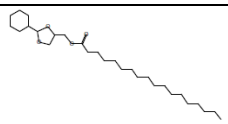
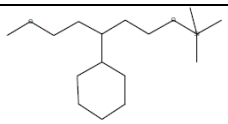
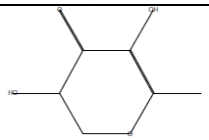
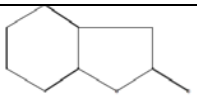
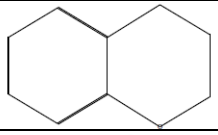
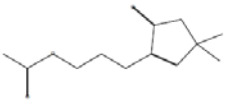
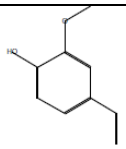
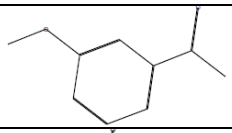
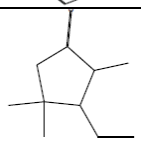
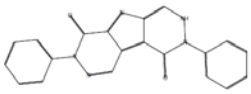
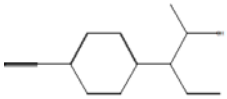
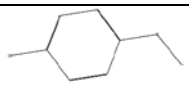
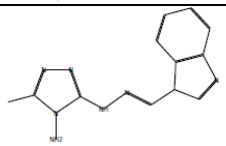
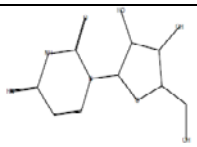
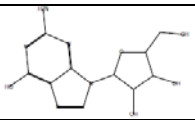
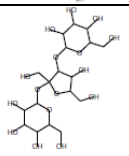
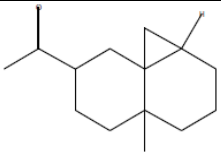


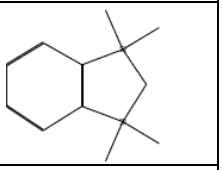
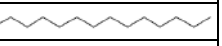
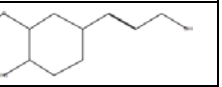
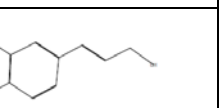
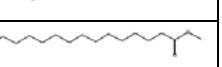
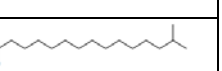
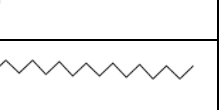
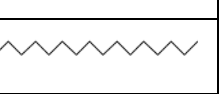
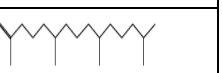
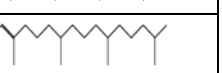
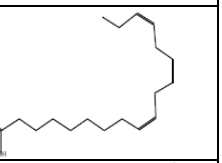
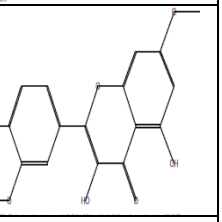
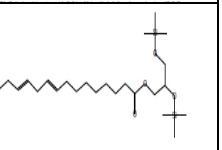
Figure - 7.2: GC-MS chromatogram of *P. fraternus* plant extract

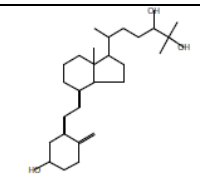
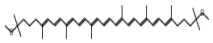
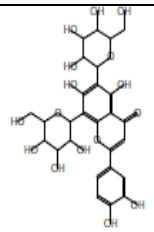
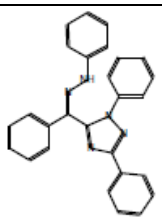
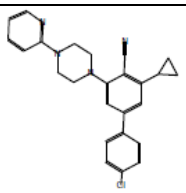
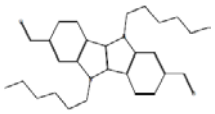
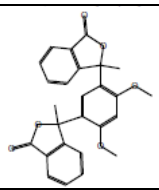
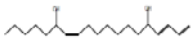
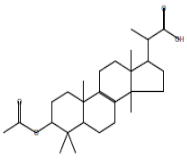
Table – 7.2: Phytochemical constituents of *P. fraternus* plant extract by GC-MS spectra

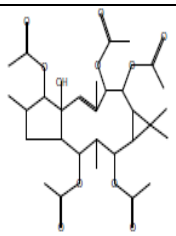
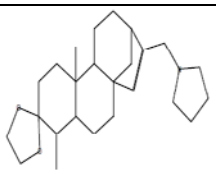
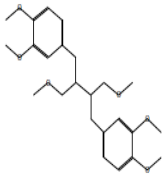
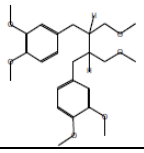
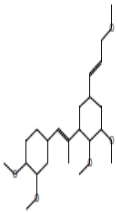
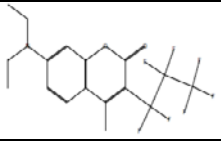
S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
1	á-D-Glucopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate	10.96	$C_{14}H_{31}BO_6Si_2$	362	0.29	
2	Hexadecanoic acid, 2,3-dihydroxypropyl ester (CAS)	10.54	$C_{19}H_{38}O_4$	330	0.29	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
3	Tetraacetyl-d-xylonic nitrile	35.90	$C_{14}H_{17}NO_9$	343	0.40	
4	Desulphosinigrin	10.36	$C_{10}H_{17}NO_6S$	279	0.40	
5	Trans-2-phenyl-1,3-dioxolane-4-methyl octadec-9,12,15-trienoate	24.09	$C_{28}H_{40}O_4$	440	0.20	
6	7,9-di(trideuteromethyl)-1,4-dioxa-7,9-diazacycloundecane-8-one	10.25	$C_9H_{12}D_6N_2O_3$	202	0.20	
7	Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	9.05	$C_{28}H_{46}O_4$	446	0.20	
8	1-Methoxy- 5-trimethylsilyloxy-3-phenylpentane	40.32	$C_{15}H_{26}O_2Si$	266	0.28	
9	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	9.66	$C_6H_8O_4$	144	0.28	
10	Dodecane	40.72	$C_{12}H_{26}$	170	0.20	
11	2-Coumaranone	12.10	$C_8H_6O_2$	134	0.39	
12	2H-1-Benzopyran, 3,4-dihydro-	9.02	$C_9H_{10}O$	134	0.39	
13	3-(3,3-Dimethyl-5-oxocyclopent-1-enyl)propyl acetate	15.24	$C_{12}H_{18}O_3$	210	0.32	
14	2-Methoxy-4-vinylphenol	14.64	$C_9H_{10}O_2$	150	0.32	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
15	3-Methoxyacetophenone	10.04	$C_9H_{10}O_2$	150	0.32	
16	2,4,4-trimethyl-3-vinylcyclopentanone	9.26	$C_{10}H_{16}O$	152	0.32	
17	6,6-Dimethyl-9-ethoxy-10-propionyl-4-propyl-2H,6 H-benzo[1,2-b:3,4:b']dipyran-2-one	42.14	$C_{22}H_{26}O_5$	370	0.18	NA
18	2,7-Diphenyl-1,6-dioxopyridazino[4,5-2',3']pyrrolo[4',5'-D]pyridazine	10.25	$C_{20}H_{13}N_5O_2$	355	0.18	
19	3-(4-Cyanophenyl)pent-4-en-2-ol	25.40	$C_{12}H_{13}NO$	187	0.22	
20	Benzene, 1-chloro-4-methoxy-	9.27	C_7H_7ClO	142	0.22	
21	3-[N'-(3H-Indol-3-ylmethylene)-hydrazino]-5-methyl-[1,2,4]triazol-4-ylamine	9.27	$C_{12}H_{13}N_7$	255	0.22	
22	Cytidine	24.43	$C_9H_{13}N_3O_5$	243	0.61	
23	Guanosine (CAS)	11.12	$C_{10}H_{13}N_5O_5$	283	0.61	
24	à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl	10.69	$C_{18}H_{32}O_{16}$	504	0.61	
25	7á-acetyl-4aá-methyl-1aá-decahydrocyclopropa[d]naphthalene	77.81	$C_{14}H_{22}O$	206	0.19	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
26	1,1,3,3-TETRAMETHYL-1,3-DISILAINDAN	11.56	$C_{11}H_{18}Si_2$	206	0.19	
27	Tetradecanal	36.83	$C_{14}H_{28}O$	212	0.31	
28	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	49.50	$C_{10}H_{12}O_3$	180	0.21	
29	Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-	28.51	$C_{10}H_{12}O_3$	180	0.21	
30	Hexadecanoic acid, methyl ester	43.73	$C_{17}H_{34}O_2$	270	0.26	
31	Pentadecanoic acid, 14-methyl-, methyl ester	38.64	$C_{17}H_{34}O_2$	270	0.26	
32	Hexadecanoic acid	83.19	$C_{16}H_{32}O_2$	256	1.11	
33	n-Hexadecanoic acid	83.19	$C_{16}H_{32}O_2$	256	1.11	
34	Phytol	65.94	$C_{20}H_{40}O$	296	1.43	
35	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-(CAS)	65.94	$C_{20}H_{40}O$	296	1.43	
36	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	31.82	$C_{18}H_{30}O_2$	278	1.54	
37	QUERCETIN 7,3',4'-TRIMETHOXY	40.84	$C_{18}H_{16}O_7$	344	0.28	
38	9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	14.91	$C_{27}H_{52}O_4Si_2$	496	0.28	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
39	9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3á,5Z,7E)-	17.48	$C_{27}H_{44}O_3$	416	0.17	
40	.psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	10.15	$C_{42}H_{64}O_2$	600	0.28	
41	Lucenin 2	54.41	$C_{27}H_{30}O_{16}$	610	0.49	
42	Phenyl (1,3-diphenyl-1,2,4-triazol-5-yl)ketone - phenylhydrazone	67.10	$C_{27}H_{21}N_5$	415	0.88	
43	4-(4-Chlorophenyl)-2-(cyclopropyl)-6-[4-(2-pyridyl) piperazinyl-1-yl]benzonitrile	22.48	$C_{25}H_{23}ClN_4$	414	0.88	
44	5,10-Dihexyl-5,10-diihydroindolo[3,2-b]indole-2,7-dicarbaldehyde	34.89	$C_{28}H_{34}N_2O_2$	430	1.12	
45	1,5-dimethoxy-2,4-bis(3-methylphthalidyl)benzol	29.47	$C_{26}H_{22}O_6$	430	1.12	
46	E,E,Z-1,3,12-Nonadecatriene-5,14-diol	7.94	$C_{19}H_{34}O_2$	294	0.24	
47	Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-	15.41	$C_{27}H_{42}O_4$	430	0.49	

S.No	Compound Name	Probability	Molecular Formula	Molecular Weight	Area	Structure
48	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate	32.48	$C_{30}H_{44}O_{11}$	580	0.49	
49	1-[(4'á)-3'-Ethylenedioxy-18'-norkaur-15'-en-17'-yl]pyrrolidine	23.69	$C_{25}H_{39}NO_2$	385	4.00	
50	3á-(Peroxymethyl)-5-vinyl-A,B-bisnor-5á-cholestane	22.77	$C28H_{48}O_2$	416	4.00	NA
51	2-Allyloxy-1-(1,1-dimethylpropyl)-4-n-pentadecylbenzene	15.62	$C_{29}H_{50}O$	414	4.00	
52	1,2-bis[3',4'-Dimethoxybenzyl]-1,2-bis(methoxymethyl)ethane	35.61	$C_{24}H_{34}O_6$	418	66.40	
53	Phyllanthin	22.29	$C_{24}H_{34}O_6$	418	66.40	
54	12,13-seco-1,12-epoxy-16-methoxy norditerpenoid alkaloid	89.76	$C_{24}H_{33}NO_6$	431	13.91	NA
55	2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)-à-methylstilbene	38.28	$C_{23}H_{28}O_5$	384	2.92	
56	7-diethylamino-3-heptafluoropropyl-4-methylcoumarin	24.72	$C_{17}H_{16}F_7NO_2$	399	2.92	

The result pertaining to GC-MS analysis leads to the identification of the number of compounds from the GC fractions of *P. fraternus* plant extract and these compounds were identified through mass spectrometry. GC-MS analysis of *P. fraternus* plant extract showed the presence of fifty six bioactive compounds that could contribute towards the medicinal properties to the plant. These constituents are á-D-Glucopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate; Hexadecanoic acid, 2,3-dihydroxypropyl ester; Tetraacetyl-d-xylonic nitrile; Desulphosinigrin; Trans-2-phenyl-1,3-dioxolane-4-methyl octadec-9,12,15-trienoate; 7,9-di(trideuteromethyl)-1,4-dioxa-7,9- diazacycloundecane-8-one; Octadecanoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis- ; 1-Methoxy- 5-trimethylsilyloxy-3-phenylpentane; 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; Dodecane; 2-Coumaranone; 2H-1-Benzopyran, 3,4-dihydro-; 3-(3,3-Dimethyl-5-oxocyclopent-1-enyl)propyl acetate; 2-Methoxy-4-vinylphenol; 3-Methoxyacetophenone; 2,4,4-trimethyl-3-vinylcyclopentanone; 6,6-Dimethyl-9-ethoxy-10-propionyl-4-propyl-2H,6 H-benzo[1,2-b:3,4:b']dipyran-2-one; 2,7-Diphenyl-1,6-dioxopyridazino[4,5-2',3']pyrrolo[4',5'-D]pyridazine; 3-(4-Cyanophenyl)pent-4-en-2-ol; Benzene, 1-chloro-4-methoxy-; 3-[N'-(3H-Indol-3-ylmethylene)-hydrazino]-5-methy l-[1,2,4]triazol-4-ylamine; Cytidine; Guanosine; à-D-Glucopyranoside, O-à-D-glucopyranosyl-(1.fwdarw.3)-á-D-fructofuranosyl; 7á-acetyl-4aá-methyl-1aá-decahydrocyclopropa[d]naphthalene; 1,1,3,3-tetramethyl-1,3-disilaindan; Tetradecanal; 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol; Phenol, 4-(3-hydroxy-1-propenyl)-2-methoxy-; Hexadecanoic acid, methyl ester;
Pentadecanoic acid, 14-methyl-, methyl ester; Hexadecanoic acid; n-Hexadecanoic acid; Phytol; 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-; 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-; Quercetin 7,3',4'-Trimethoxy;
9,12,15- Octadecatrienoic acid, 2,3-bis [(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-; 9,10-Secocholesta-5,7,10(19)-triene-3,24,25-triol, (3á,5Z,7E)-; .psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-; Lucenin 2; Phenyl (1,3-diphenyl-1,2,4-triazol-5-yl)ketone – phenylhydrazone; 4-(4-Chlorophenyl)-2-(cyclopropyl)-6-[4-(2-pyridyl); piperazinyl-1-yl]benzonitrile; 5,10-Dihexyl-5,10-diihydroindolo[3,2-b]indole-2,7-dicarbaldehyde; 1,5-dimethoxy-2,4-bis(3-methylphthalidyl)benzol; E,E,Z-1,3,12-Nonadecatriene-5,14-diol; Propanoic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17-yl)-; 7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11-hexol,
1,1a,2,3,4,4a,5,6,7,10,11,11a - dodecahydro-1,1,3,6,9-pentamethyl-, 2,4,7,10,11-pentaacetate; 1-[(4'á)-3'-Ethylenedioxy-18'-norkaur-15'-en-17'-yl]pyrrolidine; 3á-(Peroxymethyl)-5-vinyl-A,B-bisnor-5á-cholestane; 2-Allyloxy-1-(1,1-dimethylpropyl)-4-n-pentadecylbenzene; 1,2-bis[3',4'-Dimethoxybenzyl]-1,2-bis(methoxymethyl)ethane;
Phyllanthin; 12,13-seco-1,12-epoxy-16-methoxy norditerpenoid alkaloid; 2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)-à-methylstilbene; and 7-diethylamino-3-heptafluoropropyl-4-methylcoumarin.

Of these 56 constituents, the major components were 1,2-bis[3',4'-Dimethoxybenzyl]-1,2-bis(methoxymet hyl)ethane (66.40%); Phyllanthin 66.40%); 12,13-seco-1,12-epoxy-16-methoxy norditerpenoid Alkaloid (13.91%); 1-[(4'á)-3'- ethylenedioxy-18'-norkaur-15'-en-17'-yl]pyrrolidine (4.00%); 3á-(Peroxymethyl)-5-vinyl-A,B-bisnor-5á-cholestane (4.00%); 2-Allyloxy-1-(1,1-dimethylpropyl)-4-n-pentadecylbenzene (4.00%); 2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)-à-m ethylstilbene (2.92%); 7-diethylamino-3-heptafluoropropyl-4-methylcoumarin (2.92%); 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (1.54%); Phytol (1.43%); 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (1.43%); 5,10-Dihexyl-5,10-diihydroindolo[3,2-b]indole-2,7-dicarbaldehyde (1.12%); 1,5-Dimethoxy-2,4-bis(3-methylphthalidyl)benzol (1.12%); Hexadecanoic acid (1.11%); n-Hexadecanoic acid (1.11%); Lucenin 2 (0.49%); Quercetin 7,3',4'- Trimethoxy (0.28%); and psi, psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy- (0.28%).

7.4 Conclusion

The GC-MS analysis showed the existence of various compounds of medicinal importance. The screening of *M. roxburghianus* and *P. fraternus* plant extract showed occurrence of high percentage area of bioactive compounds using GC-MS.

In the present study, the phyto compounds of *M. roxburghianus* and *P. fraternus* plant extract were identified with molecular formula and structure, which may be used for drug development. This study may also enhance the traditional usage of *M. roxburghianus* due to its bioactive compounds identified by GC-MS analysis. GC-MS analysis is a step towards understanding the nature of active principles in *M. roxburghianus* and *P. fraternus.* This study will also be helpful to further pharmacological study for researchers and drug development in pharmaceutical industry.

CHAPTER 8
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 8: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF PHYTOCHEMICAL CONSTITUENTS OF MALLATUS ROXBURGHIANUS AND PHYLLANTHUS FRATERNUS PLANT EXTRACTS

Plant extracts usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities. Due to these factors, their separation remains a big challenge for the process of identification and characterization.

Recently, chromatographic fingerprint technique, as a more meaningful quality control method of herbal samples, has been attracting more and more people's attention, because the fingerprint technique emphasizes on the integral characterization of compositions of samples with a quantitative degree of reliability and focus on identifying and assessing the stability of the plants. Chromatographic fingerprint is a kind of method to show chemical information of medicines with chromatograms, spectrograms and other graphs by analytical techniques. To date, varieties of chromatographic techniques involving fingerprint include TLC, gas chromatography, HPLC, etc.

HPLC is a versatile, robust, and novel method for the isolation of natural products and for screening herbal composition for pharmaceutically active component in medicinal plants. It is used in phytochemical and analytical chemistry to identify, quantify and purify the individual components of the mixture. Because of its advantages and popularization, HPLC fingerprint analysis has been regarded as the first choice. The fingerprint analysis shows only the result of similarity calculated based on the relative value; retention time, with the selected marker compound as reference standard (Boligon and Athayde 2014).

8.1 Aim of the study: Quantification of chemical constituents in *M. roxburghianus* and *P. fraternus* plant extract by using high performance liquid chromatography (HPLC).

8.2 Materials and Methods:

(a) Reference chemicals

Beta-sitosterol, Gallic acid, Lupeol, Quercitin and Rutin

(b) Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

(c) Procedure for HPLC analysis

M. roxburghianus and *P. fraternus* plant extract were studied. Although published literature report several constituents to be present in *M. roxburghianus* and *P. fraternus*, it would be impossible to qualify and quantify all the reported phytochemical constitute present in both these plant extract. This would require conduct of further exhaustive study and is not covered under the scope of present study.

Chemical characterization of *M. roxburghianus* and *P. fraternus* plant extract was carried out by comparing the detected polyphenol peaks with respect to retention times with those of standard chemicals (Beta-sitosterol, Gallic acid, Lupeol, Quercitin and Rutin) that were monitored using the same HPLC system. Identity and purity of the chemical standards were assessed by HPLC analysis. Few phytochemical constitute such as Beta-sitosterol, Gallic acid, Lupeol, Quercitin and Rutin were analysed and quantified in the plant extracts of *M. roxburghianus* and *P. fraternus* using HPLC technique. Reference and test solution were injected. The fingerprint of extract was analyzed by interpolating data from fingerprint of extract and standard markers.

8.2.1 Quantitative estimation of Beta-sitosterol in test samples

HPLC Conditions:

Column : $C_{18,}$ 250X4.6 mm, 5 µm
Mobile phase : Methanol: 50 M Phosphate buffer (pH:3) :: (70:30 v/v)
Flow rate : 1.0 ml / min
Inj. Volume : 20 µl
Detector wavelength : 254 nm

Standard preparation:

Stock (1000 µg/ml): 10 mg of β-sitosterol in 10 ml mobile phase. Filter and inject the sample
Working solution: Prepare the concentration of 1000 µg/ml to run as a standard. Filter and inject the sample

Test Sample preparation:

Stock (1000 µg/ml): 100 mg of each test sample in 10 ml mobile phase separately. Sonicate, centrifuge, filter and run the sample
Working solution: prepare the concentration of 1000 µg/ml using mobile phase. Sonicate, centrifuge, filter and run the sample.

8.2.2 Quantitative estimation of Gallic acid in test samples

HPLC Conditions:

Column : C_{18}, 250X4.6 mm, 5 µm

Mobile phase : Acetonitrile: Water (20:80)
pH : 3.0
Flow rate : 1.0 ml / min
Inj. Volume : 20 µl
Detector wavelength : 272 nm

Standard preparation:
Stock (1000 µg/ml): 10 mg of Gallic acid in 10 ml methanol. Filter and inject the sample
Working solution: Prepare the concentration of 100 µg/ml using methanol. Filter and inject the sample.

Test Sample preparation:
Stock (1000 µg/ml): 10 mg of each test sample in 10 ml methanol separately. Sonicate, centrifuge, filter and run the sample.
Working solution: prepare the concentration of 100 µg/ml using methanol. Sonicate, centrifuge, filter and run the sample.

8.2.3 Quantitative estimation of Lupeol in test samples

HPLC Conditions:
Column : C_{18}, 250X4.6 mm, 5 µm
Mobile phase : Acetonitrile: 0.1 % Acetic acid in water:: (94:6 v/v)
pH : 6.8
Flow rate : 1.5 ml / min
Inj. Volume : 20 µl
Detector wavelength : 215 nm

Standard preparation:
Stock (1000 µg/ml): 10 mg of Lupeol in 10 ml methanol. Filter and inject the sample.
Working solution: prepare the concentration of 1000 µg/ml to run as a standard. Filter and inject the sample.

Test Sample preparation:
Stock (1000 µg/ml): 100 mg of each test sample in 10 ml methanol separately. Sonicate, centrifuge, filter and run the sample.
Working solution: prepare the concentration of 1000 µg/ml using methanol. Sonicate, centrifuge, filter and run the sample.

8.2.4 Quantitative estimation of Quercitin in test samples

HPLC Conditions:
Column : C_{18}, 250X4.6 mm, 5 µm
Mobile phase : methanol: 0.1% o-phosphoric acid in water (60:40)
Flow rate : 1.1 ml / min

Inj. Volume : 20 µl
Detector wavelength : 262 nm

Standard preparation:
Stock (1000 µg/ml): 10 mg of Quercitin in 10 ml mobile phase. Filter and inject the sample.
Working solution: Using the mobile phase, prepare a solution equivalent to a concentration 100 µg/ml. Filter and inject the sample.

Test Sample preparation:
Stock (1000 µg/ml): 10 mg of each test sample in 10 ml mobile phase separately. Sonicate, centrifuge, filter and run the sample.
Working solution: Using the mobile phase, prepare a solution equivalent to a concentration 100 µg/ml. Sonicate, centrifuge, filter and run the sample.

8.2.5 Quantitative estimation of Rutin in test samples

HPLC Conditions:
Column : C_{18}, 250 X 4.6 mm, 5 µm
Mobile phase : methanol: 0.1% o-phosphoric acid in water (60:40)
Flow rate : 1.0 ml / min
Inj. Volume : 20 µl
Detector wavelength : 255 nm

Standard preparation:
Stock (1000 µg/ml): 10 mg of rutin in 10 ml methanol. Filter and inject the sample.
Working solution: prepare the concentration of 100 µg/ml using methanol. Filter and inject the sample.

Test Sample preparation:
Stock (1000 µg/ml): 10 mg of each test sample in 10 ml methanol separately. Sonicate, centrifuge, filter and run the sample.
Working solution: prepare the concentration of 100 µg/ml using methanol. Sonicate, centrifuge, filter and run the sample.

8.3 Results and discussion

8.3.1 Quantification of Beta-sitosterol in the test samples

Beta-sitosterol was determined and quantified by HPLC in isocratic conditions. **Figure – 8.1, Figure – 8.2** and **Figure – 8.3** illustrates the separation chromatogram of the standard, *M. roxburghianus* and *P. fraternus* plant extract respectively. The peak in the

chromatogram was identified based on the retention time of standards injected separately and by addition of standard solutions. The area and quantification results are provided in **Table – 8.1**.

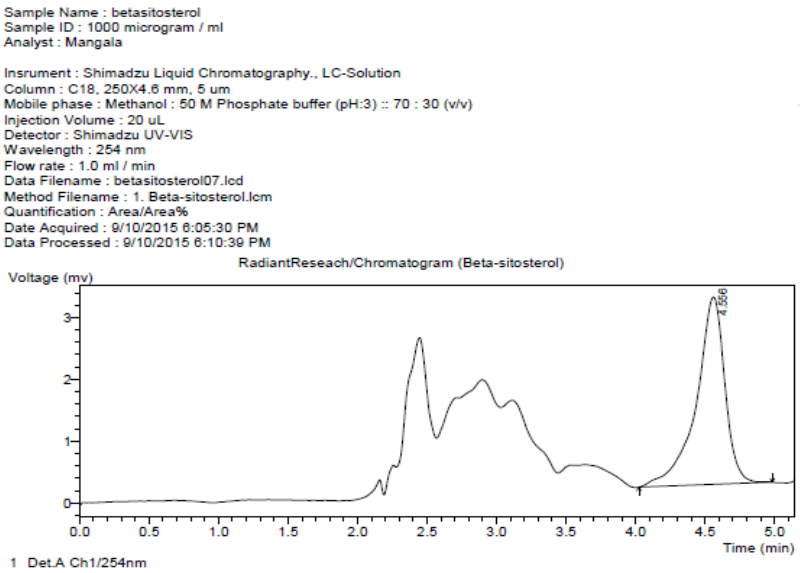


Figure - 8.1: HPLC Chromatogram of standard Beta-sitosterol

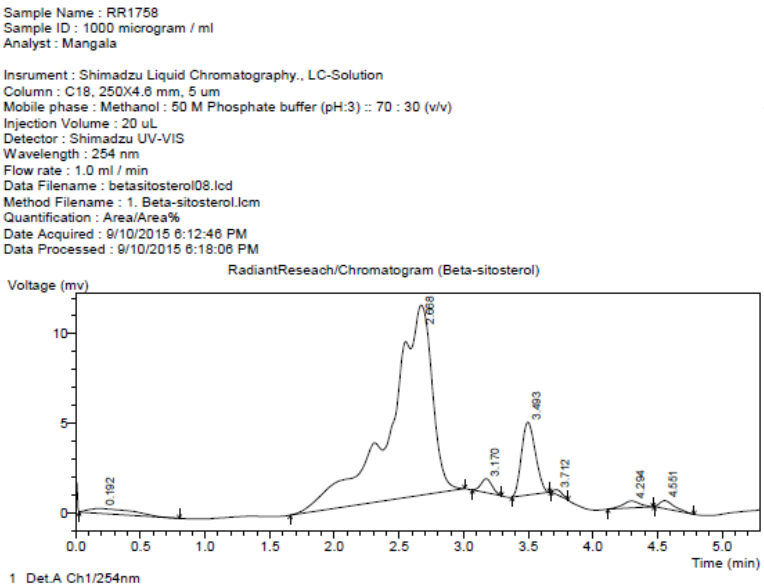


Figure – 8.2: HPLC Chromatogram of *M. roxburghianus* plant extract for Beta-sitosterol estimation

Sample Name : RR1759
Sample ID : 1000 microgram / ml
Analyst : Mangala

Insrument : Shimadzu Liquid Chromatography., LC-Solution
Column : C18, 250X4.6 mm, 5 um
Mobile phase : Methanol : 50 M Phosphate buffer (pH:3) :: 70 : 30 (v/v)
Injection Volume : 20 uL
Detector : Shimadzu UV-VIS
Wavelength : 254 nm
Flow rate : 1.0 ml / min
Data Filename : betasitosterol09.lcd
Method Filename : 1. Beta-sitosterol.lcm
Quantification : Area/Area%
Date Acquired : 9/10/2015 6:18:50 PM
Data Processed : 9/10/2015 6:24:12 PM

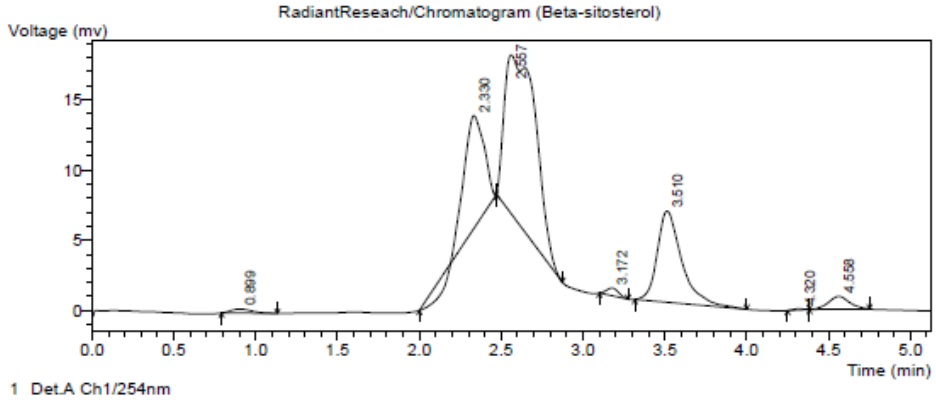


Figure – 8.3: HPLC Chromatogram of *P. fraternus* plant extract for Beta-sitosterol estimation

Table – 8.1: Quantification of Beta-sitosterol

Test sample	Conc. (µg/ml)	Ret. Time (minutes)	Area	Beta-sitosterol Content %
Standard	1000	4.556	43490	95
M. roxburghianus extract	1000	4.551	3449	7.5340
P. fraternus extract	1000	4.558	8099	17.6915

Based on the results presented in the above table it can be confirmed that both *M. roxburghianus* and *P. fraternus* plant extract contained high Beta-sitosterol content. The content in *P. fraternus* plant extract (17.69%) was higher plant than *M. roxburghianus* extract (07.53%).

8.3.2 Quantification of Gallic acid in the test samples

Gallic acid was determined and quantified by HPLC in isocratic conditions. **Figure – 8.4, Figure – 8.5** and **Figure – 8.6** illustrates the separation chromatogram of the standard, *M. roxburghianus* and *P. fraternus* plant extract respectively. The peak in the chromatogram was identified based on the retention time of standards injected separately

and by addition of standard solutions. The area and quantification results are provided in **Table – 8.2**.

Sample Name : gallic acid
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Acetonitrile : Water (20:80) pH:3.0
Injection Volume : 20 uL
Detector : UV
Wavelength : 272nm
Flow rate : 1.0 ml / min
Data Filename : galic 09.lcd
Method Filename : GALIC ACID.lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 1:55:34 PM
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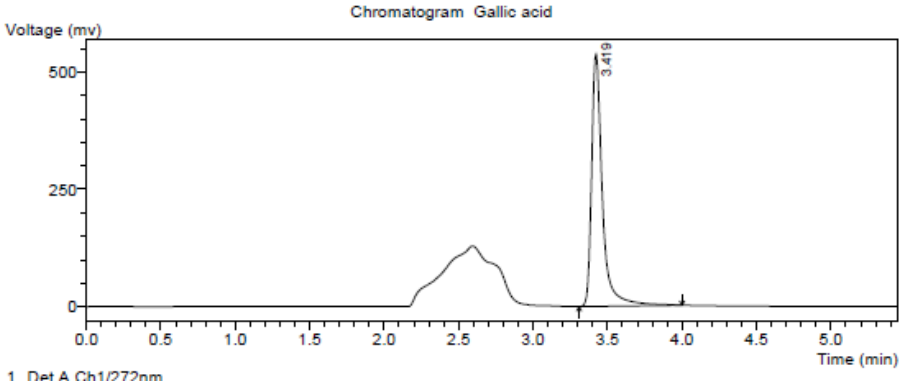

Figure - 8.4: HPLC Chromatogram of standard Gallic acid

Sample Name : rr1758
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Acetonitrile : Water (20:80) pH:3.0
Injection Volume : 20 uL
Detector : UV
Wavelength : 272nm
Flow rate : 1.0 ml / min
Data Filename : galic 10.lcd
Method Filename : GALIC ACID.lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 2:01:46 PM
Data Processed : 9/9/2015 2:06:54 PM

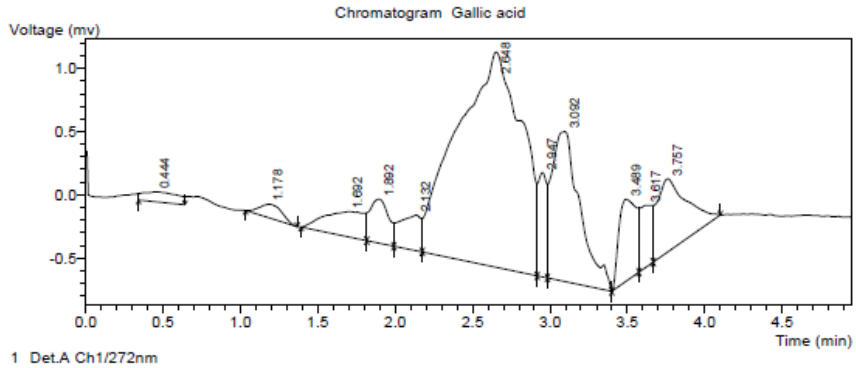

Figure – 8.5: HPLC Chromatogram of *M.roxburghianus* plant extract for Gallic acid estimation

Sample Name : rr1759
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Acetonitrile : Water (20:80) pH:3.0
Injection Volume : 20 uL
Detector : UV
Wavelength : 272nm
Flow rate : 1.0 ml / min
Data Filename : galic 11.lcd
Method Filename : GALIC ACID.lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 2:07:29 PM
Data Processed : 9/9/2015 2:13:30 PM

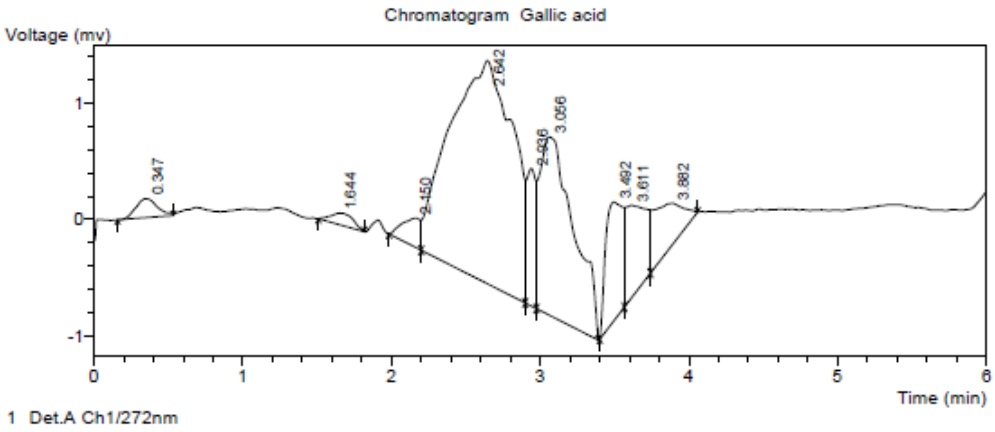

Figure - 8.6: HPLC Chromatogram of *P.fraternus* plant extract for Gallic acid estimation

Table – 8.2: Quantification of Gallic acid

Sample No.	Conc. (µg/ml)	Ret. Time (minutes)	Area	Gallic acid Content %
Standard	100	3.419	2725767	98
Mallatus roxburghianus extract	100	3.489	5212	0.1874
Phyllanthus fraternus extract	100	3.492	8330	0.2995

Based on the results presented in the above table it can be confirmed that both *M. roxburghianus* and *P. fraternus* plant extract contained Gallic acid in concentration of 0.18% and 0.29% respectively.

8.3.3 Quantification of Lupeol in the test samples

Lupeol was determined and quantified by HPLC in isocratic conditions. **Figure – 8.7, Figure – 8.8** and **Figure – 8.9** illustrates the separation chromatogram of the standard, *M. roxburghianus* and *P. fraternus* plant extract respectively. The peak in the chromatogram

was identified based on the retention time of standards injected separately and by addition of standard solutions. The area and quantification results are provided in **Table – 8.3**.

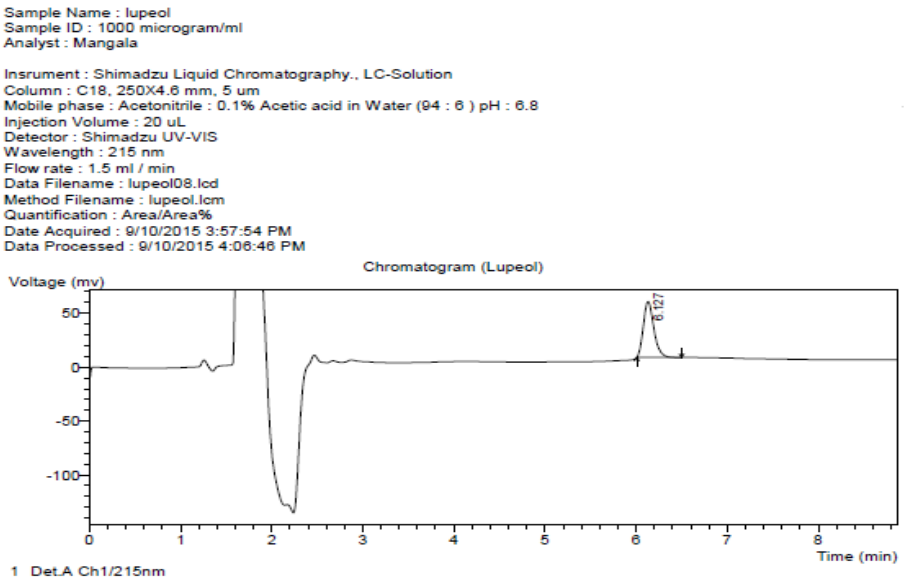

Figure - 8.7: HPLC Chromatogram of standard Lupeol

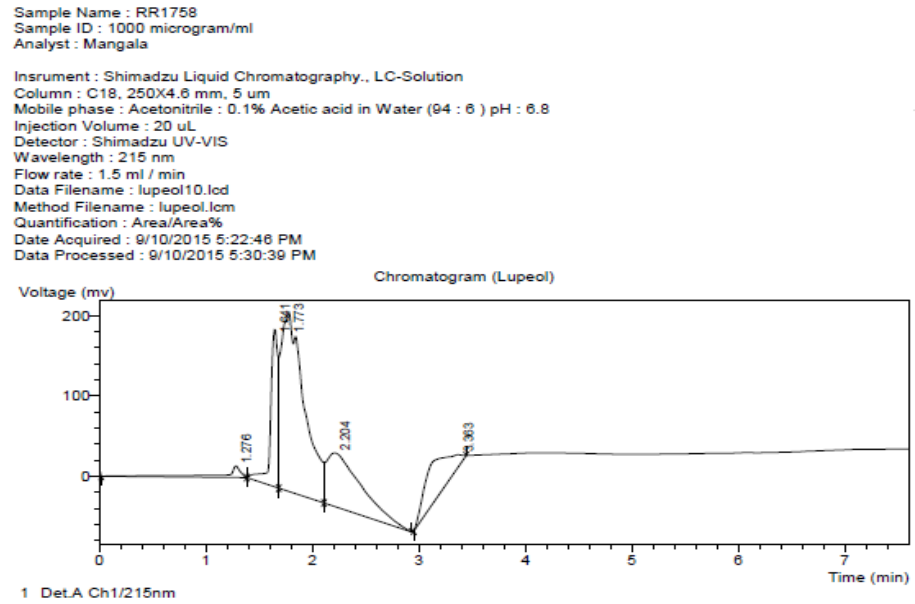

Figure - 8.8: HPLC Chromatogram of *M. roxburghianus* plant extract for Lupeol estimation

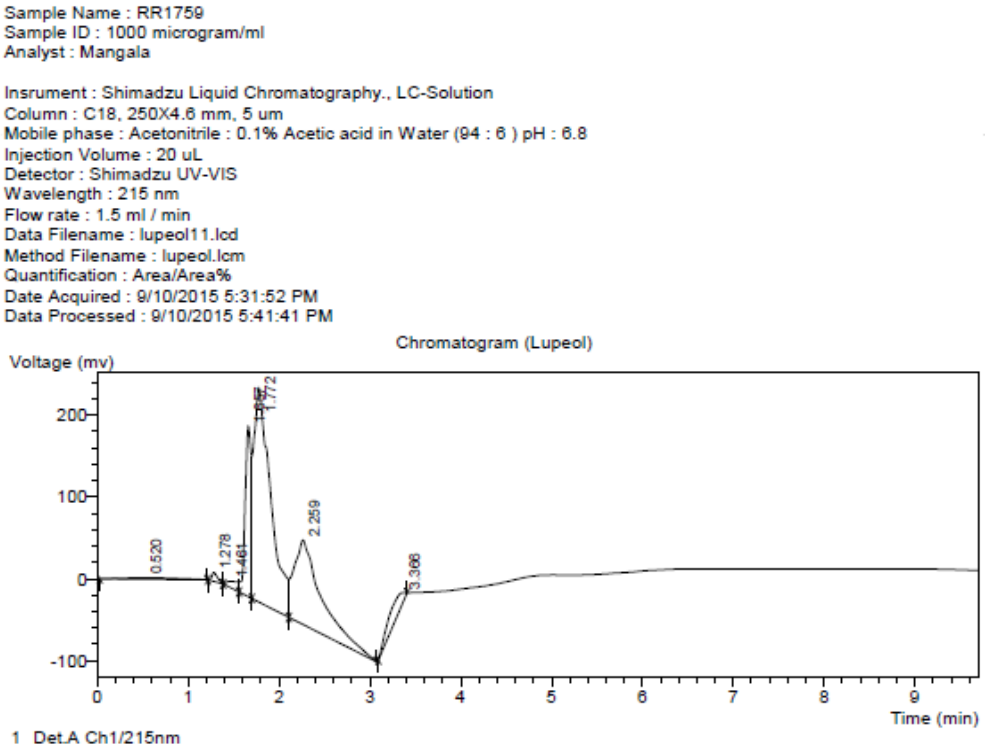

Figure – 8.9: HPLC Chromatogram of *P. fraternus* plant extract for Lupeol estimation

Table – 8.3: Quantification of Lupeol

Sample No.	Conc. (µg/ml)	Ret. Time (minutes)	Area	Lupeol Content %
Standard	1000	6.127	413578	94
M. roxburghianus extract	1000	---	---	0
P. fraternus extract	1000	----	---	0

Based on the results presented in the above table it can be confirmed that both *M. roxburghianus* and *P. fraternus* plant extract did not contain Lupeol.

8.3.4 Quantification of Quercitin in the test samples

Quercitin was determined and quantified by HPLC in isocratic conditions. **Figure – 8.10, Figure – 8.11** and **Figure – 8.12** illustrates the separation chromatogram of the standard, *M. roxburghianus* and *P. fraternus* plant extract respectively. The peak in the chromatogram was identified based on the retention time of standards injected separately and by addition of standard solutions. The area and quantification results are provided in **Table – 8.4**.

Sample Name : quercitin
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Methanol : Methanol : 0.1% Phosphoric acid in water :: 60 : 40 (v/v)
Injection Volume : 20 uL
Detector : UV
Wavelength : 262 nm
Flow rate : 1.1 ml / min
Data Filename : quercitin 07.lcd
Method Filename : quercitin (22-01-15).lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 10:25:27 AM
Data Processed : 9/9/2015 10:35:33 AM

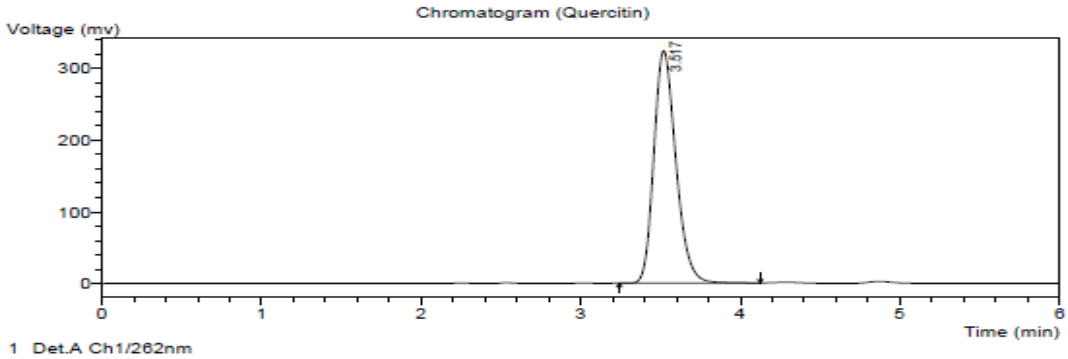

Figure – 8.10: HPLC Chromatogram of standard Quercitin

Sample Name : RR1758
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Methanol : Methanol : 0.1% Phosphoric acid in water :: 60 : 40 (v/v)
Injection Volume : 20 uL
Detector : UV
Wavelength : 262 nm
Flow rate : 1.1 ml / min
Data Filename : quercitin 08.lcd
Method Filename : quercitin (22-01-15).lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 10:40:30 AM
Data Processed : 9/9/2015 10:50:34 AM

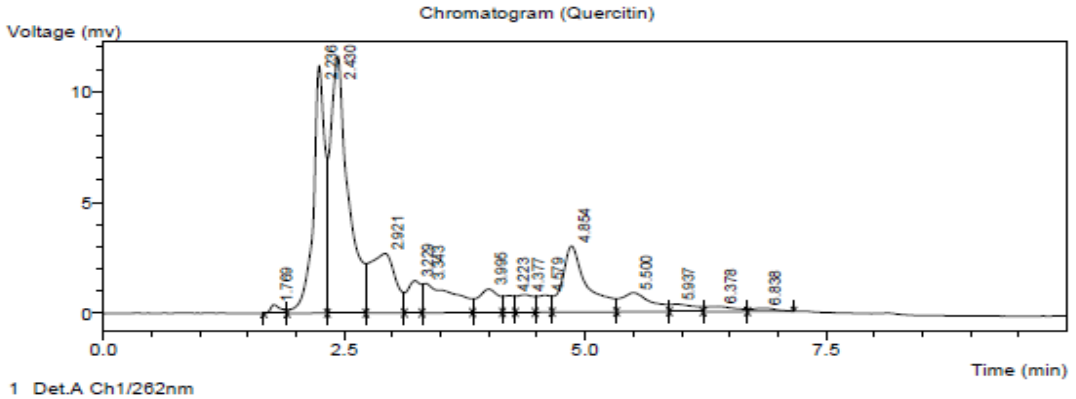

Figure – 8.11: HPLC Chromatogram of *M. roxburghianus* plant extract for Quercitin estimation

Sample Name : RR1759
Sample ID : 100 microgram/ml

Column : C18
Mobile phase : Methanol : Methanol : 0.1% Phosphoric acid in water :: 60 : 40 (v/v)
Injection Volume : 20 uL
Detector : UV
Wavelength : 262 nm
Flow rate : 1.1 ml / min
Data Filename : quercitin 09.lcd
Method Filename : quercitin (22-01-15).lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 10:51:44 AM
Data Processed : 9/9/2015 11:01:46 AM

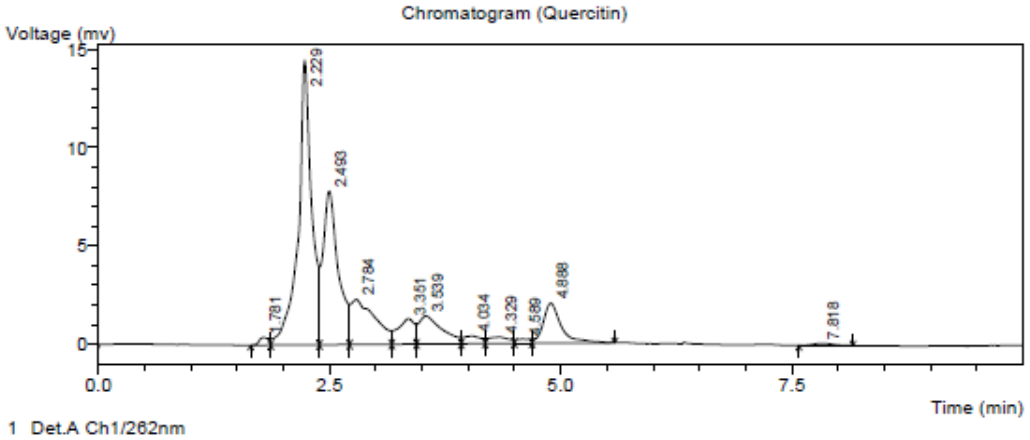

Figure – 8.12: HPLC Chromatogram of *P. fraternus* plant extract for Quercitin estimation

Table – 8.4: Quantification of Quercitin

Sample No.	Conc. (µg/ml)	Ret. Time (minutes)	Area	Quercitin Content %
Standard	100	3.517	3111554	98
M. roxburghianus extract	100	-	-	0
P. fraternus extract	100	3.539	24829	0.782002176

Based on the results presented in the above table it can be confirmed that *M. roxburghianus* plant extract did not contain Quercitin while *P. fraternus* plant extract did contain Quercitin (0.78%).

8.3.5 Quantification of Rutin in the test samples

Rutin was determined and quantified by HPLC in isocratic conditions. **Figure – 8.13, Figure – 8.14** and **Figure – 8.15** illustrates the separation chromatogram of the standard, *M. roxburghianus* and *P. fraternus* plant extract respectively. The peak in the chromatogram was identified based on the retention time of standards injected separately

and by addition of standard solutions. The area and quantification results are provided in **Table – 8.5**.

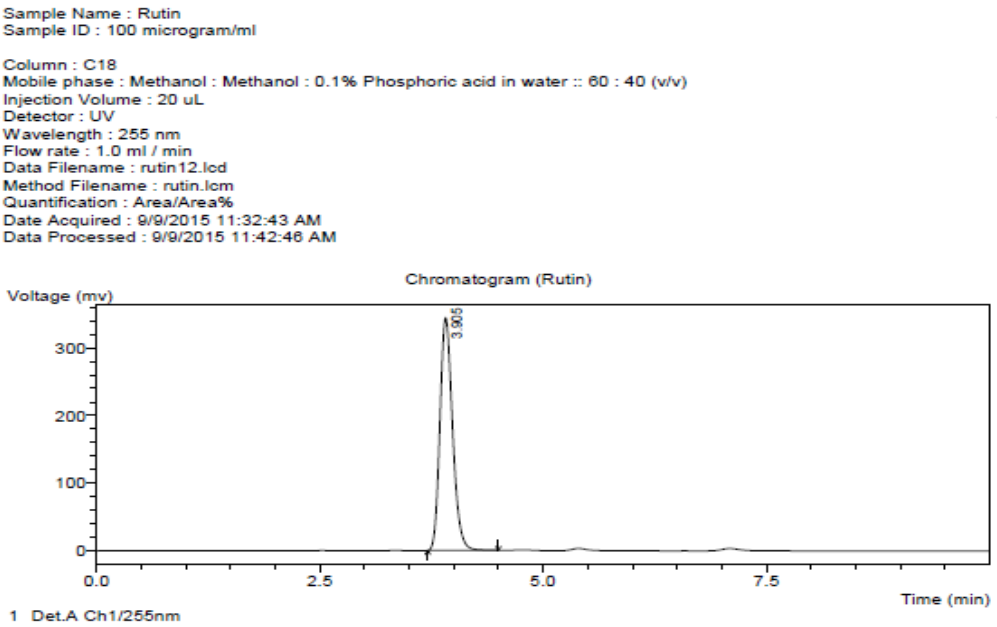

Figure – 8.13: HPLC Chromatogram of standard Rutin

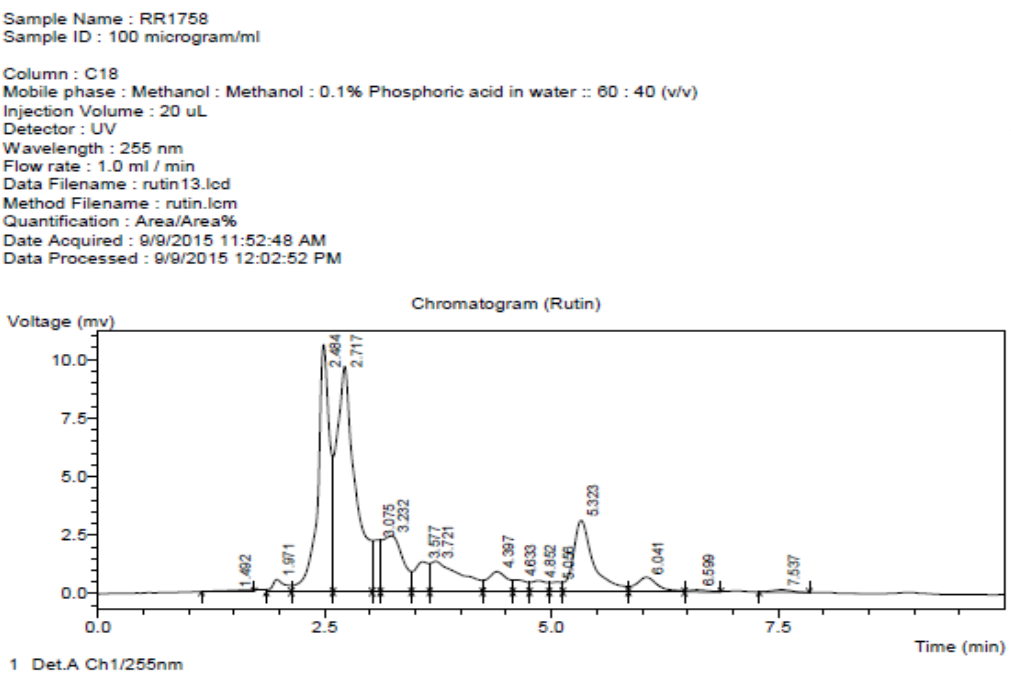

Figure – 8.14: HPLC Chromatogram of *M. roxburghianus* plant extract for Rutin estimation

Sample Name : rr1759
Sample ID : 100 microgrmam/ml

Column : C18
Mobile phase : Methanol : Methanol : 0.1% Phosphoric acid in water :: 60 : 40 (v/v)
Injection Volume : 20 uL
Detector : UV
Wavelength : 255 nm
Flow rate : 1.0 ml / min
Data Filename : rutin 07.lcd
Method Filename : rutin.lcm
Quantification : Area/Area%
Date Acquired : 9/9/2015 1:24:15 PM
Data Processed : 9/9/2015 1:34:18 PM

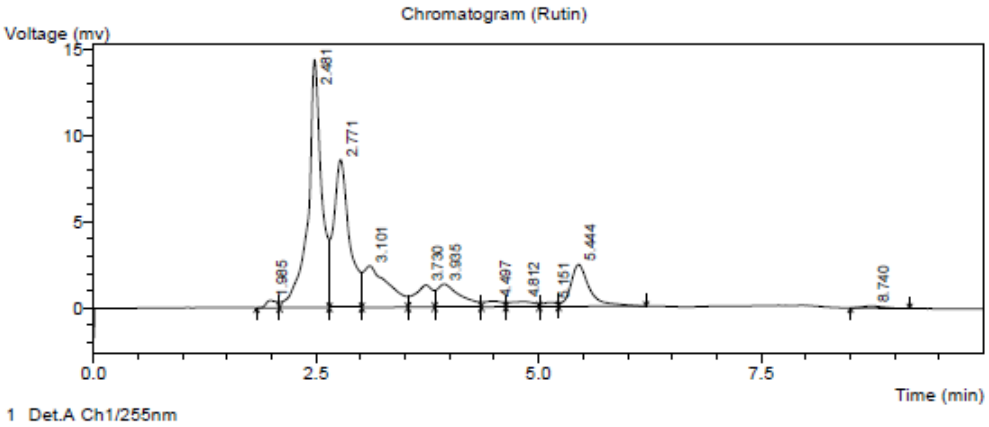

Figure – 8.15: HPLC Chromatogram of *P. fraternus* plant extract for Rutin estimation

Table – 8.5: Quantification of Rutin

Sample No.	Conc. (µg/ml)	Ret. Time (minutes)	Area	Rutin Content %
Standard	100	3.905	3369880	95
M. roxburghianus extract	100	-	-	0
P. fraternus extract	100	3.935	24110	0.6797

Based on the results presented in the above table it can be confirmed that *M. roxburghianus* plant extract did not contain Rutin while *P. fraternus* plant extract did contain Rutin (0.67%).

8.4 Conclusion

A HPLC method was successfully developed for fingerprint analysis of *M. roxburghianus* and *P. fraternus* plant extract. The HPLC fingerprint of Beta-sitosterol, Gallic acid, Lupeol, Quercitin and Rutin represent the characteristic markers of the plant extract constituents. The fingerprint obtained provides a good repeatability in separation pattern which demonstrated that the fingerprint presented is a rapid, reliable and effective

method suitable for either qualitatively or quantitatively determination of the constituents present in *M. roxburghianus* and *P. fraternus* plant extract.

The result of this study demonstrated that *M. roxburghianus* and *P. fraternus* plant extract does contain bioactive compounds according to a few peaks pattern that were shown in HPLC profiling analysis result. The peaks of *M. roxburghianus* plant extract confirmed the presence of the potential chemical content of Beta-sitosterol and Gallic acid, while the peaks of *P. fraternus* plant extract confirmed the presence of the potential chemical content of Beta-sitosterol, Gallic acid, Quercitin and Rutin.

CHAPTER 9
IN VITRO ANTIOXIDANT ACTIVITIES OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS AGAINST DPPH, ABTS, LIPID PEROXIDATION ASSAY, AND NITRIC OXIDE

CHAPTER 9: *IN VITRO* ANTIOXIDANT ACTIVITIES OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS AGAINST DPPH, ABTS, LIPID PEROXIDATION ASSAY AND NITRIC OXIDE

In both animals and plants, reactive oxygen species (ROS) are produced during normal cell metabolism. ROS is generated from metabolism and or by the environmental factors interact directly to the biological systems (Sudha *et al.,* 2011). Free radical contains one or more unpaired electrons and due to this they are highly unstable. They cause damage to other molecules by extracting electrons from them to attain stability (Vadivelan P *et al.*, 2009). Excess production of ROS generation leads to oxidative stress, direct damage to biological molecules such as DNA, proteins, lipids, carbohydrates leading to tumor development and progression and have been implicated with variety of chronic diseases including cancer, diabetes, atherosclerosis, neurodegenerative disorders, arthritis, cardiovascular, atherosclerosis, reperfusion injury, cataractogenesis, rheumatoid arthritis, inflammatory disorders and cancer (Sudha *et al.,* 2011; Aseervatham *et al.,* 2012).

Antioxidants are compounds that inhibit the propagation of oxidizing chain reactions thereby slowing down or totally inhibit the oxidation of lipid or other molecules. The body's antioxidant defense system helps to protect the cells from excess reactive oxygen species production. They are useful in the prevention and treatment of many human diseases which originate from free radical derived oxidative stress (Aseervatham *et al.,* 2012). Natural compounds have been reported to possess antioxidant properties and bioactivities and are considered as the main sources of antioxidants, which constitute a rich diversity of compounds such as flavonoids (anthocyanins, flavonols, flavones) and several classes of nonflavonoids (phenolic acids, lignins, stilbenes, terpenoids, etc.).. Natural antioxidant molecules from phytochemicals of plant origin may directly inhibit either their production or limit their propagation or destroy them to protect the system (Moukette *et al.,* 2015). Several epidemiological studies have indicated a relationship between the plant antioxidants and reduction of chronic diseases. The antioxidant components of plant origin, vitamins, flavonoids, and carotenoids are thought to be responsible for these results. Herbal products could turn out to be a better option to find a suitable treatment for reducing the free radicals generation (Aseervatham *et al.,* 2012).

In the present study, *M. roxburghianus* and *P. fraternus* plant extract was determined for its antioxidant activities by *in vitro* methods, including DPPH, ABTS, NO and lipid peroxide assay. The scavenging ability of DPPH free radical is widely used to analyze the antioxidant potential of naturally derived foods and plants (Moukette *et al.,* 2015). Several complementary methods have been proposed to assess the antioxidant activity of plant extracts and pure compounds. In vitro assays for the free radical scavenging

capacity are usually based on the inactivation of radicals, such as hydroxyl (OH) and nitric oxide (NO) radicals (Moukette *et al.*, 2015).The ABTS method is known to be a rapid method for the determination of the antioxidant activity and could be a useful tool to screen samples and cultivars in order to obtain high content of natural antioxidants in foods (Moukette *et al.*, 2015).The inhibitory effect of the extracts was investigated by lipid peroxidation assay. In the biological system a number of end products of lipid peroxidation such as MDA constitute a significant source of cell membrane obliteration and cell injuries. The inhibition of lipid peroxidation by antioxidant compounds is a crucial property by which they can diminish the induction and/or propagation of oxidative stress (Moukette *et al.*, 2015).

9.1 Aim of the study: Determination of antioxidant activity of test substances against DPPH, ABTS, Lipid peroxidation assay, Nitric oxide.

9.2 Materials and Methods

9.2.1 Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

9.2.2 DPPH Assay

(i) Reagents

2, 2-Diphenly 1-picryl hydrazyl solution (DPPH, 100 μM, Aldrich Chemistry-D913-2): 22 mg of DPPH was accurately weighed and dissolved in 100 ml of methanol. From this stock solution, 18 ml was taken and diluted to 100 ml using methanol to obtain 100 μM DPPH solution.

Preparation of test solutions:

21 mg each of the extracts was dissolved in distilled DMSO separately to obtain solutions of 21 mg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.

Preparation of standard solutions:

10 mg each of ascorbic acid and rutin were weighed separately and dissolved in 0.95 ml of Dimethyl sulfoxide (DMSO) to get 10.5 mg/ml concentrations. These solutions were serially diluted with DMSO to get lower concentrations.

(ii) Principle

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in color and upon reaction with hydrogen donor changes to yellow color. It is a discoloration assay, which is evaluated by the

addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490nm.

(iii) Procedure:

The assay was carried out in a 96 well micro-titre plate. To 200 µl of DPPH solution, 10 µl of each of the test sample or the standard solution was added separately in wells of the micro-titre plate. The final concentration of the test and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812 µg/ml. The plates were incubated at 37^{o}C for 30 min and the absorbance of each solution was measured at 490 nm, using a micro plate reader (Radovanović T B *et al.*, 2010, Sinha AK, 1972).

9.2.3 Scavenging of 2, 2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt) (ABTS) radical cation Assay

(i) Reagents

2,2'-Azino-bis (3-Ethylbenzothiazolin-6-Sulfonic acid) diammonium salt (1096 µg/ ml, Sigma-Aldrich, SLBG 1958):
10.96 mg weighed and dissolved in 10 ml of distilled water. 60 µl of Potassium persulfate (17mM) is added to above solution. Reagent is stored in dark for overnight before the experiment.

Preparation of test and standard solutions: 13.5 mg of each of the extracts and the standards, ascorbic acid and rutin were accurately weighed and separately dissolved in 2ml of DMSO. These solutions were serially diluted with DMSO to obtain the lower dilutions.

(ii) Principle:

ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and, is often used for screening complex antioxidant mixture such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS radical for the estimation of the antioxidant activity (Re *et al.*, 1999; Nenadis *et al.*, 2004)

(iii) Procedure:

ABTS (54.8 mg) was dissolved in 50 ml of distilled water to 2 mM concentration and potassium persulphate (17 mM, 0.3 ml) was added. The reaction mixture was left to stand

at room temperature overnight in dark before use. To 0.2 ml of various concentrations of the extracts or standards, 1.0 ml of distilled DMSO and 0.16 ml of ABTS solution was added to make a final volume of 1.36 ml. Absorbance was measured spectrophotometrically, after 20 min at 734 nm (Radovanović T B *et al.,* 2010, Sinha A K, 1972).

9.2.4 Lipid peroxidation inhibitory activity

(i) Reagents

Preparation of egg lecithin: Separate the egg yolk and wash it with acetone until yellow colour disappears. The creamy white powder thus obtained is used for the procedure by dissolving in phosphate buffer pH 7.4 (3mg/ml).

(ii) Principle:

Lipid peroxidation can be initiated by ROS such as hydroxyl radicals by extracting a hydrogen atom from lipids and forming a conjugated lipid radical. This reacts rapidly with oxygen to form a lipid radical until the chain reaction is terminated. The lipid peroxidation adducts may induce the oxidation of biomolecules such as DNA, proteins and other lipids resulting in cellular damage.

(iii) Procedure

The reaction mixture containing egg lecithin (1ml), ferric chloride (0.02ml), ascorbic acid (0.02ml) and extract or standard (0.1ml) in DMSO at various concentrations was kept for incubation for 1 hour at 37ºC. After incubation 2 ml of 15% TCA and 2ml of 0.37% TBA were added. Then the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm (Radovanović T B *et al.,* 2010, Sinha A K, 1972).

9.2.5 Scavenging of Nitric Oxide radical

(i) Reagents

Sodium nitroprusside solution: Weighed accurately 0.2998 g of sodium nitroprusside and dissolved in phosphate buffer to make up the volume to 100 ml in a volumetric flask (10 mM).

Naphthyl ethylene diaminedihydrochloride (NEDD, 0.1%)
Weighed accurately 0.1 g of NEDD and dissolved in 100 ml of distilled water.

Sulfanilamide (1 % w/v) reagent

Weighed accurately 1 g of sulfanilamide and dissolved in 5 % phosphoric acid and made up the volume to 100 ml in a volumetric flask.

Preparation of sample solutions

6 mg each of the extracts was dissolved in distilled DMSO separately to obtain solutions of 6 mg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.

Preparation of standard solutions

Weighed accurately 10 mg of ascorbic acid and rutin and dissolved in 1 ml of DMSO separately. From these solutions, serial dilutions were made to obtain lower concentrations using DMSO.

(ii) Principle

Sodium nitroprusside in aqueous solution at physiological pH, spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of modified GriessIlosvay reaction. In the present investigation, GriessIlosvay reagent is modified by using Naphthyl ethylene diaminedihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Nitrite ions react with Griess reagent, which forms a purple azo dye. In presence of test components, likely to be scavengers, the amount of nitrites will decrease. The degree of decrease in the formation of purple azo dye will reflect the extent of scavenging. The absorbance of the chromophore formed was measured at 540 nm.

(iii) Procedure

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and extract in DMSO at various concentrations or standard was incubated at 25^{o} C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulfanilamide reagent was added, mixed well and allowed to stand for 5 min for completion of diazotization. Then, 1 ml of NEDD was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance of these solutions was measured at 540 nm (Radovanović T B *et al.*, 2010, Sinha A K, 1972).

9.3 Results and Discussion

The results obtained in the in vitro antioxidant assays of M. roxburghianus and *P. fraternus* plant extract against DPPH, ABTS, Lipid peroxidation assay, Nitric oxide are discussed below:

9.3.1 DPPH Assay

The *M. roxburghianus* plant extract have shown the significant result in DPPH scavenging assay with IC_{50} 64.58 compared to *P. fraternus* plant extract which showed IC_{50} of 116.67. Quercitin was used as reference standards in this study. These results are depicted in **Table – 9.1**, **Table – 9. 2**, **Figure – 9.1** and **Figure – 9.2** below.

Table – 9.1: DPPH assay of *M. roxburghianus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
1000	98.50	96.85	102.83	99.39	3.09
500	86.52	79.59	80.19	82.10	3.84
250	72.10	70.32	68.49	70.30	1.80
125	56.37	60.30	54.53	57.06	2.95
62.5	49.81	49.72	49.81	49.78	0.05
IC_{50}	**68.75**	**62.5**	**62.5**	**64.58**	**3.61**

Table – 9.2: DPPH assay of *P. fraternus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
1000	92.02	91.83	92.02	91.96	0.11
500	73.80	70.58	67.65	70.68	3.07
250	67.47	64.34	64.40	65.40	1.79
125	51.96	51.71	52.73	52.13	0.53
62.5	39.91	40.12	41.65	40.56	0.95
IC_{50}	**118.75**	**118.75**	**112.5**	**116.67**	**3.61**

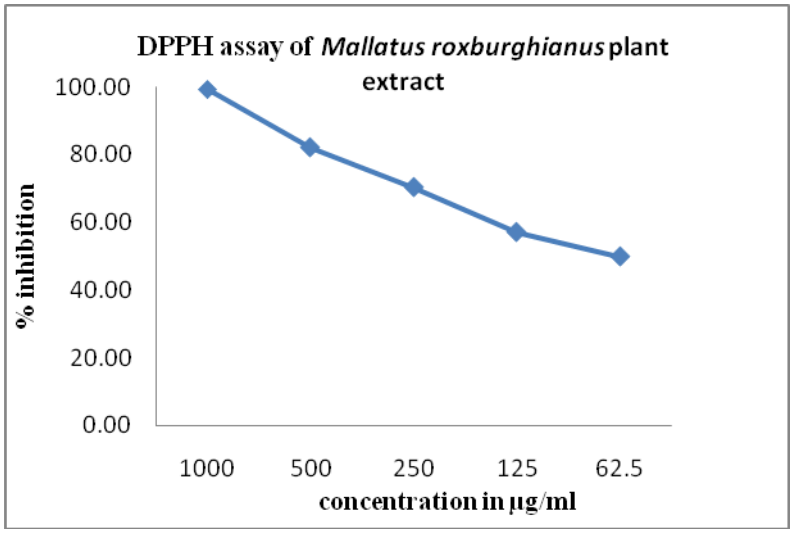

Figure – 9.1: DPPH assay of *M. roxburghianus* plant extract

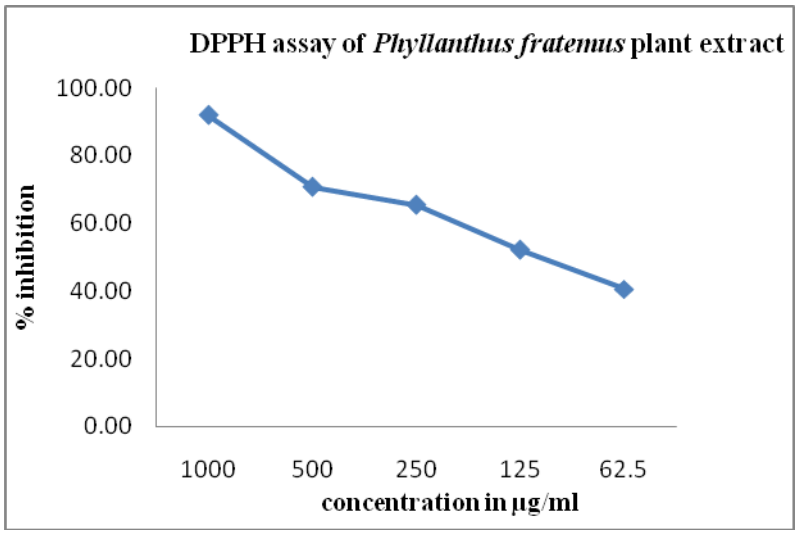

Figure – 9.2: DPPH assay of *P. fraternus* plant extract

9.3.2 Scavenging of 2, 2'-azino-bis (3-ethylbezothiazoline-6-sulfonic acid) diammonium salt) (ABTS) radical cation Assay

In the ABTS scavenging assay, both the plant extract sample showed an IC_{50}value which ranged less than 6.25 µg/ml. ABTS was used as reference standards in this study. These results are depicted in **Table – 9.3**, **Table – 9.4**, **Figure – 9.3** and **Figure – 9.4** below.

Table – 9.3: ABTS assay of *M. roxburghianus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
100	96.30	96.34	97.50	96.71	0.68
50	95.06	93.90	96.25	95.07	1.17

25	95.06	91.46	93.75	93.43	1.82
12.5	92.59	90.24	92.50	91.78	1.33
6.25	92.59	90.24	91.25	91.36	1.18
IC_{50}	**<6.25**	**<6.25**	**<6.25**	**<6.25**	

Table – 9.4: ABTS assay of *P. fraternus* plant extract

Concentration (µg/ml)	**% of Inhibition**				
	1	**2**	**3**	**Average**	**Standard deviation**
100	98.85	98.85	98.85	98.85	0.00
50	94.25	95.40	95.40	95.02	0.66
25	94.25	93.10	96.55	94.64	1.76
12.5	94.25	94.25	94.25	94.25	0.00
6.25	89.66	89.66	98.85	92.72	5.31
IC_{50}	**<6.25**	**<6.25**	**<6.25**	**<6.25**	

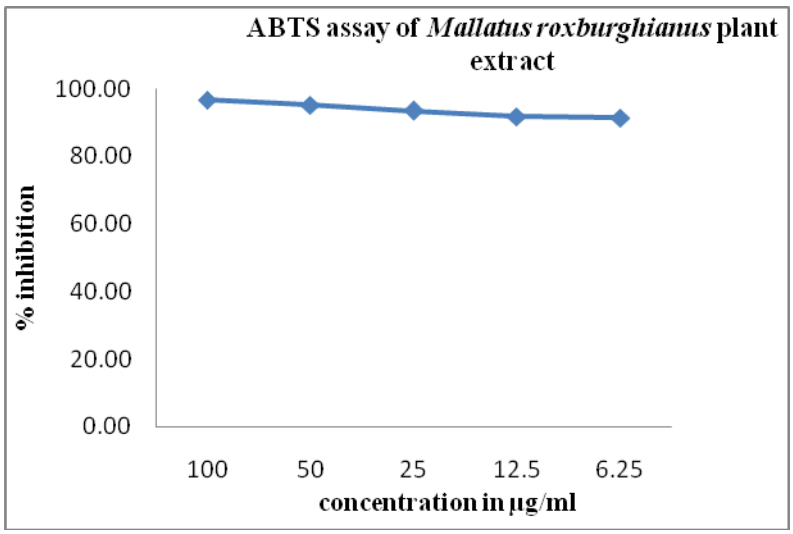

Figure – 9.3: ABTS assay of *M. roxburghianus* plant extract

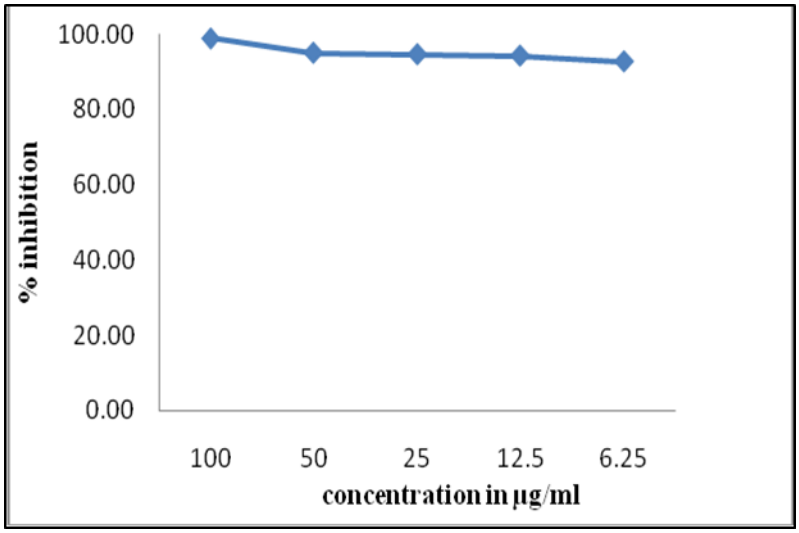

Figure – 9.4: ABTS assay of *P. fraternus* plant extract

9.3.3 Lipid peroxidation inhibitory activity

In the lipid peroxidation assay, both the plant extract samples have IC_{50} which ranges above 1000 µg/ml. BHA was used as reference standards in this study. These results are depicted in **Table 5**, **Table 6**, **Figure 5** and **Figure 6** below.

Table – 9.5: LPO assay of *M. roxburghianus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
1000	40.91	44.25	42.34	42.50	1.67
500	38.18	41.59	36.04	38.60	2.80
250	8.18	7.08	11.71	8.99	2.42
125	6.36	0.00	0.00	2.12	3.67
62.5	0.00	0.00	0.00	0.00	0.00
IC_{50}	**>1000**	**>1000**	**>1000**	**>1000**	

Table – 9.6: LPO assay of *P. fraternus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
1000	46.77	47.15	46.72	46.88	0.24
500	33.87	34.15	31.15	33.05	1.66
250	29.03	29.27	28.69	29.00	0.29
125	10.48	8.13	5.74	8.12	2.37
62.5	7.26	3.25	4.10	4.87	2.11
IC_{50}	**>1000**	**>1000**	**>1000**	**>1000**	

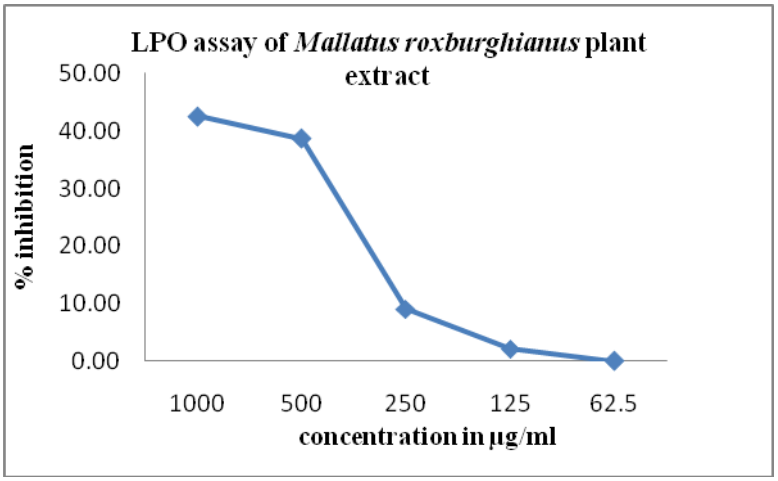

Figure – 9.5: LPO assay of *M. roxburghianus* plant extract

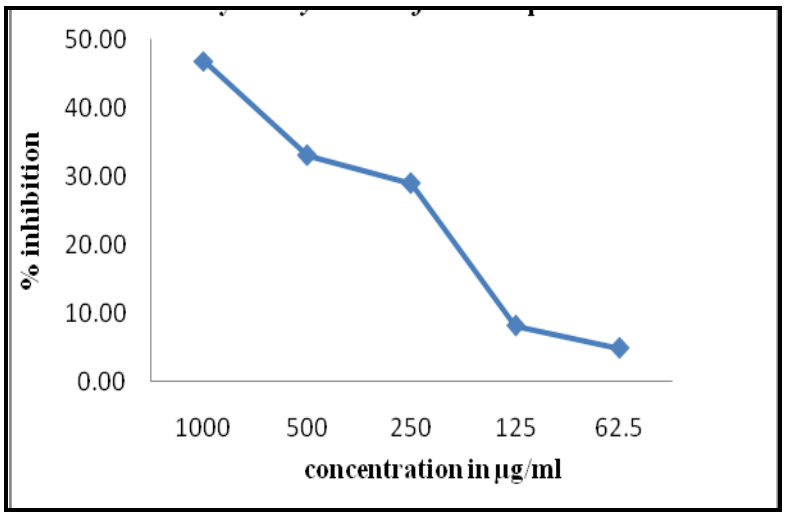

Figure – 9.6: LPO assay of *P. fraternus* plant extract

9.3.4 Scavenging of Nitric Oxide radical

In nitric oxide scavenging assay, the *M. roxburghianus* plant extract showed significant result with IC_{50} 208.33 compared to *P. fraternus* plant extract with an IC_{50} of 394.17. Ascorbic acid was used as reference standards in this study. These results are depicted in **Table – 9.7**, **Table – 9.8**, **Figure – 9.7** and **Figure – 9.8** below.

Table – 9.7: NO scavenging assay of *M. roxburghianus* plant extract

Concentration (µg/ml)	% of Inhibition				
	1	2	3	Average	Standard deviation
1000	66.51	67.15	63.86	65.84	1.75
500	65.51	65.87	64.41	65.26	0.76
250	56.45	55.81	55.63	55.96	0.43

125	36.96	36.69	36.96	36.87	0.16
62.5	27.54	27.72	27.72	27.66	0.11
IC_{50}	**207.5**	**208.75**	**208.75**	**208.33**	**0.72**

Table – 9.8: NO scavenging assay of *P. fraternus* plant extract

Concentration (µg/ml)	**% of Inhibition**				
	1	**2**	**3**	**Average**	**Standard deviation**
1000	63.78	63.41	63.26	63.48	0.27
500	56.80	56.42	56.00	56.41	0.40
250	40.32	40.13	40.37	40.27	0.13
125	19.93	19.83	19.91	19.89	0.05
62.5	17.60	16.01	17.02	16.88	0.80
IC_{50}	**392.5**	**397.5**	**392.5**	**394.17**	**2.89**

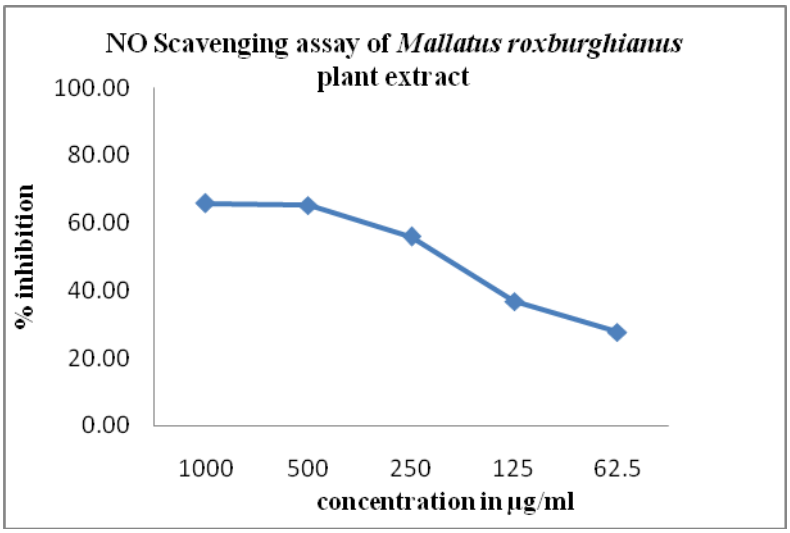

Figure – 9.7: NO scavenging assay of *M. roxburghianus* plant extract

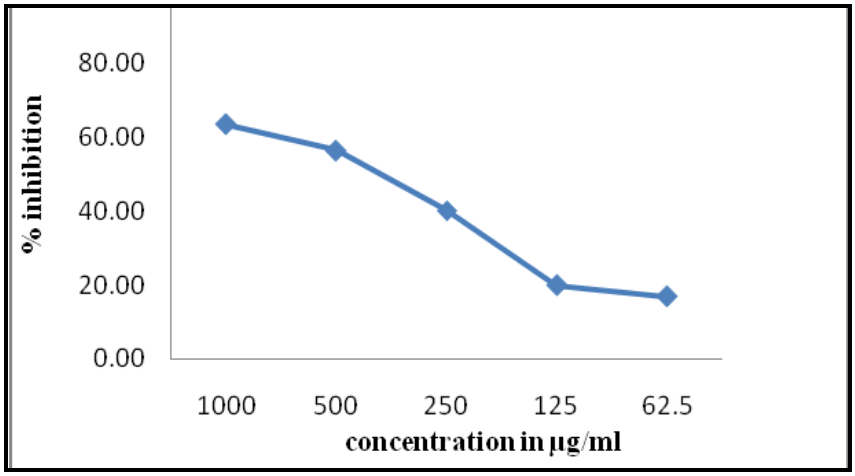

Figure – 9.8: NO scavenging assay of *P. fraternus* plant extract

The overall antioxidant study results are summarized in **Table – 9.9** below.

Table – 9.9: Antioxidant investigations of *M. roxburghianus* and *P. fraternus* extracts

Samples	**Different methods of investigations, and their IC_{50} values (μg/ml)**			
	DPPH	**ABTS**	**LPO**	**Nitric oxide**
***M. roxburghianus* plant extract**	64.58 ±3.61	<6.25	>1000	208.33 ± 0.72
***P. fraternus* plant extract**	116.67± 3.61	<6.25	>1000	394.17 ± 2.89
Standards	Quercitin 20.3 ±0.64	ABTS 1.87±0.06	BHA 27.33 ± 0.53	Ascorbic acid 64.67 ± 1.15

9.4 Conclusion

Based on the observations recorded in **Table – 9.1** to **Table – 9.9** as well as **Figure – 9.1** to **Figure – 9.8**; it can be concluded that both *M. roxburghianus* and *P. fraternus* plant extract exhibited significant antioxidant property. *M. roxburghianus* plant extract were found to possess significant radical scavenging and antioxidant property compared to *P. fraternus* plant extract. Besides the traditional uses of the plant extract, the plant extract can also be used as an accessible source of natural antioxidants to attenuated oxidative stress via its antioxidant properties with consequent health benefits. The observation shows that antidiabetic activity of the plant extract may also be attributed to the antioxidant property of the plant extract.

CHAPTER 10
IN VITRO ∝ - GLYCOSIDASE INHIBITORY ACTIVITY OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 10: *IN VITRO* ALPHA - GLUCOSIDASE INHIBITORY ACTIVITY OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS

Blood glucose level management is an important approach in the controlling the complications of diabetes. ∝-amylase and ∝-glucosidase are two important carbohydrates hydrolyzing enzymes. In patients with type-2 diabetes mellitus, inhibitors of ∝ -amylase and ∝-glucosidase have been helpful for the control of hyperglycemia.

Inhibition of these enzymes holds of carbohydrate digestion and extends the total carbohydrate digestion time. This leads to a decrease in the rate of glucose absorption, thereby reducing the postprandial plasma glucose rise. For hypoglycemic agents to elicit their pharmacological effects and minimize the side effects of these synthetic drugs, medicinal plants should be mild inhibitors of α-amylase and ∝-glucosidase (Dastjerdi *et al.,* 2015).

10.1 Aim of the study: Determination of alpha glucosidase inhibitory activity of test substances.

10.2 Materials and Methods

10.2.1 Reagents

α-Glucosidase enzyme (Sigma-Aldrich- 1001918139)
Phosphate buffer (pH-6.8) (HiMedia-TS1101-54)
0.2 M Sodium carbonate (HighMedia-RM254) and
5 mM p-Nitrophenyl α-D glucopyronosidase (PNPG, HiMedia-RM10294).

10.2.2 Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

10.2.3 Preparation of test solutions

2 mg each of the test substance was dissolved in 750 µL of phosphate buffer separately to obtain solutions of 1000 µg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.

10.2.4 Procedure

In vitro ∝ - glucosidase inhibition was studied by the method of Kim *et al.*, 2005 and Kaskoos RA, 2013. In brief, 50 µL of the α-glucosidase was preincubated with 60 µL of different concentration of test substance for 20 minutes. Then the reaction is initiated by adding 50 µL of p-Nitrophenyl α-D glucopyronosidase (pNPG) as a substrate. The

reaction mixture was incubated at 37 °C for 20 minutes and stopped by adding 160 µL of Sodium carbonate. The α-glucosidase activity was determined by measuring the yellow-colored paranitrophenol released from pNPG at 405 nm and the percentage inhibition of α-glucosidase enzyme was calculated using the formula below:

$$\text{Inhibition } (\%) = 100\left(\frac{\text{control} - \text{test}}{\text{control}}\right).$$

10.3 Results and Discussion

The ∝ - glucosidase inhibitory activity of methanolic extracts of *M. roxburghianus* and *P. fraternus* at various concentrations was investigated in this study and the results are shown in **Table 10.1** and **Table 10.2**. In the ∝ - glucosidase inhibitory assay, *M. roxburghianus* (193.62 µg/ml) and *P. fraternus* (96.85 µg/ml) showed 50% alpha amylase inhibition activity at the mentioned concentrations.

Percent ∝ - glucosidase inhibition of both the plants extracts was plotted as a function of concentration in comparison with % inhibition as shown in **Figure 10.1** and **Figure 10.2**. The results indicate that out of the two methanolic plant extracts, *P. fraternus* exhibited good anti ∝ - amylase activity and *M. roxburghianus* showed appreciable inhibition activity. Our findings also revealed that that the methanolic extracts of the plants efficiently inhibited ∝ - glucosidase enzyme *in vitro*. There was a dose dependent increase in percentage inhibitory activity against ∝ - glucosidase by both the plant extracts.

Table – 10.1: ∝ - glucosidase inhibitory activity of *M. roxburghianus* plant extract

Plant extract	Test Conc (µg/ml)	% inhibition	IC_{50} (µg/ml)
M. roxburghianus plant extract	62.5	25.79 ± 1.03	193.62 ± 0.12
	125	37.20 ± 0.81	
	250	58.98 ± 0.15	
	500	76.68 ± 0.15	
	1000	85.16 ± 0.17	

Table – 10.2: ∝ - glucosidase inhibitory activity of *P. fraternus* plant extract

Plant extract	Test Conc (µg/ml)	% inhibition	IC_{50} (µg/ml)
P. fraternus plant extract	62.5	36.33 ± 0.97	96.85 ± 0.05
	125	60.09 ± 0.05	
	250	68.04 ± 0.05	
	500	73.18 ± 0.54	
	1000	78.48 ± 0.07	

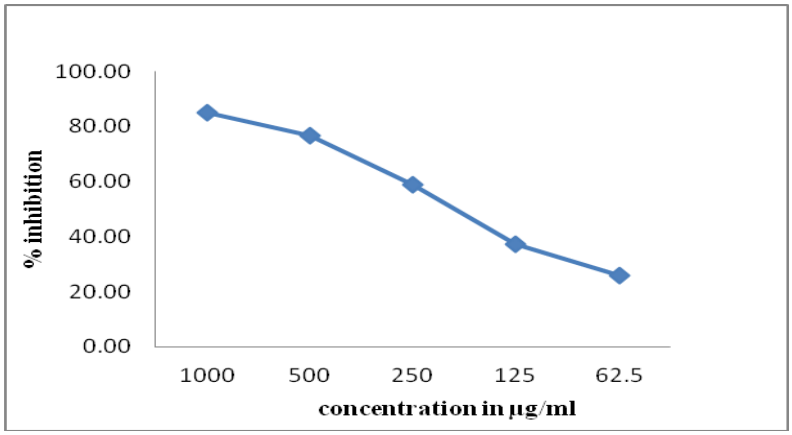

Figure – 10.1: ∝ - glucosidase inhibition of *M. roxburghianus* plant extract

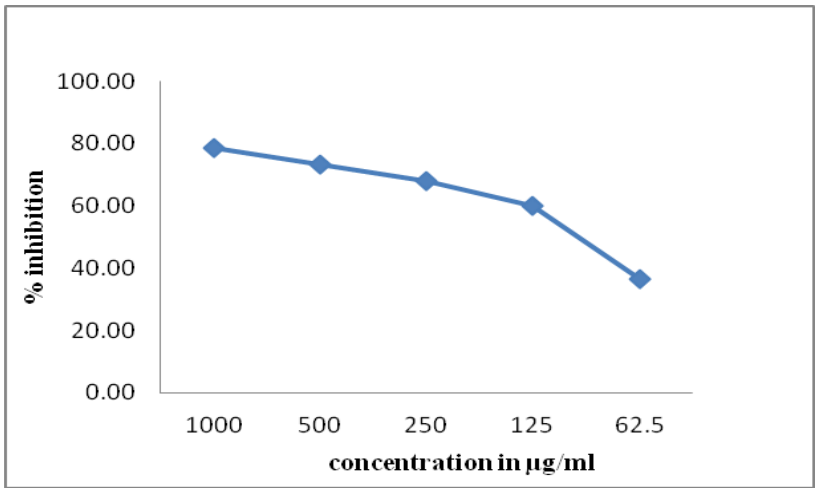

Figure – 10.2: ∝ - glucosidase inhibition of *P. fraternus* plant extract

The plant extracts *M. roxburghianus* and *P. fraternus* showed an IC_{50} value of 193.62 ± 0.12 and 96.85 ± 0.05 µg/ml respectively in the ∝ - glucosidase inhibition assay. *P. fraternus* showed the greater % inhibition of the ∝ - glucosidase enzyme compared to *M.*

roxburghianus plant extracts. The present study indicated that *P. fraternus* could be useful in management of postprandial hyperglycemia.

10.4 Conclusion

∝ - glucosidase inhibitors regulate postprandial hyperglycemia (PPHG) by impeding the rate of carbohydrate digestion in the small intestine and thereby hampering the diet associated acute glucose excursion. PPHG is a major risk factor for diabetic vascular complications leading to disabilities and mortality in diabetics (Shihabudeen *et al.*, 2011). *P. fraternus* and *M. roxburghianus* has been used in traditional medicine for treating diabetes. In this study we have evaluated the ∝-glucosidase inhibitory potential of *P. fraternus* and *M. roxburghianus* extract.

The results of the present study indicate that out of both the plant extracts, methanolic extracts of *P. fraternus* showed the maximum ∝ - glucosidase inhibitory activity. The plants may essentially contain herbal bioactive compounds inhibiting enzyme activity. One of the mechanism of action of the plant extract as anti-diabetic agent could be by inhibiting the activity of intestinal ∝ - glucosidase. In conclusion, more research is required for developing a potential and valuable anti diabetic therapy using ∝ - glucosidase inhibitors of plant origin.

CHAPTER 11
IN VITRO ∝ - AMYLASE INHIBITORY ACTIVITY OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 11: *IN VITRO* ALPHA - AMYLASE INHIBITORY ACTIVITY OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS

Blood glucose level management is an important approach in the controlling the complications of diabetes. ∝ - amylase and ∝ - glucosidase are two important carbohydrates hydrolyzing enzymes. In patients with type-2 diabetes mellitus, inhibitors of ∝ - amylase and ∝ - glucosidase have been helpful for the control of hyperglycemia.

Inhibition of these enzymes holds off carbohydrate digestion and extends the total carbohydrate digestion time. This leads to a decrease in the rate of glucose absorption, thereby reducing the postprandial plasma glucose rise (Dastjerdi *et al.,* 2015). α - Amylase is one of the main enzymes in human body responsible for the breakdown of starch to more simple sugars. α - amylases hydrolyzes complex polysaccharides to produce oligosaccharides and disaccharides. These are then hydrolyzed by α - glycosidase to monosaccharide which are then absorbed through the small intestines into the hepatic portal vein. This then increases postprandial glucose levels. Slowing the digestion and breakdown of starch may have beneficial effects on insulin resistance and glycemic index control in people with diabetes (Uddin *et. al.,* 2014). For hypoglycemic agents to elicit their pharmacological effects and minimize the side effects of these synthetic drugs, medicinal plants should be mild inhibitors of α - amylase and ∝ - glucosidase (Dastjerdi *et al.,* 2015).

11.1 Aim of the study: Determination of α - amylase inhibitory activity of test substances.

11.2 Materials and Methods

11.2.1 Reagents

α-amylase enzyme
phosphate buffer (pH-6.9)
1% starch solution
Dimethyl sulfoxide (DMSO)
Dinitrosalicylic acid reagent and
HCl

11.2.2 Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

11.2.3 Preparation of test solutions

45 mg each of the extracts was dissolved in 1% DMSO separately to obtain solutions of 45 mg/ml concentrations. Each of these solutions was serially diluted separately to obtain lower concentrations.

11.2.4 Procedure

In vitro amylase inhibition was studied by the method of Tamil *et al.,* 2011, Xiao *et al.,* 2006 and Bernfeld P, 1955. In brief, 100 µL of the test extract was allowed to react with 100 µL of α-amylase enzyme (Hi media Rm 638). After 15-minute incubation, 100 µL of 1% starch solution was added. The same was performed for the controls where 100 μL of the test extract was replaced by buffer. After incubation for 15 minutes, 100 μL of HCl and 100 μL dinitrosalicylic acid reagent was added to both control and test. These were kept in boiling water bath for 5 minutes. Next the absorbance was recorded at 540 nm using spectrophotometer and the percentage inhibition of α-amylase enzyme was calculated using the below formula:

$$\text{Inhibition } (\%) = 100\left(\frac{\text{control} - \text{test}}{\text{control}}\right).$$

Suitable reagent blank and inhibitor controls tests were simultaneously carried out. Ascorbic acid was used as standard.

11.3 Results and Discussion

The $\propto$ − amylase inhibitory activity of methanolic extracts of *M. roxburghianus* and *P. fraternus* at various concentrations was investigated in this study. The IC_{50} value of both the extract was compared with that of ascorbic acid. The results are shown in **Table – 11.1, Table – 11.2** and **Table – 11.3**. In the alpha-amylase inhibitory assay, *M. roxburghianus* showed >10000 µg/ml while *P. fraternus* showed 4066 µg/ml of alpha amylase inhibition activity at the mentioned concentrations. Ascorbic acid showed an inhibition of 4375 µg/ml.

Percent α-amylase inhibition of both the plants extracts and ascorbic acid was plotted as a function of concentration in comparison with % inhibition as shown in **Figure – 11.1**, **Figure – 11.2** and **Figure – 11.3**. The results indicate that out of the two methanolic plant

extracts, *P. fraternus* exhibited good anti-alpha amylase activity than *M. roxburghianus* when compared to the results of ascorbic acid inhibition activity. Our findings also revealed that that the methanolic extracts of the plants efficiently inhibited α-amylase enzyme in vitro. There was a dose dependent increase in percentage inhibitory activity against α-amylase by both the plant extracts.

The plant extracts of *M. roxburghianus* and *P. fraternus* showed an IC_{50} value of >10000 µg/ml and 4066.67 ± 28.87 µg/ml respectively in the α-amylase inhibition assay. *P. fraternus* showed the greater % inhibition of the $\propto$ − amylase enzyme compared to *M. roxburghianus* plant extracts and a comparable inhibition when compared with ascorbic acid. The present study indicated that *P. fraternus* could be useful in management of postprandial hyperglycemia.

Table – 11.1: $\propto$ − amylase inhibitory activity of *M. roxburghianus* plant extract

Name of test substance	Test Conc (µg/ml)	% inhibition	IC_{50} (µg/ml)
M. roxburghianus plant extract	625	0	>10000
	1250	4.00 ± 4.02	
	2500	6.67 ± 2.31	
	5000	10.67 ± 2.29	
	10000	25.33 ± 2.31	

Table – 11.2: $\propto$ − amylase inhibitory activity of *P. fraternus* plant extract

Name of test substance	Test Conc (µg/ml)	% inhibition	IC_{50} (µg/ml)
P. fraternus plant extract	625	26.32 ± 0.01	4066.67 ± 28.87
	1250	35.09 ± 3.03	
	2500	45.61 ± 3.04	
	5000	52.63 ± 0.01	
	10000	91.23 ± 3.04	

Table – 11.3: $\propto$ − amylase inhibitory activity of test substances Ascorbic acid

Name of test substance	Test Conc (µg/ml)	% inhibition	IC_{50} (µg/ml)
Ascorbic acid	625	9.76 ± 0.02	4375 ± 25.01
	1250	17.07 ± 2.44	
	2500	24.39 ± 0.05	
	5000	56.91 ± 1.41	
	10000	73.17 ± 4.22	

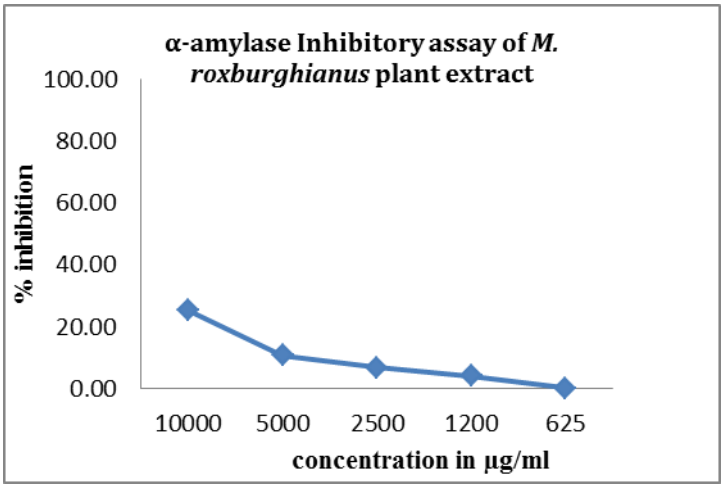

Figure – 11.1: ∝ − amylase inhibition assay of *M. roxburghianus* plant extract

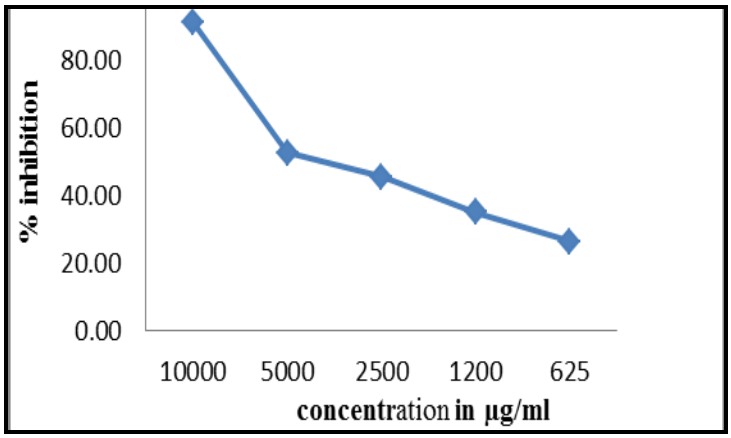

Figure – 11.2: ∝ − amylase inhibition assay of *P. fraternus* plant extract

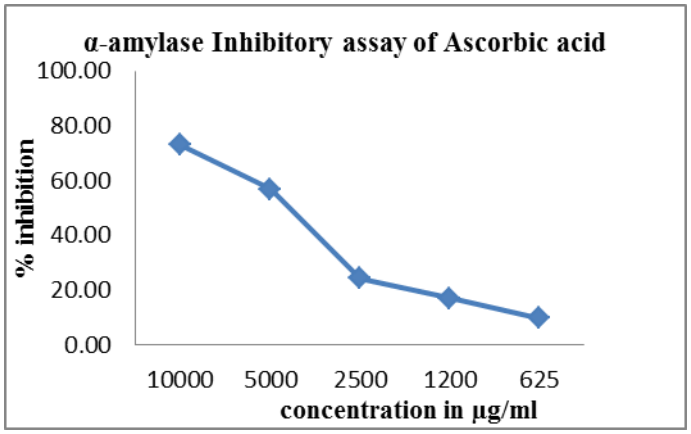

Figure – 11.3: ∝ − amylase inhibition for the test substance Ascorbic acid

11.4 Conclusion

∝ - Amylase inhibitors regulate postprandial hyperglycemia (PPHG) by impeding the rate of carbohydrate digestion in the small intestine and thereby hampering the diet

associated acute glucose excursion. PPHG is a major risk factor for diabetic vascular complications leading to disabilities and mortality in diabetics (Tamil *et al*, 2011). *M. roxburghianus* and *P. fraternus* has been used in traditional medicine for treating diabetes. In this study we have evaluated the ∝-amylase inhibitory potential of *M. roxburghianus* and *P. fraternus* extracts.

The results of the present study indicate that out of both the plant extracts, methanolic extracts of *P. fraternus* showed the maximum ∝ - amylase inhibitory activity which was comparable to ascorbic acid. The plants may essentially contain herbal bioactive compounds inhibiting enzyme activity. One of the mechanism of action of the plant extract as anti-diabetic agent could be by inhibiting the activity of intestinal ∝ - amylase. In conclusion, more research is required for developing a potential and valuable anti-diabetic therapy using ∝ - amylase inhibitors of plant origin.

CHAPTER 12
IN VITRO EFFECT OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS FOR THEIR EFFECT ON GLUCOSE UPTAKE IN L6 CELL LINE

CHAPTER 12: *IN VITRO* EFFECT OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS FOR THEIR EFFECT ON GLUCOSE UPTAKE IN L6 CELL LINE

Diabetes mellitus is associated with insulin deficiency and decreased glucose uptake in skeletal muscles. Hyperglycemia generally occurs due to increased plasma free radicals observed in diabetes mellitus may impair insulin action defects in GLUT-4 and GLUT-1 may explain the insulin- resistant glucose transport. Skeletal muscle is a major tissue for blood glucose utilization and a primary target tissue for insulin action. Insulin increases glucose uptake in skeletal muscle by increasing functional glucose transport molecules (GLUT-4) in the plasma membrane. Glucose transport in skeletal muscle can also be stimulated by contractile activity. Free radical impairs insulin- stimulated GLUT-4 translocation and exerts an inhibitory effect on muscle contractility that is major pathological feature of diabetes (Dachani *et al.,* 2012).

The establishment of *in vitro* cell culture systems is a useful approach in biomedical research studies to assist understanding of *in vivo* events. Several muscle cell lines, such as C2C12, L6, and H9c2 and the adipocyte cell line 3T3-L1, have been used extensively for *in vitro* cell culture studies of glucose transport and signaling mechanisms. Each individual cell line exhibits unique properties in response to different stimuli. The commonly-used L6 cells respond to insulin-induced glucose transport via PI 3-kinase (Purintrapiban and Ratanachaiyavong, 2003). L6 cells represent a good model for glucose uptake because they have been used extensively to elucidate the mechanisms of glucose uptake in muscle, have an intact insulin signaling pathway, and express the insulin-sensitive GLUT-4 (Dachani *et al.,* 2012). L6 cells represent a more extensively studied model. They not only have an intact insulin pathway but also express both α and β adrenoceptors (Tandon *et al.,* 2013).

12.1 Aim of the study: To evaluate the *in vitro* antidiabetic effect of *M. Roxburghianus* and *P. Fraternus* plant extracts for their effect on glucose uptake in L6 cell lines.

12.2 Materials and methods

12.2.1 Chemicals

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), D-glucose, Dulbecco's Modified Eagle's Medium (DMEM), Metformin and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Antibiotics from Hi-Media Laboratories Ltd., Mumbai. Insulin (Torrent Pharmaceuticals, 40IU/ml) was purchased

from a drug store. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

12.2.2 Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

12.2.3 Cell lines and Culture medium

L-6 (Rat, Skeletal muscle) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of L-6 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

12.2.4 Preparation of Test Solutions

For *in vitro* studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

12.2.5 Cytotoxicity studies

The 24 hours cell cultures with 70-80% confluence in 96 well plates were used for the study. 100 µl of each dilution of the test drugs were added in quadruplicate in 96 well plate and cell controls maintained in same number. The cultures were incubated at 37^0 C with 5% CO_2 for 24 hours and the cultures were observed microscopically for any visible change in morphology of cells and observations were recorded. The cell viability assay was determined by MTT assay (Francis and Lang, 1986). The percentage cytotoxicity caused by each dilution of the drug was determined and Cytotoxic $Concentration_{50}$ (CTC_{50}) values determined by interpolation method. The non-toxic concentrations of test drugs, i.e. concentrations below CTC_{50} value were taken for glucose uptake studies.

12.2.6 In vitro glucose uptake assay

Glucose uptake activity of test drugs were determined in **d**ifferentiated L6 cells (Koivisto *et al.,* 1991; Walker *et al.,* 1990). In brief, the 24 hours cell cultures with 70-80% confluence in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multi-nucleation of cells. The differentiated cells were serum starved overnight and at the time of experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30min at

37^0C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37^0C. 20µl of D-glucose solution was added simultaneously to each well and incubated at 37^0C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (Biovision Inc, USA). Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls (Imamura *et al.,* 2003, Bergandi *et al.,* 2003, Yap *et al.,* 2007).

12.3 Results and discussion

The *in vitro* antidiabetic effect of *M. roxburghianus* and *P. fraternus* plant extract for their effect on glucose uptake in L6 cell lines were evaluated. The results obtained from the study are discussed herewith.

12.3.1 Cytotoxicity studies

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. The cytotoxicity of extracts was evaluated by MTT assay. The results obtained from the cytotoxicity studies are presented in **Table – 12.1** and **Figure – 12.1.**

Table – 12.1: Cytotoxic properties of *M. roxburghianus* and *P. fraternus* plant extract against L-6 cell line

Plant extract	Concentration (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
M. Roxburghianus plant extract	62.5	13.90±1.7	>1000
	125	21.01±3.3	
	250	27.02±1.6	
	500	30.60±3.8	
	1000	39.39±5.5	
P. Fraternus plant extract	62.5	12.86±2.7	593.33±5.8
	125	14.39±2.6	
	250	19.13±7.9	
	500	45.73±0.8	
	1000	69.41±2.3	

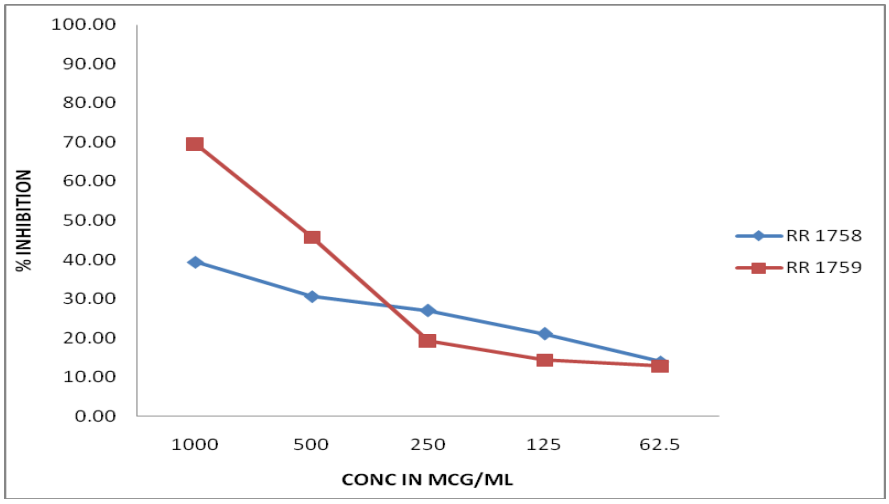

Figure – 12.1: Cytotoxic effect of *M. roxburghianus* and *P. fraternus* plant extract on L6 Cell line

[Note : 1758: M. roxburghianus plant extract; 1759: *P. fraternus* plant extract]

Both *M. roxburghianus* and *P. fraternus* plant extract exhibited dose dependent cytotoxicity against L6 cell line. The cytotoxicity of extracts were non selective as reflected by uniform CTC_{50} values. Among them, test substance *M. roxburghianus* plant extract exhibited poor cytotoxicity with CTC_{50} value higher than 1000 µg/ml. Whereas, *P. fraternus* plant extract exhibited moderate toxicity with CTC_{50} value 593.33±5.8 µg/ml and showed promising *in vitro* cytotoxicity activity. From the study it was observed that the extracts were moderately toxic. Toxicity from plants may be attributed to majorly from alkaloids, glycosides, saponins, polyacetylenes, and key phytochemicals.

12.3.2 In vitro glucose uptake assay

Peripheral tissue such as skeletal muscle is important to maintain the postprandial plasma glucose levels (Ishiki *et al.*, 2005). Rat L6 myotubes were employed as an in vitro system to investigate the glucose uptake activity of test drugs were determined in differentiated L6 cells. Based on the results of cytotoxicity studies, for *in vitro* glucose uptake studies, a test doses 500 µg/ml and 250 µg/ml was selected.

The results obtained from the *in vitro* glucose uptake studies are presented in **Table – 12.2** and **Figure – 12.2**.

Table – 12.2: *In vitro* antidiabetic effect of *M. roxburghianus* and *P. fraternus* plant extract on glucose uptake in L6 cell lines

Name of the extract	Concentration (µg/ml)	Glucose uptake percentage (%)
Control	-	0.00
Standard Rosiglitazone	50	36.23±3.4
M. roxburghianus plant extract	500 250	16.79±3.1 7.65±1.5
P. fraternus plant extract	500 250	23.33±3.7 14.23±2.2

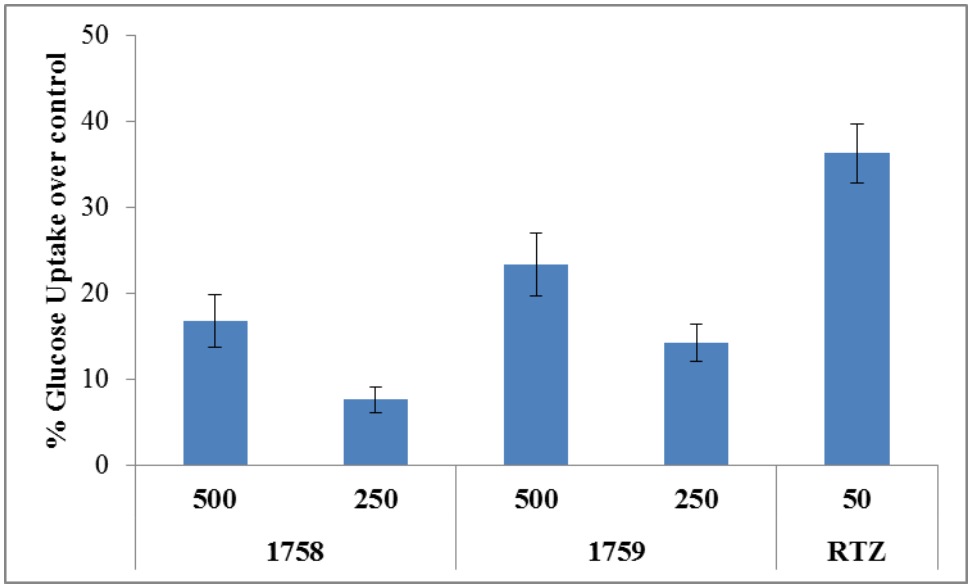

Figure – 12.2: Percentage glucose uptake enhancement by treated groups (*M. roxburghianus*, *P. fraternus* plant extract and standard Rosiglitazone)
[Note : 1758: M. roxburghianus plant extract; 1759: *P. fraternus* plant extract]

In vitro glucose uptake studies revealed that, *P. Fraternus* plant extract has better glucose uptake enhancement potential with 23.33±3.7 and 14.23±2.2 % at 500 and 250 µg/ml, respectively compared to the standard. *M. roxburghianus* plant extract exhibited at 16.79±3.1 and 7.65±1.5 % at 500 and 250 µg/ml, respectively compared to the standard.

GLUT-4, which is an important component of the insulin signal transduction network that regulates glucose transport, might be involved in the increased uptake of glucose by L6 cell lines in presence of *M. roxburghianus* and *P. fraternus* plant extract. These extracts enhance glucose uptake under in vitro conditions which may be due to its effect on the number of receptors located in the skeletal muscle.

12.4 Conclusion

The *in vitro* cytotoxicity assays offers quick, simple and cost-efficient way of testing the toxicity and forms an important tool for high throughput screening of plant extracts. In addition, they have significant impact in the implementation of the three R's; the reduction of number of animals used, refinement of animal test models and replacement of animal in research.

While both the plant extract have *in vitro* cytotoxicity activity and an effective glucose uptake potential, but *P. fraternus* plant extract shows better potential compared to *M. roxburghianus* plant extract. From the present findings, it can be concluded that *P. fraternus* plant extract shows moderate toxicity against L6 cell line.

The results obtained in the present study clearly demonstrate that the *P. fraternus* plant extract enhances glucose uptake under *in vitro* conditions. This may be due to its effect on the number of receptors located in the skeletal muscle cell line. The glucose uptake activity of *P. fraternus* plant extract may be attributed to the GLUT4 translocation.

CHAPTER 13
IN VITRO STUDY OF THE EFFECT OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS ON GLUT-4 GENE EXPRESSION IN L-6 MYOTUBES CELL LINE

CHAPTER 13: *IN VITRO* STUDY OF THE EFFECT OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS ON GLUT-4 GENE EXPRESSION IN L-6 MYOTUBES CELL LINE

Even after huge caloric ingestions, elevated glucose levels are rapidly returned to normal (5–6 mM) and they are maintained at only slightly lower levels during long term starvation. It is due to this control system that severe dysfunctions such as loss of consciousness due to hypoglycemia and toxicity to peripheral tissues in response to the chronic hyperglycemia of diabetes are prevented. The major cellular mechanism for disposal of an exogenous glucose load is insulin-stimulated glucose transport into skeletal muscle. Skeletal muscle stores glucose as glycogen and oxidizes it to produce energy following the transport step (Huang and Czech, 2007).

Glucose diffusion through the plasma membrane into the cell interior is facilitated by glucose transporters. Several isoforms of glucose transporters have been identified and they are distributed in a tissue-specific manner. Insulin's effect to increase glucose uptake is exerted by stimulating the translocation of glucose transporters from an intracellular pool to the plasma membrane (Yu *et al.,* 2001). Glucose transporters (GLUT) play a pivotal roles in energy metabolism. Around six different eukaryotic GLUT isoforms have been identified. GLUTS possess 12 transmembrane helical segments with the N- and C-termini and a large central loop exposed to the cytoplasm. GLUT transporters are responsible for the facilitative uptake of D-glucose and its analogues.

In adipocytes, there is also another isoform of glucose transporter, the so called erythrocyte/brain glucose transporter isoform (GLUT1) which is located mainly at the plasma membrane and considered to be responsible for basal glucose transport (Yu *et al.,* 2001).GLUT 1 is most abundant in erythrocytes and brain micro vessels, and is present in most tissues and cell cultures. GLUT 2 is expressed mainly in pancreatic beta cells, liver, and enterocytes basolateral membranes. GLUT 3 is found in brain neurons, fetal muscle, skeletal and cardiac myoblasts (Broydell *et al.,* 1998).

GLUT 4 is present in insulin-sensitive tissues, such as brown and white adipose tissues, cardiac and skeletal muscles (Broydell *et al.,* 1998). GLUT 4 is the main mediator of glucose transport activation in the target tissues of insulin is the adipose tissue/muscle glucose transporter isoform (Yu *et al.,* 2001). The GLUT 4 glucose transporter (Figure 1) is the principal glucose transporter protein that mediates glucose removal from the circulation and a key regulator of whole-body glucose homeostasis (Huang and Czech, 2007).

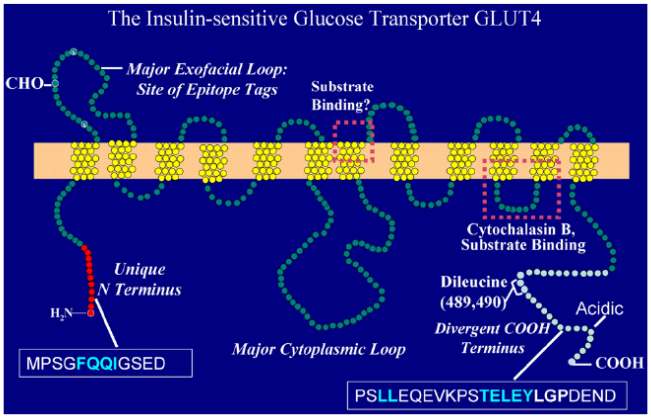

Figure 13.1. Structural features of the insulin-regulated GLUT 4 glucose transporter protein

GLUT 5 is actually a fructose transporter detected in human enterocyte luminal membranes, adipocytes, skeletal muscle and sperm. GLUT 7 is located in liver microsomes.

Valuable information on the molecular mechanisms and the signal transduction pathways by which glucose transport processes can be regulated has been obtained based on the studies on the effects of insulin, tumor necrosis factor, glucose starvation, exercise and development on the expression, intracellular location, and activities of GLUT 1 and GLUT 4 transporters (Broydell *et al.*, 1998).

The abnormal glucose transport associated with deficient GLUT-4 translocation, and/or faulty insulin signaling cascade are manifested as among the dominant defects in insulin resistance in type 2 diabetes. The intense rise in GLUT-4 and P13 kinase, mRNA levels in euglycemic and hyperinsulinemic clamp in the presence of insulin refined the role of GLUT-4, and P13 kinase in insulin-mediated glucose transport.

L6 muscle cell line was a suitable in *vitro* model to study glucose transport activity. Skeletal muscle is the major site for primary glucose clearance and glucose utilization. Earlier reports of L6 myotubes evidenced the enhanced glucose uptake in L6 cells is mostly mediated through elevated GLUT-4 levels (Kumar et al., 2014).

13.1 Aim of the study: To evaluate the *in vitro* effect of *M. roxburghianus* and *P. fraternus* plant extract on GLUT 4 expression in L-6 myotubes cell line.

13.2 Materials and Methods:

13.2.1 Test substance

M. roxburghianus and *P. fraternus* plant extract were studied.

13.2.2 Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT) - Sigma Aldrich Co, St Louis, USA,
Fetal Bovine serum (FBS) - Sigma Aldrich Co, St Louis, USA,
Phosphate Buffered Saline (PBS) - Sigma Aldrich Co, St Louis, USA,
Dulbecco's Modified Eagle's Medium (DMEM) - Sigma Aldrich Co, St Louis, USA,
Trypsin - Sigma Aldrich Co, St Louis, USA,
EDTA - Hi-Media Laboratories Ltd., Mumbai,
Glucose - Hi-Media Laboratories Ltd., Mumbai
Antibiotics from Hi-Media Laboratories Ltd., Mumbai
Dimethyl Sulfoxide (DMSO) - E. Merck Ltd., Mumbai, India
Propanol - E. Merck Ltd., Mumbai, India

13.2.3 Cell lines and Culture medium

L-6 (Rat Skeletal Muscle) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

13.2.4 Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

13.2.5 Determination of cell viability by MTT Assay

(i) Principle:

The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) by mitochondrial enzyme succinate dehydrogenase. The

number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

(ii) Procedure:

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. 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 of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line (Francis and Lang, 1986).

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of Control}} X\ 100 \right)$$

13.2.6 RT-PCR procedure

The mRNA expression levels Glut 4 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, the L6 cells were cultured in 60 mm petridish and maintained in DMEM medium for 5 days. The DMEM medium was supplemented with FBS and amphotericin. To the dish was added the required concentration of *M. roxburghianus* plant extract (1000 µg/ml and 500 µg/ml) and *P. fraternus* plant extract (600 µg/ml and 300 µg/ml) incubated for 24 hr. Total cellular RNA was isolated from the untreated (Control) and treated cells using Tri Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufactures instructions (Thermo scientific). Then 20µl of the reaction mixture was subjected to PCR for amplification of Glut 4 cDNA using specifically designed primers

procured from Eurofins India, as an internal control, the house keeping gene GAPDH was co-amplified with each reaction (Zanchi *et al.*, 2012).

(i) Amplification conditions for Glut 4 gene:

95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 minute , annealing at 55°C for 1 minute and extension at 72°C for 1 min. This was followed by final extension at 72°C for 10 min.

(ii) Primer used:

For Ist strand synthesis:

oligo dT primer

For IInd strand synthesis:

Forward : 5′-GGGCTGTGAGTGAGTGCTTTC-3′
Reverse : 5′-CAGCGAGGCAAGGCTAGA-3′

(iii) Product size: 150 bp

13.3 Results and Discussion

MTT assay was performed to determine whether *M. roxburghianus* and *P. fraternus* plant extracts treatment affects cell viability. The percentage growth inhibition was calculated and concentration of drug or test extract needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for the cell line. Dose-dependent anti-proliferative effect on the cell viability was observed. The results have been summarized in **Table - 14.1**.

Table - 13.1: Cytotoxic properties of *M. Roxburghianus* and *P. Fraternus* plant extracts on L-6 cell line

Name of test sample	Concentration (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
M. roxburghianus plant extract	62.5 125 250 500 1000	13.90±1.7 21.01±3.3 27.02±1.6 30.60±3.8 39.39±5.5	>1000
P. fraternus plant extract	62.5 125 250	12.86±2.7 14.39±2.6 19.13±7.9	593.33±5.8

	500 1000	45.73±0.8 69.41±2.3	

M. roxburghianus plant extract showed CTC_{50} value of >1000 µg/ml in L6 cell lines. These value indicated that the maximum cytotoxic effect of *M. roxburghianus* plant extract showed 50% reduction in cell viability upon treatment with highest dose (>1000 µg/ml). *P. fraternus* plant extract showed CTC_{50} value of 593.33 µg/ml in L6 cell lines. These values indicated that the maximum cytotoxic effect of *P. fraternus* plant extract showed 50% reduction in cell viability upon treatment with highest dose (593 µg/ml). Relatively less value of CTC_{50} indicates the sample is more cytotoxic and may possess antidiabetic activity. The results indicate that *P. fraternus* plant extract showed a potent activity against the *in vitro* L-6 cell line compared to *M. roxburghianus* plant extract.

The semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) test to estimate the mRNA expression Glut 4 levels was performed. The concentrations of plant extract to be used were based on the results of cytotoxic study. The results of RT-PCR are illustrated in **Figure - 13.2, Figure - 13.3, Figure - 13.4 and Table - 13.5**. The aim of this study was to characterize the interaction between these agents and insulin with respect to adipose tissue/muscle glucose transporter isoform (GLUT4) gene regulation.

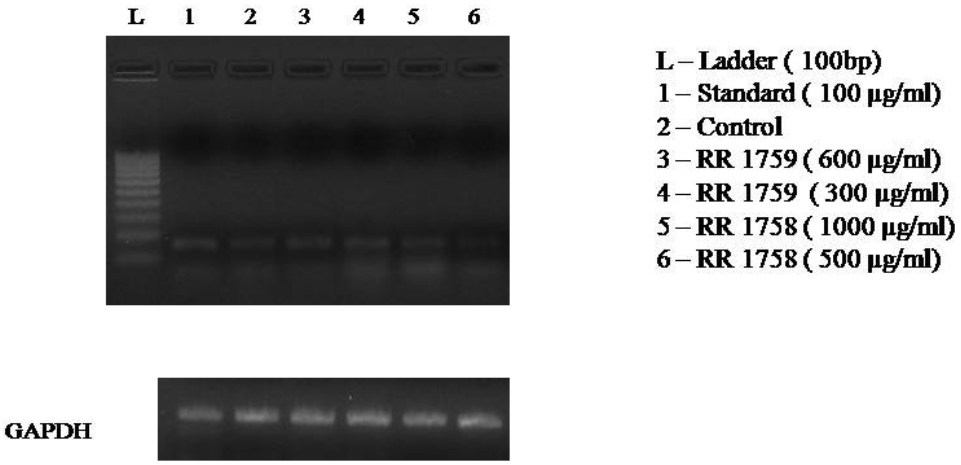

Figure - 13.2: RT-PCR profile of Glut 4 gene amplified from *M. roxburghianus*, *P. fraternus* plant extracts and standard on L6 Myotubes cells

[Note : *1758: M. roxburghianus* plant extract; 1759: *P. fraternus* plant extract, Standard: Rosiglitazone]

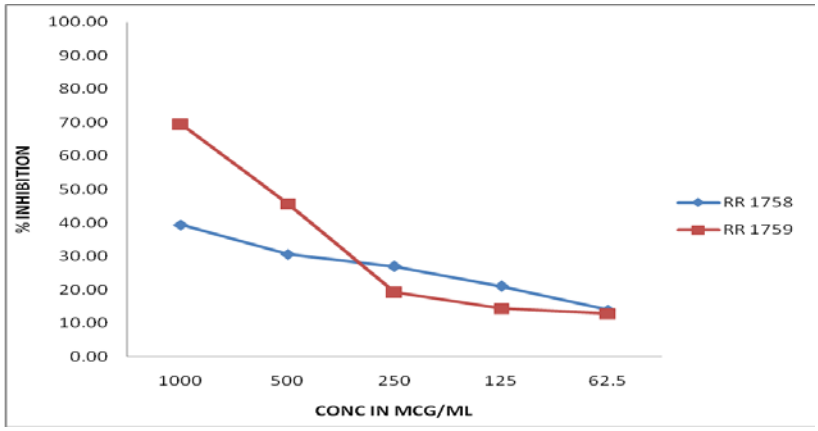

Figure - 13.3: Cytotoxic effect of the sample *M. roxburghianus* and *P. fraternus* plant extracts on L6 Cell line

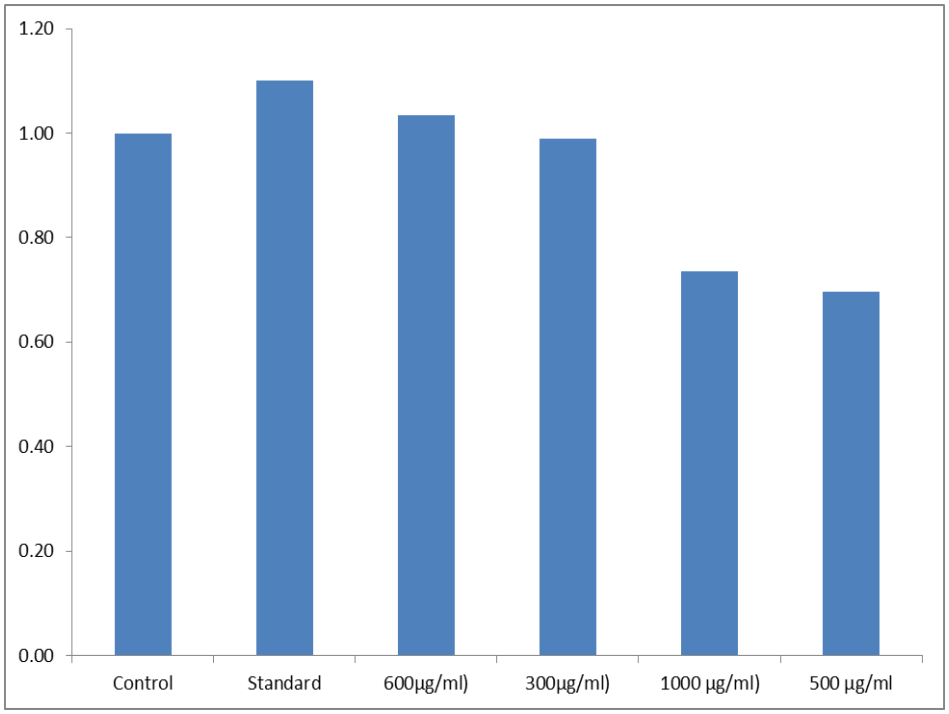

Figure - 13.4: Densitometry analysis of gene transcripts of *M. roxburghianus* and *P. fraternus* plant extracts compared to control and standard

The relative level of Glut 4 gene expression in **Figure - 13.4** is normalized to GAPDH. Values shown depict arbitrary units.

Table - 13.2: Relative level of Glut 4 gene expression of *M. roxburghianus* and *P. fraternus* plant extracts against control and standard

Test sample	Concentration	Regulation in terms of folds
Control	NA	1.00
Standard - Rosiglitazone	100 µg/ml	1.10
***M. roxburghianus* plant extract**	500 µg/ml	0.70
	1000 µg/ml	0.74
***P. fraternus* plant extract**	300 µg/ml	0.99
	600 µg/ml	1.04

In this densitometry analysis study the results show that when compared with the control, the standard drug rosiglitazone, at a concentration of 100µg/ml show a 0.10 folds up-regulation of GLUT4 gene expression in L6 cell lines. *P. fraternus* plant extract at a concentration of 300µg/ml didn't show any modulation in GLUT4 expression while at a concentration of 600 µg/ml, it showed a 0.04 folds up- regulation of GLUT4 gene expression in L6 cell lines when compared with the control L6 cell lines. On the other hand *M. roxburghianus* plant extract at a concentration of 500 µg/ml and 1000 µg/ml showed 0.3 and 0.26 folds down regulation respectively of GLUT4 gene expression when compared with the control.

The *P. fraternus* plant extract in a dose of 600 µg/ml produced up-regulation of GLUT4 and the effects were only slightly lesser than that produced by rosiglitazone. Based on the gene expression study outcomes it was hypothesized that *P. fraternus* plant extract could activate the glucose transport by up-regulation of GLUT-4. It can be inferred that one of the mechanisms of action of *P. fraternus* plant extract may be similar to that of thiazolidinedione's.

The findings in the present study are in agreement with the literature reports where there is a concomitant increase in glucose uptake along with the enhanced GLUT-4 levels in L6 myotubes, the *P. fraternus* plant extract has shown enhanced glucose uptake, and assisted the enhanced GLUT4 expression.

13.4 Conclusion

In the current study, the unifying approach of medicinal chemistry and *in vitro* screenings assays was manifested, which assured the evidence of a set of targets on glucose transport. Also this study demonstrated the importance of GLUT-4 up-regulation by *P. fraternus* plant extract in enhancing glucose transport. *P. fraternus* is a plant with great potential in the management of type2 DM since it promotes the up-regulation of GLUT4 glucose transporter genes. The study proved that the plant extract can be used to reduce insulin resistance associated with type 2 DM since the plant possesses good insulin sensitizing properties. The plant promotes glucose uptake too, thereby improving glucose utilization and disposal in skeletal muscles.

CHAPTER 14
IN VIVO ACUTE ORAL TOXICITY STUDY OF *M. ROXBURGHIANUS* AND *P. FRATERNUS* PLANT EXTRACTS

CHAPTER 14: *IN-VIVO* ACUTE ORAL TOXICITY STUDY OF *MALLATUS ROXBURGHIANUS* AND *PHYLLANTHUS FRATERNUS* PLANT EXTRACTS

Acute toxicity is defined as the ability of a substance to cause adverse effects within a short time of dosing or exposure of a test substance or substances (Walum E, 1998). As per the IUPAC Recommendations 2003, it is defined as the adverse effects of finite duration occurring within a short time (up to 14 days) after administration of a single dose (or exposure to a given concentration) of a test substance or after multiple doses (exposures), usually within 24 hours of a starting point (which may be exposure to the toxicant, or loss of reserve capacity, or developmental change, etc.).

An adverse effect is "any effect that results in functional impairment and/or biochemical lesions that may affect the performance of the whole organism or that reduce the organ's ability to respond to an additional challenge". A chemical that enters the organism via the oral route during a restricted time and produces any adverse effect with little delay is orally and acutely toxic (Walum E, 1998).

14.1 Aim of the study: To perform acute oral toxicity study of *M. Roxburghianus* and *P. fraternus* plant extracts in rats.

14.2 Materials and methods

Acute oral toxicity study was carried out as per the OECD Limit test 423 for Acute Oral Toxicity (OECD Limit test 423, 2001).

14.2.1 Animals

Breed	Wistar Rat
Source	In-house breeded animals
Number of animals	3 + 3 females
Total number of animals	06 females
Age when treated	8 - 12 weeks
Body weight when treated	153.0 to 185.0 grams
Identification	By unique cage number and individual animal numbers marked with indelible marker pen on the tail. The animals were marked (towards the tip of tail) with the temporary animal numbers at start of acclimatization. The animals were marked with permanent animal numbers (towards the base of tail) with different color indelible marker pen before the start of test item administration.
Conditions	Standard Laboratory Conditions. The animal room (Room no.01) was air-conditioned with adequate air changes per hour.

	The animals were provided with a light cycle of 12 hours light and 12 hours dark.
Accommodation	Housed in groups of three in Polycarbonate cages (approximate internal dimensions of 365 mm x 202 mm x 180 mm height) with paddy husk bedding.
Diet	Rodent feed was provided *ad libitum*.
Water	Genpure RO water was provided *ad libitum*.

14.2.2 Test Facility

Radiant Research Services Pvt. Ltd
99/A, 8th main, 3rd phase, Peenya industrial area
Bangalore – 560 058

The experiments were performed in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi, India (Registration No: 1803/PO/RcBi/S/2015/CPCSEA) and the project approval number is RR/IAEC/04/1-2015.

14.2.3 Test substance

M. roxburghianus and *P. fraternus* plant extracts were studied.

14.2.4 Dose selection

Both the test drug is of natural origin and non-toxic, therefore a test dose of 2000 mg/kg, as per the OECD guideline 423 “Acute oral toxicity”.

14.2.5 Preparation of dosing solution

A known amount of test substance was taken in a mortar and small quantity of vehicle (quantity sufficient to make a uniform suspension) was added slowly with continuous trituration to obtain a uniform suspension. Sufficient volume of vehicle was then added to make up the appropriate dose concentration and stirred for 15 – 20 minutes to get uniform suspension.

14.2.6 Experimental conditions

Mode of application	Per oral, via gastric tube. Oral administration was considered as recommended by the guideline.
Frequency of treatment	Single dose
Dosage level	2000mg/kg

Vehicle	Carboxy methylcellulose sodium
Post treatment examination period	14 days
Safety precautions	Routine hygienic procedures: protective gloves, face mask, aprons/protective suit and goggles were used to ensure the health and safety of the personnel.

14.2.7 Procedure

Acute toxicity test was performed according to the Organization of Economic Co-operation and Development (OECD) guidelines for testing of chemicals. The OECD guideline 423 recommends use of three animals to determine the lethal dose of test substance. According to OECD guidelines 423, three animals should be used for a particular dose (5, 50, 300, 2000 mg/kg body weight) to know the lethal dose and further three animals required for conformation of the same. Three animals were used to study each test substance. This guideline does not recommended use of statistics to fix the dose level.

For acute oral toxicity study, animals were selected and grouped manually. No computer generated randomization program was used. The allocations of animals were done as below followed by the following steps for selection of animals:

SL.NO	NO. OF ANIMALS	ANIMAL NUMBER		
1	3	RRAC 001	-	RRAC 003
2	3	RRAC 004	-	RRAC 006

Step I: Acclimatization under laboratory conditions for 5, 7 and 9 days to screen the health after veterinary examination.

Step II: Observation for visible illness after veterinary examination.

Step III: Observation for body weight after veterinary examination.

Only animals without any visible signs of illness were used for the study.

The animals received a single dose of the test item by oral administration at 2000 mg/kg body weight after being fasted for approximately 18.0 hours but with free access to water. Food was provided again at approximately 3.0 hours after dosing. The administration volume was 10 mL / Kg body weight.

The following observations were recorded:

Mortality / Viability: Daily during the acclimatization period, during the first 30 minutes and at approximately 1, 2, 3 and 4 hours after administration on test day 0 (in common with the clinical signs) and twice daily during days 1-14 (at least once on day of sacrifice) or more.

Body weights: On test days 0 (prior to administration), day 7, day 14or more.

Clinical signs: Daily during the acclimatization period, during the first 30 minutes and at approximately 1, 2, 3 and 4 hours after administration on test day 0 and once daily during days one to fourteen.

Body weights, clinical signs, mortality/viability, and macroscopic findings were recorded on data sheets. Column statistics were applied for the data analysis. Column statistics is a method to analyze body weight (statistics) data in a graphical representation. Graph pad prism software to calculate the body weight.

All surviving animals were sacrificed at the end of the observation period and discarded after the gross/macroscopic pathological changes were observed and recorded. No organs or tissues were retained.

14.3 Results and discussion

The effects of oral administration of single doses of *M. roxburghianus* and *P. fraternus* plant extract. The body weights, clinical signs, mortality/viability, symptoms of toxicity and macroscopic findings were observed and recorded. These observations are presented below:

Mortality: No mortalities were observed in the animals (2000 mg/kg body weight).

Clinical signs and behavioral observation: All the animals appeared normal throughout the experimental period (as per Annexure - I). Clinical signs and behavioral parameters were observed carefully after the dose administration. There were not any abnormal signs observed and even throughout the study in all the animals.

Body weights: All surviving animals had gained body weight by day 14 as compared to day 0 (Refer **Table - 14.1**, **Table - 14.2**, **Figure - 14.1** and **Figure - 14.2**). Increased body weight in animals during the study was observed in all the animals and it is a normal pattern with healthy animals.

Macroscopic findings and observation: No abnormalities were detected for any of the animals at terminal sacrifice (**Table - 14.3**, **Table - 14.4**, **Table - 14.5**, **Table - 14.6** and **Figure - 14.3**, **Figure - 14.4**).

Heart, liver, lungs, kidney, spleen and intestinal organs were observed macroscopically and found to be normal and healthy animals at sacrificed terminally.

Table - 14.1: Clinical and behavioral observation during the study in rats with *M. roxburghianus* plant extract

Animal identification number	*After Treatment**	Days of post treatment examination													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RRAC 001	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RRAC 002	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RRAC 003	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01

* Observation of first four hours after treatment

01 – NAD (02-85 codes observations are not seen)

Table - 14.2: Clinical and behavioral observation during the study in rats with *P. fraternus* plant extract

Animal identification number	*After Treatment**	Days of post treatment examination													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RRAC 004	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RRAC 004	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01
RRAC 005	01	01	01	01	01	01	01	01	01	01	01	01	01	01	01

* Observation of first four hours after treatment 01 – NAD (02-85 codes observations are not seen)

Table - 14.3: Individual body weight of Rats during the study with *M. roxburghianus* plant extract

Animal ID No.	Plant extract	Dose	Treatment		
			Before	After	
			Day 0	Day 7	Day 14
RRAC 001	*M. roxburghianus*	2000 mg/kg	173	177	182
RRAC 002			176	181	185
RRAC 003			167	171	175

Table - 14.4: Individual body weight of Rats during the study with *P. fraternus* plant extract

Animal ID No.	Plant extract	Dose	Treatment		
			Before	After	
			Day 0	Day 7	Day 14
RRAC 004	*P. fraternus*	2000 mg/kg	185	195	198
RRAC 005			153	156	163
RRAC 006			164	172	173

Table - 14.5: The result of pathological examinations with *M. roxburghianus* plant extract

Animal ID No	Plant extract	Dose	Macroscopic lesions
RRAC 001	*M. roxburghianus*	2000 mg/kg	No macroscopic alteration occurred
RRAC 002			No macroscopic alteration occurred
RRAC 003			No macroscopic alteration occurred

Table - 14.6: The result of pathological examinations with *P. fraternus* plant extract

Animal ID No	Plant extract	Dose	Macroscopic lesions
RRAC 004	*P. fraternus*	2000 mg/kg	No macroscopic alteration occurred
RRAC 005			No macroscopic alteration occurred
RRAC 006			No macroscopic alteration occurred

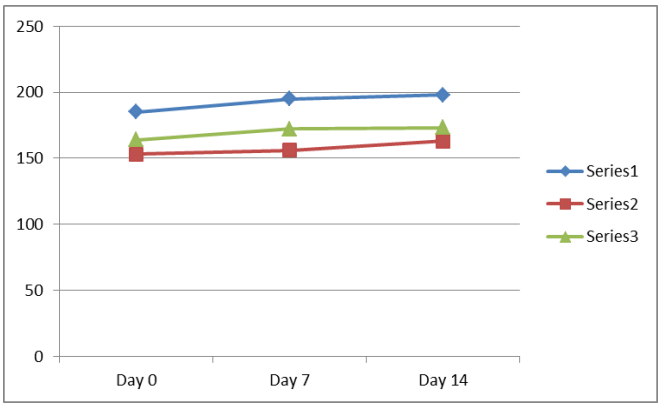

Figure - 14.1: Increased body weight of animals from Day 0 to Day 14 with *M. roxburghianus* plant extract

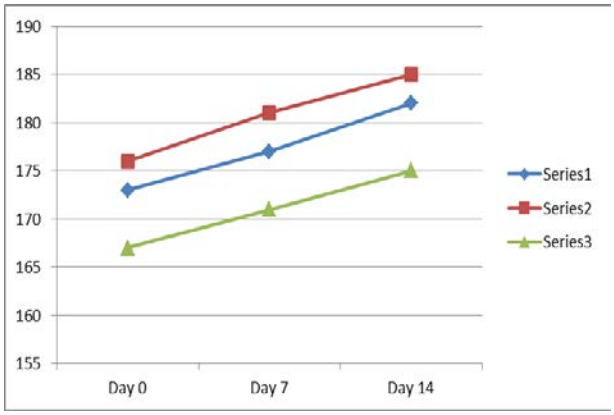

Figure - 14.2: Increased body weight of animals from Day 0 to Day 14 with *P. fraternus* plant extract

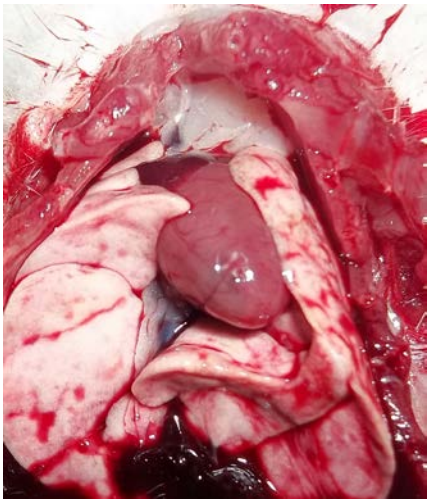

Figure - 14.3a: Heart and lungs

Figure - 14.3b: Liver

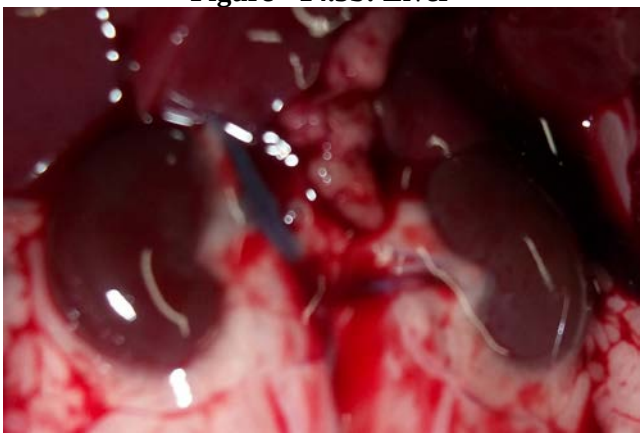

Figure - 14.3c: Kidney

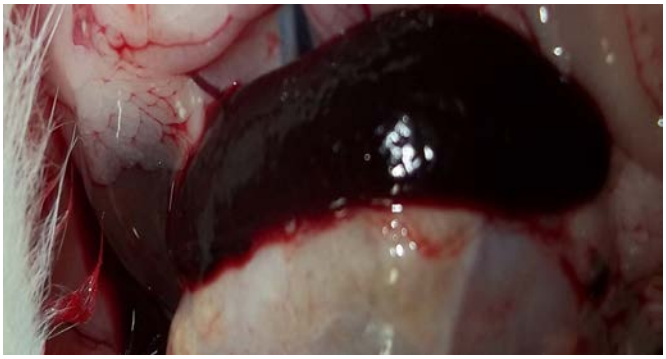

Figure - 14.3d: Spleen

Figure - 14.3: Gross pathological examination (Macroscopic observation) of animals treated with M. roxburghianus plant extract

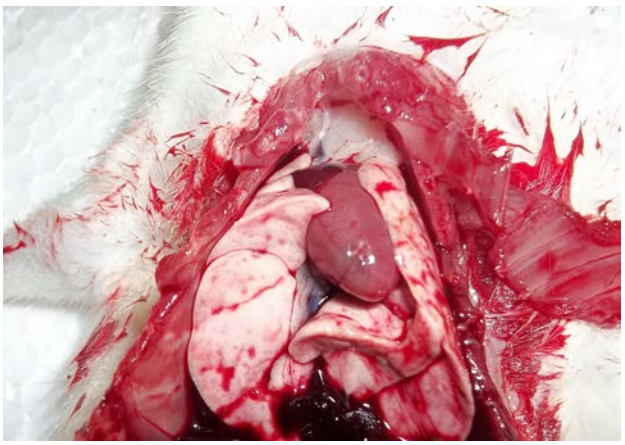

Figure - 14.4a: Heart and lungs

Figure - 14.4b: Liver

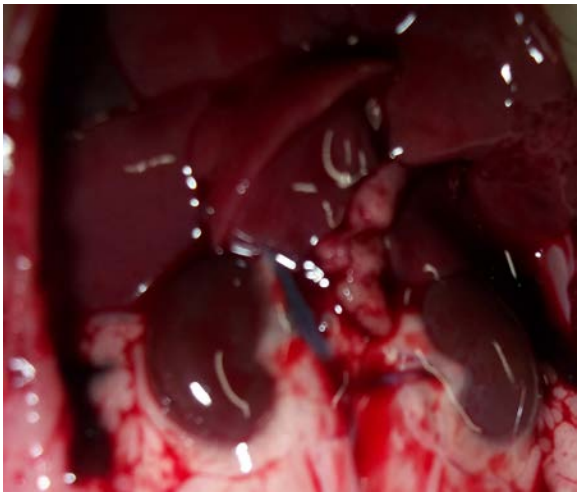

Figure - 14.4c: Kidney

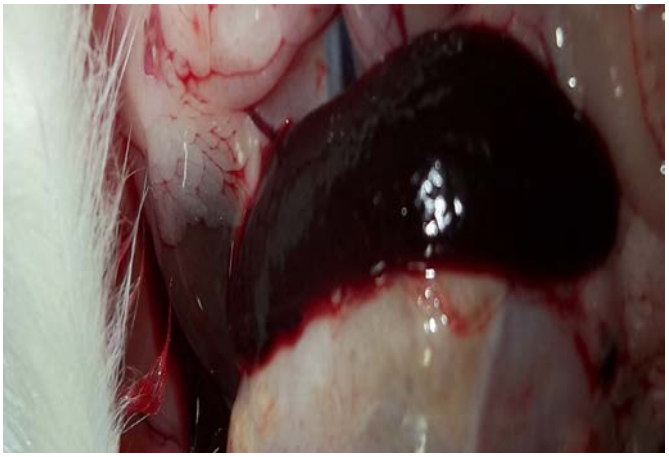

Figure - 14.4d: Spleen

Figure - 14.4: Gross pathological examination (Macroscopic observation) of animals treated with P. fraternus plant extract

From the experiment performed as per the OECD Guidelines 423, the results reveal that the methanolic extract of *M. roxburghianus* and *P. fraternus* plant extracts have not been found to be toxic at 2000 mg/kg body weight of experimental animals. The test group at a single oral dose of 2000 mg/kg of *M. roxburghianus* and *P. fraternus* plant extracts did not cause death or signs of toxicity in rats observed over a period of 14 days. No significant changes were observed in body weight and wellness parameters used for evaluation of toxicity. Skin, fur, eyes, mucous membrane, behavioral pattern, salivation, sleep of the treated as well as the control animals were found to be normal. Tremors, lethargy, diarrhea and coma did not occur in any of the animal. The histology examination revealed no changes in the architecture of the internal organs of rats. The LD_{50} of each plant extract was determined. The median lethal dose of test substance (LD_{50}) more than 2000mg/kg body weight.

14.4 Conclusion

In conclusion, acute toxicity studies on *M. roxburghianus* and *P. fraternus* plant extracts were carried out as a prerequisite to exploration of antidiabetic potential of these plants. The LD_{50} value of *M. roxburghianus* and *P. fraternus* plant extracts in female rats after single oral treatment is above 2000 mg/kg body weight and is classified as Category 5. According to the OECD guideline 423, if the test compound could not be toxic in the level 2000 mg/kg of body weight in an animal, such level classified as **Category 5** and hence the plant extracts were found to be safe.

The selected plants can be further explored for their therapeutic potential in different chronic diseases.

ANNEXURE – 1

CLINICAL SIGNS AND BEHAVIOURAL OBSERVATION

CODES	OBSERVATIONS	SIGNS/SYMPTOMS
01	NAD	02-85 codes observations are not seen
02	Accidental death	
03	Partial Cannibalism	An animal of a species consuming part of another animal of the same species
04	Total Cannibalism	An animal of a species consuming the major organs of another animal of the same species
05	Dead	Irreversible cessation of all body functions, manifested by absence of spontaneous breathing and total loss of cardiovascular and cerebral functions
06	Moribund condition	Approaching death animal will not be available for examination for next day
07	Emaciation	Extreme loss of subcutaneous fat that results in an abnormally lean body
08	Weakness	A weak bodily state as expressed by difficulty in rising, a shuffling, disinclination to move, eating slowly and a drooping posture
09	Lethargy	A level of consciousness characterized by decreased interaction with objects in the environment, sluggishness, abnormal drowsiness
10	Salivation	Flow of saliva, Drooling(Abnormally abundant flow of saliva)
11	Lacrimation	Flow of tears
12	Discharge	Abnormal discharge
13	Snuffling (Unusual respiratory pattern)	A bubbling sound from the nasal cavities
14	Bronchial rales	An abnormal respiratory sound (crackles) in auscultation of lungs
15	Sneezing	Sudden, reflex, noisy expiration through the nasal cavities
16	Cough	A forceful release of air from the lungs
17	Dyspnea (Unusual respiratory pattern)	Shortness of breath
18	Corneal opacity	Opaque white spot on the cornea
19	Cataract	Opacity of the crystalline lens of the eye
20	Microsphthalmia	Abnormal smallness of the eyeball
21	Exophthalmia	Protrusion of the eyeballs
22	Anophthalmia	Absence of the eyes or the presence of vestigial eyes
23	Fecal staining	Excessive and frequent evacuation of watery feces
24	Diarrhea	Diarrhea is the frequent passage of loose, watery, soft stools
25	Urine staining	The urinary stains around urinary opening
26	Perineum wet with urine	The perineal region becomes wet with urine
27	Hematuria	Presence of blood in the urine

28	Prolapse	The slipping or falling out of place of an organ
29	Prolonged parturition	Process of giving birth that lasts longer than the usual period
30	Abortion	Expulsion of the fetus before it is viable
31	Rough coat	Roughness of coat
32	Piloerection	Erection of hair
33	Response to handling	Normal response to approach
34	Tremors	Involuntary, rhythmical alternating movement that may affect the muscles of any part of the body
35	Convulsions	Violent involuntary contraction of a muscle or muscles
36	Repetitive Circling	Continuous circling
37	Head tilted on one side	Head facing towards some other direction other than straight
38	Ataxia	Inability to control voluntary muscle movement
39	Gait Characteristics	The pattern of movement of the limbs
40	Pain or Palpation	Unpleasant sensory and emotional experience
41	Dermatitis	Inflammation of the skin
42	Vesicle	A small sac containing liquid
43	Blister	A local swelling of the skin that contains watery fluid
44	Papule	A small, solid, usually inflammatory elevation of the skin that does not contain pus
45	Pustule	A small inflamed elevated area of skin containing pus
46	Urticaria	An itchy skin eruption characterized by weal's with pale interiors and well-defined red margins
47	Scale formation	Insoluble scale coats on the skin
48	Scab formation	A crust that forms over a sore or wound during healing
49	Erosion	The superficial destruction of a surface area of tissue (as mucous membrane) by inflammation, ulceration, or trauma
50	Fissure	A break or slit in tissue usually at the junction of skin and mucous membrane
51	Necrosis	Death of a portion of tissue differentially affected by local injury
52	Eschar	A scab formed especially after a burn
53	Malocclusion	Improper occlusion especially abnormality in the coming together of teeth, jaw
54	Peeling/Desquamation	loss of bits of outer skin by peeling or shedding or coming off in scales
55	Parchment paper like	Untanned skin
56	Erythema	Redness of the skin
57	Oedema	A swelling from effusion of watery fluid in the cellular tissue beneath the skin or mucous membrane
58	Cyanosis	Bluish discoloration of the skin and mucous membranes
59	Paralysis	Loss of sensation over a region of the body
60	Edema	An excessive accumulation of serous fluid in tissue spaces or a body cavity
61	Crepitation	A dry, crackling sound or sensation
62	Dehydration	Loss of water and salts. The skin turns pale and cold, the mucous membranes lining lose their natural moisture

63	Dull	Lacking responsiveness or alertness
64	Posture	position of the body or of body parts
65	Epistaxis	Bleeding from the nose
66	Urine dribbling	Leaking of urine
67	Lame	Incapable of normal locomotion
68	Arched back	postural abnormality, the back is depressed
69	Warts	small, benign growths
70	Fistula	Permanent abnormal passageway between two organs in the body or between an organ and the exterior of the body
71	Abscess	Collection of pus has accumulated in a cavity formed by the tissue
72	Injury due to biting (specify location)	Injury caused by biting
73	Distended abdomen	Enlarged abdomen
74	Palpable mass Location	Abnormal morphologic structure (malformation)
75	Subcutaneous mass	Mass formation in hypodermis
76	Injury or wound (specify location)	An injury caused by external source
77	Obesity	Excess body fat that has accumulated
78	Incisor elongation/ unworn teeth	Abnormal increase in incisor teeth
79	Mucous membrane	Abnormal nature of mucous membrane that lines the cavities
80	Pupillary response absent	Constriction of a dilated pupil in response to an increase in light intensity and a dilatation of a constricted pupil in response to a decrease in the intensity
81	Clonic or tonic Movements	Sustained muscular contraction without intervals of relaxation
82	Excessive grooming	Excessive grooming
83	Self mutilation	Self injury
84	Walking backwards	Abnormal walking
85	Bloody stools	Blood stained faces

CHAPTER 15
IN VIVO ANTI-DIABETIC ACTIVITY *OF P. FRATERNUS* PLANT EXTRACT AGAINST STREPTOZOTOCIN INDUCED DIABETES IN RATS

CHAPTER 15: IN VIVO ANTI-DIABETIC ACTIVITY OF *PHYLLANTHUS FRATERNUS* PLANT EXTRACT AGAINST STREPTOZOTOCIN INDUCED DIABETES IN RATS

Streptozotocin (STZ) is an antibiotic produced by Streptomyces achromogenes (Alimohammadi *et al.,* 2013). STZ is frequently used to induce diabetes in experimental animals through its toxic effects on pancreatic β –cell and as a potential inducer of oxidative stress. STZ induced diabetic rats are a type of animal models of type 1 diabetes mellitus and is the commonly used model for the screening of anti-hyperglycemic activities. It is well known for its selective pancreatic islet beta cell cytotoxicity and has been extensively used to induce type 1 diabetes in experimental rat model (Gandhi and Sasikumar, 2012). The mechanism by which STZ destroys β-cells of the pancreas and induces hyperglycemia is still not clear. Various actions attributed to the diabetogenic action STZ include damage to pancreatic β-cell membranes and depletion of intracellular nicotinamide adenine dinucleotide (NAD) in islet cells, induction of DNA strand breaks, an increase in the intracellular methylation reaction in pancreatic islet cells and the production of nitric oxide (NO) and free radicals (Alimohammadi *et al.,* 2013).

15.1 Aim of the study: The present study has been undertaken with the aim to evaluate the antidiabetic activity of *P. fraternus* plant extract.

15.2 Materials and Methods:

a) Reagents and kits

Streptozotocin procured from Spectrchem Private Limited, Mumbai, India
Glibenclamide procured from Sigma Aldrich, Bangalore, India
Citrate buffer, pH 4.5 Sodium carboxy methyl cellulose
Accu-Chek active kits
Diagnostics kits

b) Test substance

P. fraternus plant extract was studied.

c) Animals

Breed	Albino Wistar Rat
Source	In-house breed animals
Number of animals	24 diabetic surviving rats + 6 normal rats
Total number of animals	30 females rats
Age when treated	6 to 8 weeks

Body weight when treated	150–200 g
Identification	By unique cage number and individual animal numbers marked with indelible marker pen on the tail. The animals were marked (towards the tip of tail) with the temporary animal numbers at start of acclimatization. The animals were marked with permanent animal numbers (towards the base of tail) with different color indelible marker pen before the start of test item administration.
Conditions	Standard Laboratory Conditions. The animal room (Room no.01) was air-conditioned with adequate air changes per hour. The animals were provided with a light cycle of 12 hours light and 12 hours dark.
Accommodation	Housed in groups of three in Polycarbonate cages (approximate internal dimensions of 365 mm x 202 mm x 180 mm height) with paddy husk bedding.
Diet	Rodent feed was provided *ad libitum*.
Water	Genpure RO water was provided *ad libitum*.

d) Test Facility

Radiant Research Services Pvt. Ltd
99/A, 8th main, 3rd phase, Peenya Industrial Area
Bangalore – 560 058

The experiments were performed in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi, India (Registration No: 1803/PO/RcBi/S/2015/CPCSEA) and the project approval number is RR/IAEC/04/1-2015.

e) Dose selection

The acute oral toxicity study showed that the plant extract of *P. fraternus* was devoid of any toxicity even at the dose of 2000 mg/kg, hence 200mg/kg and 400 mg/kg body weight doses were selected for the study.

f) Preparation of citrate buffer solution

Citrate buffer: Dissolve 1.47 gm of sodium citrate in 50 ml distilled water. Prepare the fresh buffer solution for every group of injection.

g) Preparation of streptozotocin solutions

Weigh required quantity of streptozotocin. A fresh solution of streptozotocin (50 mg/kg) was prepared in citrate buffer. Adjust the pH with monohydrate sodium citrate solution to obtain a pH of 4.5.

h) Preparation of test solutions

Suspension *P. fraternus plant extract* in sodium carboxy methyl cellulose to give a dose of 200 mg/ml and 400 mg/ml

Standard solution 10mg/ml

i) Induction of experimental diabetes

A freshly prepared solution of streptozotocin solution was injected intraperitonially to the rats. After 48 hours of streptozotocin administration, rats with moderate diabetes (i.e. with blood glucose more than 250 mg/dL) were selected for the experiment.

Diabetes was induced in the overnight fasted rats by a single intra-peritoneal injection of 60 mg/kg body weight streptozotocin dissolved in sodium citrate buffer, pH 4.5. After the injection they had free access to food and water. The animals were allowed to drink 5% glucose solution overnight to overcome hypoglycaemic shock. The development of diabetes was confirmed after 48 hours of streptozotocin injection. The animals having blood glucose level more than 200 mg/dl were considered as diabetic and were used for the experimentation.

j) Experimental procedure

In the experiment a total of 30 rats (24 diabetic surviving rats, 6 normal rats) were used. The rats were divided into five groups of 6 rats each:

Group I - Normal control rats

Group II - Diabetic control rats

Group III - Diabetic rats treated with plant extract of *P. fraternus* (200 mg/kg body weight in sodium carboxy methyl cellulose suspension administered with an intragastric tube)

Group IV - Diabetic rats treated with plant extract of *P. fraternus* (400 mg/kg body weight in sodium carboxy methyl cellulose suspension administered with an intragastric tube)

Group V - Diabetic rats given Glibenclamide (10mg/kg body weight in sodium carboxy methyl cellulose suspension administered with an intragastric tube).

Glibenclamide is often used as a standard antidiabetic drug in STZ induced diabetes to compare the efficacy of variety of hypoglycemic drugs.

All drug treatment was given for 21 days. During treatment period daily food & water intake of rats in each group was checked. After the last treatment (21stday of drug treatment) rats were fasted overnight and sacrificed by cervical decapitation. Blood was collected and serum was used for the estimation of biochemical parameters. Liver and pancreas tissues were excised immediately and stored in ice-cold containers. The tissues

were homogenized with appropriate buffer, centrifuged and the supernatant was collected. Tissue antioxidant estimations were carried out in the homogenates.

The ***biochemical parameters*** analysed included fasted blood glucose levels were estimated by glucose strips (Accu-Chek active). The biochemical parameters evaluated were serum lipid profiles, liver biomarkers such as, SGPT, SGOT and ALP using diagnostics kits.

The ***tissue antioxidant enzymes assays*** analysed included the activities of antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione (GSH) and thiobarbituric acid reactive in liver (TBARS) were assayed by standard methods.

For ***histopathological studies***, rats from control and experimental groups were perfused with 10 per cent neutral formalin solution. Pancreas was removed immediately from the rat. A portion of pancreatic tissue was dissected out and fixed out at 10% buffered neutral formal saline. After fixation, tissues were embedded in paraffin. Fixed tissues were cut at 5 µm and stained with hematoxylin and eosin (H&E). These were examined under light microscope and photomicrographs taken (Onkaramurthy *et al.,* 2013, Ahmed and Urooj, 2008, Das *et al.,* 2015 Patel and Sachdeva, 2014 and Emmanuel *et al.,* 2010).

15.3 Statistical analysis

Statistical differences between groups were assessed by analysis of variance (ANOVA) followed by Dunnett test. $P < 0.05$ was considered statistically significant. All the results were expressed as mean ± SEM.

15.4 Results and discussion

The results obtained from the in vivo antidiabetic activity evaluation of *P. fraternus* plant extract against Streptozotocin induced diabetes in rats is discussed and presented below:

a) Effect on body weight

The study results of the effects on body weight are presented in **Table -15.1** and **Figure - 15.1.**

Table -15.1: Effect of *P. fraternus* plant extract on body weight of STZ induced diabetic rats

Groups	Body weight (grams)			
	0th Day	**7th Day**	**14th Day**	**21st Day**
Group I	163.3±3.93	181.2±3.65	199.7±4.14	213.8±3.51

Group II	156.2±3.22	147±3.651	132±2.76	115.3±2.40
Group III	152.5±3.07	158.3±3.073	165.2±3.19	171.7±3.41
Group IV	155±2.23	158±2.033	165.2±2.05	167.7±2.06
Group V	155.3±2.70	165.8±3.341	176±3.04	187.2±3.04

Values are expressed as Mean ± SEM (n=6)

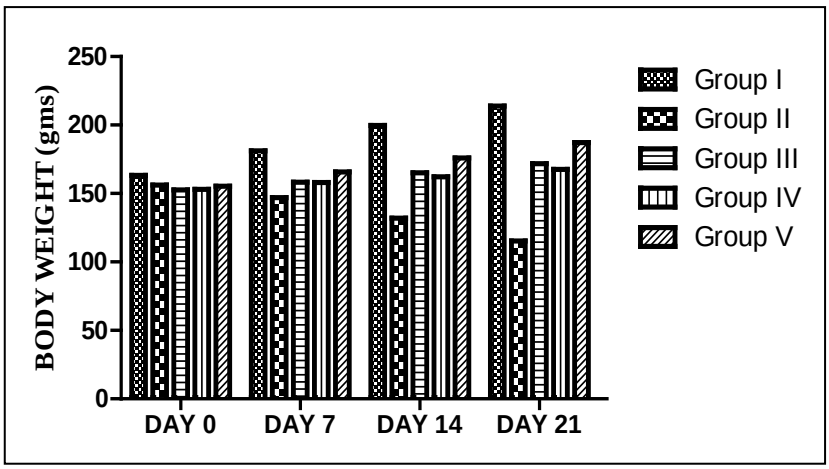

Figure -15.1: Effect of *P. fraternus* plant extract on body weight in STZ induced diabetic rats.

As is evident from the results presented in **Table 1**, the mean body weight of diabetic control animals on day 0 was 156.2 ± 3.22 g which reduced to 115.3 ± 2.40 g (- 26.%) on day 21 of induction of diabetes. This decrease in body weight in diabetic animals was statistically significantly as compared to normal control animals.

Administration of *P. fraternus* plant extract in both doses (200mg/kg & 400mg/kg) showed significant improvement in body weight compared to diabetic groups. Mean body weight of animals in test group-1 and test group-2 increased from 152.5 ± 3.07 g & 155 ± 2.23gm on 0 day to 171.7 ± 3.41 g (+ 12.5 %) and 167.7 ± 2.06 g (+ 8 %) on day 21 of induction of diabetes respectively.

Similar results of significant improvement in body weight as compared to diabetic control group of animals were obtained with administration of standard antidiabetic drug Glibenclamide (10 mg/kg), which increased body weight from 155.3 ± 2.70 g to 187.2 ± 3.04 g (+ 20%) at the end of the study.

From the results obtained, decrease in body weight gain was observed in STZ-induced diabetic rats when compared to controls. Decreased body weight observed in diabetic control rats in comparison to normal rats indicates that loss of body weight is a result of excessive breakdown of tissue proteins. However, *P. fraternus* plant extract treated diabetic rats showed improved body weight to a certain extent when compared with STZ

treated rats, indicating that control over muscle wasting resulted from glycemic control. This suggests the hypoglycemic effect of *P. fraternus* plant extract in diabetic rats.

b) Effect on blood glucose level (BGL)

The study results of the effects on blood glucose level are presented in **Table -15.2** and **Figure -15.2.**

Table -15.2 – Effect of *P. fraternus* plant extract on blood glucose level of STZ induced diabetic rats

Groups	Blood glucose (mg/dl)			
	0th Day	7th Day	14th Day	21st Day
Group I	98 ± 3.71	95 ± 2.20	110 ± 3.59	92 ± 1.61
Group II	304 ± 22.55	297 ± 20.57	305 ± 18.97	301 ± 20.49
Group III	296 ± 12.23	290 ± 12.65	261 ± 12.27	226.7 ± 6.40
Group IV	308 ± 16.12	251 ± 10.76*	232 ± 7.09*	189 ± 4.17**
Group V	338 ± 10.98	233 ± 5.06*	185 ± 2.87**	131 ± 4.04***

Values are expressed as Mean ± SEM.(n=6)

Values are expressed as Mean ± SEM.(n=6); * p<0.05, ** p<0.01, *** p<0.001.

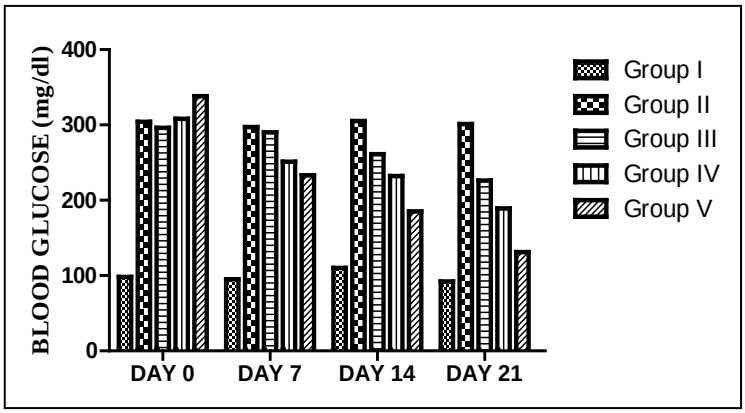

Figure -15.2: Effect of *P. fraternus* plant extract on blood glucose level in STZ induced diabetic rats

Table -15.2 shows variation in BGL from day 1 to day 21 in each group. Mean BGL in non-diabetic control animals ranged from 98 ± 3.71 mg/dl on 0 day to 92 ± 1.61 mg/dl on day 21 of the study.

In STZ-induced diabetic animals the BGL changes from 304 ± 22.55 mg/dl on 0 day to 301 ± 20.49 mg/dl (-0.98%) on day 21 of the study. With standard drug Glibenclamide (10mg/kg) the BGL reduced from 338 ± 10.98 mg/dl on 0 day to 131 ± 4.04 mg/dl (-

61%) on day 21. This difference was found to be highly significant when compared with diabetic control group.

P. fraternus plant extract in the dose of 200 mg/kg reduced BGL from 296 ± 12.23 mg/dl on day 0 to 226.7 ± 6.40 mg/dl (-23.4%) on day 21. 400mg/kg dose of test drug also significantly reduced BGL from 308±16.12 on 0 day to 189 ± 4.17 mg/dl (-38.4%) on day 21.

The blood glucose level (BGL) was higher in STZ-diabetic rats as compared to normal rats. *P. fraternus* plant extract treated groups and the Glibenclamide treated group shows significant decrease in the fasting blood glucose levels when compared with diabetic control, which was determined on the day 7 and day 14 and day 21 of the experiment. Treatment of STZ-diabetic rats with *P. fraternus* plant extract reduced the hyperglycemia when compared with STZ alone treated rats. This effect was more significant in higher dose of plant extract.

(c) Effect on antioxidant enzymes

It is well known that diabetes mellitus is associated with an increased production of reactive oxygen species and a reduction in anti-oxidative defenses. This defense includes the enzymes SOD, CAT and GSH. The diabetogenic action of STZ can be prevented by the superoxide dismutase and catalase; hence there is evidence to suggest that the incidence of diabetes involves superoxide anion and hydroxyl radicals. The enzyme SOD scavenges superoxide radicals (O2 -) by catalysing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen (Emmanuel S, Rani MS and Sreekanth M R, 2010).

The study results of the effects on antioxidant enzymes are presented in **Table -15.3** and **Figure -15.3**, **Figure -15.4**, **Figure -15.5** and **Figure -15.6**.

Table -15.3 – Effect of Test substance on tissues antioxidant enzymes and lipid peroxidation levels in STZ induced diabetic rats

Groups	LPO (TBARS) (µM/mg protein)	GSH (µM/mg protein)	CAT (µM of H_2O_2 utilized/min/mg protein)	SOD (U/mg protein)
Group I	0.12±0.002	7.07±0.06	0.35±0.01	0.38±0.028
Group II	0.39±0.02	2.90±0.07	0.21±0.01	0.14±0.032
Group III	0.34±0.03	3.30±0.05	0.24±0.004	0.17±0.007
Group IV	0.27±0.03**	3.48±0.24*	0.26±0.005**	0.28±0.066*
Group V	0.19±0.01***	5.56±0.13***	0.30±0.006***	0.36±0.026***

Values are expressed as Mean ± SEM.(n=6); * p<0.05, ** p<0.01, *** p<0.001.
LPO - lipid peroxidation; GSH-reduced glutathione; MDA-malondialdehyde; SOD-Super Oxide Dismutase CAT-Catalase

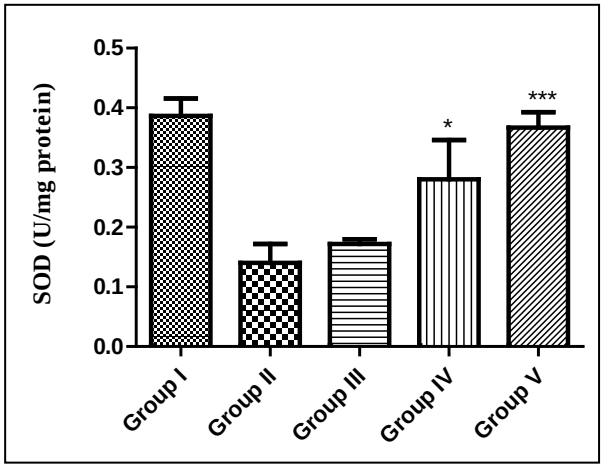

Figure -15.3: Effect of test substance on SOD level in STZ induced diabetic rats

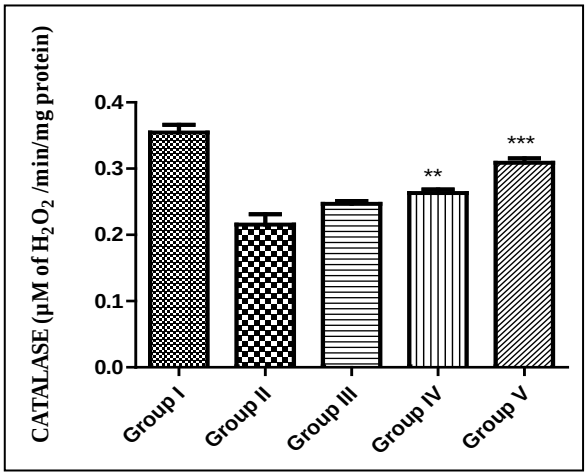

Figure -15.4: Effect of test substance on Catalase level in STZ induced diabetic rats

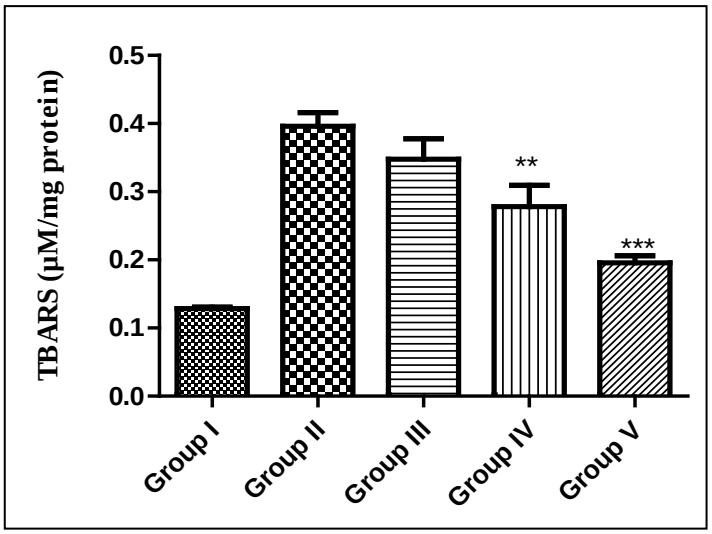

Figure -15.5: Effect of test substance on TBARS level in STZ induced diabetic rats

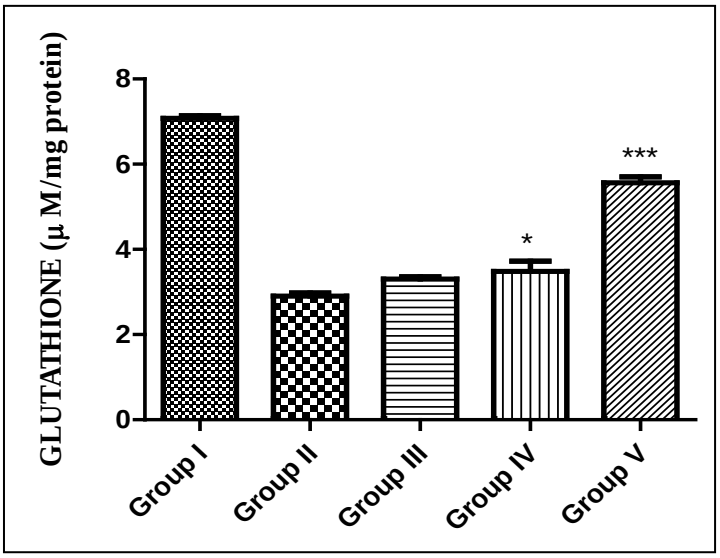

Figure -15.6: Effect of test substance on Glutathione level in STZ induced diabetic rats

Oxidative stress is considered as a major pathogenesis in diabetes-related complications. The antioxidant enzymes SOD, CAT and non-enzymatic GSH levels were determined in all the groups. In the normal, Glibenclamide (standard) and *P. fraternus* plant extract (400mg/kg) treated groups, highest antioxidant levels were found in the liver compared to the antioxidant levels were found in the liver of *P. fraternus* plant extract (200mg/kg) treated group and diabetic control rat group.

STZ induced diabetic rats were found to have decreased SOD, GSH and CAT enzyme level in liver as compared to control. Administration of *P. fraternus* plant extract (400mg/kg) to the diabetic rats resulted in significant increase in the activities of SOD

($p<0.05$) , GSH ($p<0.05$) and CAT ($p<0.01$). Glibenclamide (standard) group was able to reverse the altered peroxidative damage to near normal values.

STZ diabetic rats were found to exhibit significant increase in LPO (TBARS) level in liver as compared to control rats. Treatment with *P. fraternus* plant extract (400mg/kg) produced significant decrease ($p<0.01$) in LPO (TBARS).

(d) Effect on liver enzymes

Liver plays an important role in the maintenance of blood glucose level by regulating its metabolism. Hepatotoxicity is another risk associated with long term use of anti-diabetic agents.
The study results of the effects on liver enzymes (SGOT, SGPT and ALP) are presented in **Table -15.4** and **Figure -15.7**, **Figure -15.8** and **Figure -15.9**.

Table -15.4 – Effect of test substance on serum biochemical parameters on streptozotocin induced diabetic rats

Groups	**SGPT** (IU/ml)	**SGOT** (IU/ml)	**ALP** (IU/ml)
Group I	46.7±1.03	66.39±3.33	164.5±2.83
Group II	75.96±3.77	270.6±22.18	254.2±4.64
Group III	71.46±8.60	222.8±23.37	253.4±5.08
Group IV	58.52±1.88*	183.4±24.85*	231.4±5.16*
Group V	53.45±2.02***	139±7.36***	190.1±6.5***

Values are expressed as Mean ± SEM.(n=6); * $p<0.05$, ** $p<0.01$, *** $p<0.001$.
SGOT-Serum Glutamate Oxalo Transferase; SGPT -Serum Glutamic Pyruvic Transaminase; ALP-Alkaline phosphatase

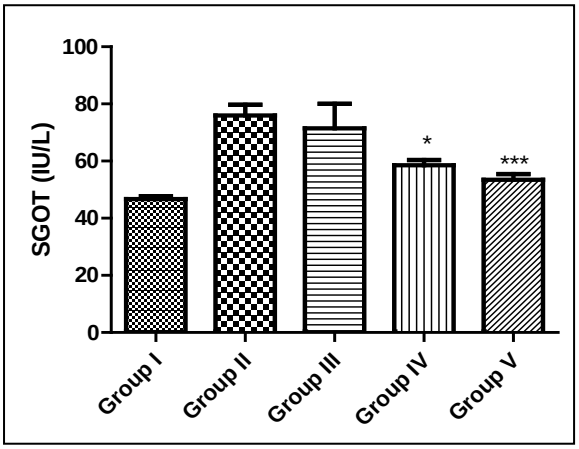

Figure -15.7: Effect of test substance on SGOT level in STZ induced diabetic rats

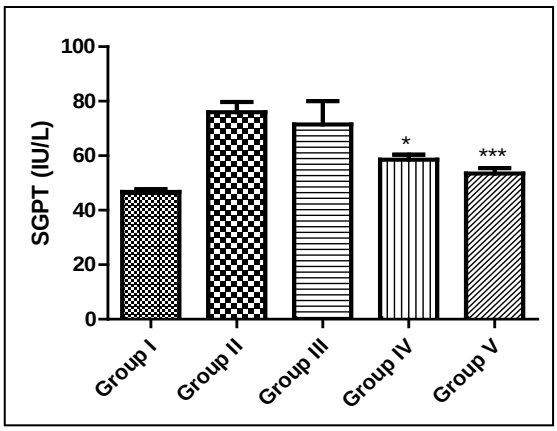

Figure -15.8: Effect of test substance on SGPT level in STZ induced diabetic rats

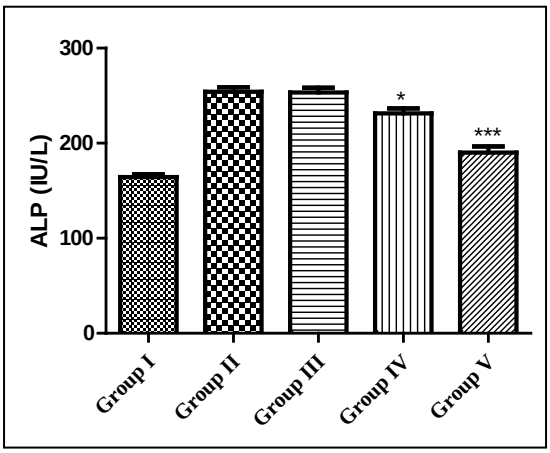

Figure -15.9: Effect of test substance on Alkaline phosphatase level in STZ induced diabetic rats

The effect is more pronounced in Glibenclamide (standard) group (10mg/kg) group, followed by *P. fraternus* plant extract (400mg/kg, 200mg/kg) group. *P. fraternus* plant extract (400mg/kg) group shown significantly lower levels of SGOT, SGPT and ALP than *P. fraternus* plant extract (200mg/kg) in comparison to the diabetic control group.

(e) Effect on pancreas by histopathology

The study results of the effects on pancreas were studied from histopathogical studies. The observations found in each group are discussed below:

Group I:

The histological observation of normal control group (non-diabetic) is presented **Figure -15.11** and **-15.12**. These figures showed normal islets of Langerhans and β cells in pancreas.

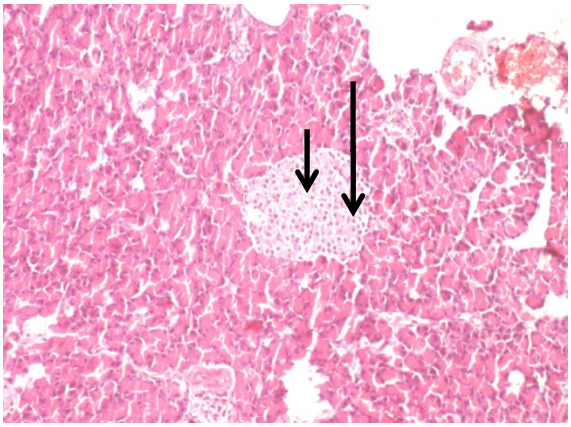

Figure -15.11: Histological observations of pancreas (H&E; 10 X) in Normal control group (presence of normal pancreatic islet cells); Beta cells – 60 %, Alpha cells 40 %

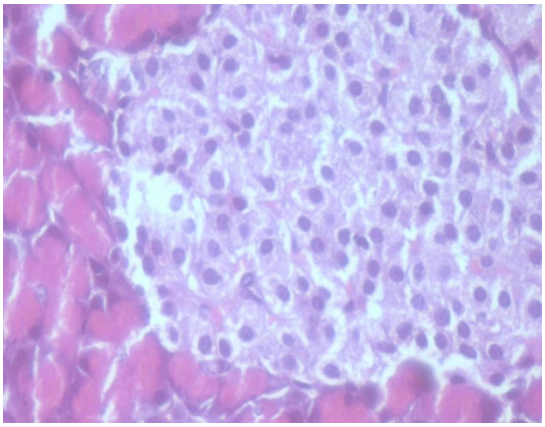

Figure -15.12: Histological observations of pancreas (H&E; 40 X) in Normal control group (presence of normal pancreatic islet cells); Beta cells – 60 %, Alpha cells 40 %

10X: 10 times magnification
40X: 40 times magnification

Section studied shows pancreatic lobules with acini separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. Adequate numbers of islets are seen. The center of islet cells consists of Beta-cells (60%, Fig. Short-Arrow), while the periphery comprises of Alpha-cells (40%, Fig. Long-Arrow).

Group II:

The histological observation of positive control group (diabetic) is presented **Figure -15.13**. This figure showed that the number of pancreatic islets as well as β-cells is reduced as compared to control group with most of β-cells being destroyed.

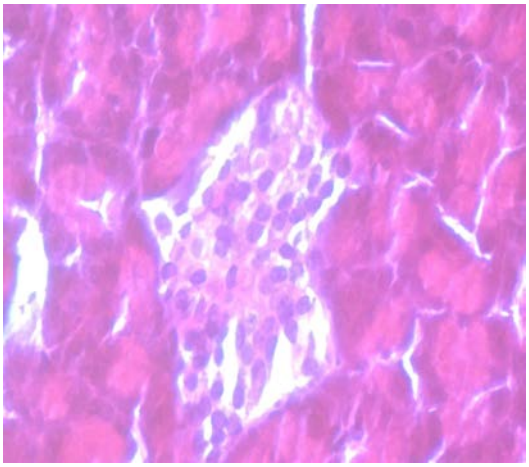

Figure -15.13: Histological observations of pancreas (H&E; 40 X) in Diabetic control group (expansion and dilated islet cells); Beta cells – 30 %, Alpha cells 65 %

Section studied shows pancreatic lobules with acini separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The numbers of islets are drastically reduced. Islet size is reduced. The beta cells which are placed in the center of the islet are reduced in number (30%). Alpha cells constitute 65% of the cells. Aggregates of inflammatory cells like lymphocytes are seen in the periphery of the pancreatic lobules.

Group III:

The histological observation of *P. fraternus* plant extract group (200mg/kg) group (diabetic + extract 200mg/kg) is presented **Figure -15.14** and **Figure -15.15**. These figure showed that the number of pancreatic islets as well as β-cells is reduced as compared to control group with most of β-cells being destroyed.

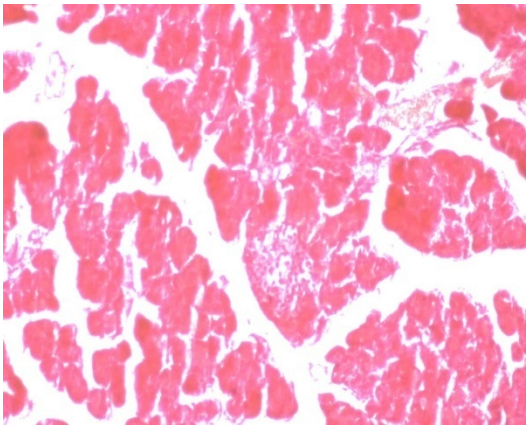

Figure -15.14: Histological observations of pancreas (H&E; 10 X) in *P. fraternus* plant extract group (200mg/kg) (expansion and dilated islet cells); Beta cells – 35 %, Alpha cells 60 %

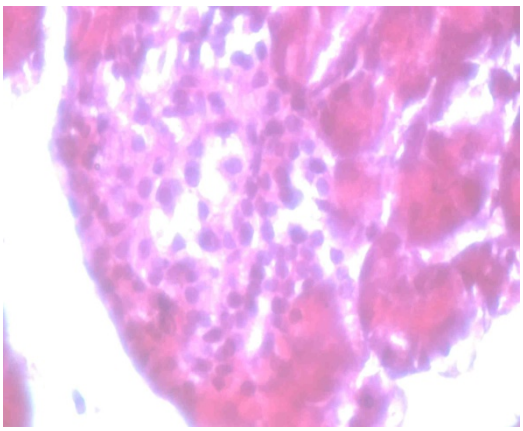

Figure -15.15: Histological observations of pancreas (H&E; 40 X) in *P. fraternus* plant extract group (200mg/kg) (expansion and dilated islet cells); Beta cells – 35 %, Alpha cells 60 %

Section studied shows pancreatic lobules with acini separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The numbers of islets are drastically reduced. Islet size is reduced. The beta cells which are placed in the center of the islet are reduced in number (35%). Alpha cells constitute 60% of the cells Hemorrhage and congestion is seen in some parts of the exocrine pancreas. Aggregates of inflammatory cells like lymphocytes are seen in the periphery of the pancreatic lobules.

Group IV:

The histological observation of *P. fraternus* plant extract group (400mg/kg) group (diabetic + extract 400mg/kg) is presented **Figure -15.16** and **Figure -15.17**. These figure showed increase in pancreatic islets & number of β-cells in the pancreas.

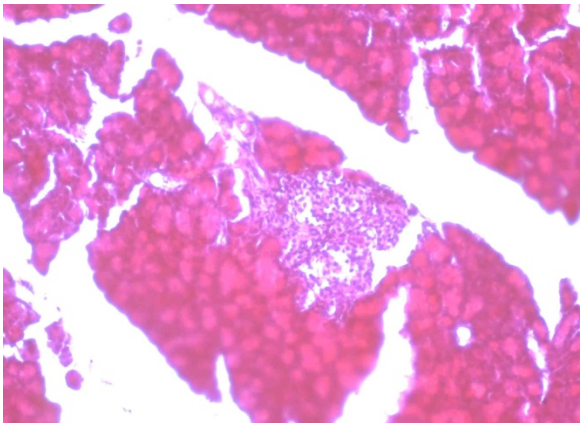

Figure -15.16: Histological observations of pancreas (H&E; 10 X) in *P. fraternus* plant extract group (400mg/kg) (absence of dilation and prominent hyperplastic of islets); Beta cells – 50 %, Alpha cells 40 %

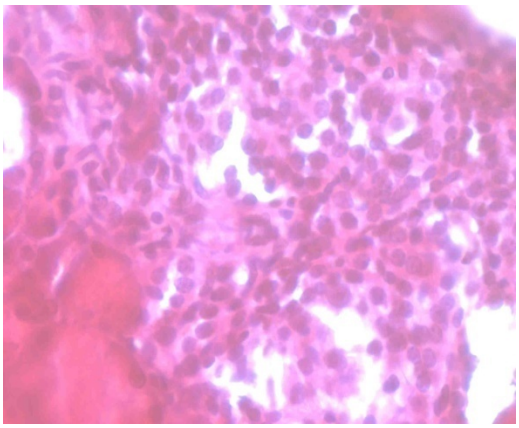

Figure -15.17: Histological observations of pancreas (H&E; 40 X) in *P. fraternus* plant extract group (400mg/kg) (absence of dilation and prominent hyperplastic of islets); Beta cells – 50 %, Alpha cells 40 %

Section studied shows pancreatic lobules with acini separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. Adequate numbers of islets are seen. The center of islet cells consists of Beta-cells (50%), while the periphery comprises of Alpha-cells (40%).

Treatment with *P. fraternus* plant extract group resulted in normalizing the pancreatic histoarchitecture quite appreciably. The damaged β-cell seen after induction of diabetes was no longer observed after treatment with extract. This indicates that the test drug causes regeneration of β-cell of islets of Langerhans of pancreas and restores normal cellular appearance and size of islet with hyperplasia. The increase in secretory granules in the cells indicates that the cells were stimulated for insulin synthesis.

Group V:

The histological observation of Glibenclamide group (10mg/kg) group (diabetic + Glibenclamide) is presented **Figure -15.18**. This figure showed increase in pancreatic islets & number of β-cells in the pancreas.

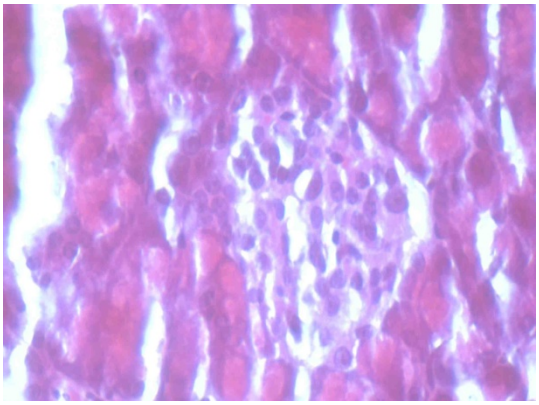

Figure -15.18: Histological observations of pancreas (H&E; 40 X) in Glibenclamide group (10mg/kg) (absence of dilation and prominent hyperplastic of islets); Beta cells – 55 %, Alpha cells 45 %

Section studied shows pancreatic lobules with acini separated by fibrovascular septa. The pancreatic lobules consist of intact acinar cells with their intralobular ducts. The numbers of islets are decreased. The center of islet cells consists of Beta-cells (55 %,), while the periphery comprises of Alpha-cells (45 %).

The damaged β-cell seen after induction of diabetes was no longer observed after treatment with Glibenclamide. β-cell of islets of Langerhans of pancreas were regenerated and normal cellular appearance and size of islet with hyperplasia was restored.

15.5 Conclusion

The findings of the present study indicates that the *Phyllanthus fraternus* plant extract (400mg/kg) possesses strong antidiabetic activity against STZ induced diabetes in Wistar rats. The results suggests that the *P. fraternus* plant extract has beneficial effect on blood glucose level and ameliorative effect on regeneration of pancreatic islets and may be used as a therapeutic agent in the management of diabetes mellitus after detail in-vivo investigation. Further preclinical research into the utility of *P. fraternus* treatment may indicate its usefulness as a potential treatment in diabetic patients.

SUMMARY

SUMMARY

Diabetes mellitus, a dreadful endocrine disorder, is one of the most common global diseases afflicting many from various walks of life in different countries. It is a serious health threat affecting millions of people worldwide. Its major cause includes either complete absence of insulin hormone due to auto-immune disorder/genetic defects/abnormal physiology (Type I) or inadequate biological response towards insulin due to down-regulation of receptors (Type II), leading to elevated blood glucose levels. Elevated blood glucose levels if not controlled may cause deleterious effects on multiple organs like kidney, heart, eyes or nerves. At a later stage, diabetic people show chronic metabolic disorder. The chronic impact of untreated diabetes significantly affects vital organs. The complexity of chronic diabetes or lack of awareness leads to sudden onset of diabetes poses a significant risk of occurrence of ketoacidosis and diabetic coma, if untreated/unnoticed respectively. The multi-organ dysfunction syndrome arises through this metabolic disorder can be mitigated/delayed by utilizing holistic approach of herbal drugs.

Allopathic medicines, or otherwise insulin in Type I diabetes, targeting insulin secretion, decreasing effect of glucagon, sensitization of receptors for enhanced glucose uptake etc and in addition, diet management, increased food fiber intake, resistant starch intake and routine exercise aids in managing diabetes mellitus. With the recent resurgence of interest in natural medicines people are turning towards use of medicinal plants and phyto-chemicals in health care. India has one of the oldest cultural traditions of use of its medicinal flora since Vedic period. Ayurveda, Unani, Siddha and other traditional systems of medicine are one of the oldest systems and utilize large number of medicinal plants.

Plants have been the major source of medicine since ancient times. Ayurveda and other Indian literature mentioned the used of plants in treatment of various ailments. Even today, in rural areas there is a lavish amount of medicinal plants. Folklore medicinal plants are mostly used in such areas. Western medicine are often limited in efficacy and

carry the risk of adverse effects. In addition they are often too costly, especially for the developing world. Though there are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to negligible side effects and low cost. Treating diabetes mellitus with more accessible plant derived compounds is a highly attractive segment.

Herbal drugs have been used since the inception of human beings on this. Various geographical locations worldwide have been using their own folklore medicinal plants for the treatment of diabetes mellitus. Use of plant derived compounds for treatment diabetes mellitus, which are easy accessible, are highly attractive option. These plant are time tested and do not require the usual laborious pharmaceutical synthesis seems.

Many herbal plants exhibit significant clinical & pharmacological activity. Researchers are exploring herbal plants and trying to decode its utility for enhancing health standards of human beings. Plants contains multiple active constituents in complex chemical mixtures developed during its growth under various environmental stresses providing a plethora of chemical families with medicinal utility.

Isolation & identification of active constituents from these plants, preparation of standardized dose & dosage regimen can play a significant role in improving the hypoglycemic action. Effective treatment of diabetes is increasingly dependent on active constituents of medicinal plants capable of controlling hyperglycemia as well as its secondary complications. In the past few years, many new bioactive drugs isolated from plants have demonstrated better antidiabetic activity than oral hypoglycemic agents.

The present thesis entitled; **"Pharmacological studies on novel anti-diabetic bioactive constituents of some ethno medicinal plants of Mizoram"** deals with screening of few common anti-diabetic ethno medicinal plants of Mizoram and the selection and identification of two potential plant(s) for detailed investigation. It also deals with pharmacognostic, phytochemical, pharmacological, antioxidant, α-glucosidase, α-amylase, glucose uptake, GLUT-4 gene expression and pharmacological evaluations.

Among the many plants ethno medicinal widely used by Mizo traditional folklore and tribal populations for the management and treatment of diabetes mellitus, the medicinal plants *M. roxburghianus and P. fraternus* were selected for the investigation to explore the potential to treat diabetes mellitus.

The pharmacognostic and phytochemical evaluation of *M. roxburghianus* and *P. fraternus* plant extracts reveals the standard parameters for the quality and purity of herbal drug and also gives information regarding the authenticity of crude drug. The studies showed presence of steroids, saponins, terpenoids, tannins, flavonoids, glycosides and sugars in *M. roxburghianus* and *P. fraternus* plant extract showed the presence of carbohydrates, glycosides, saponins, alkoloids, tannins, fixed oils and fats. Many of these compounds have been shown to produce potent hypoglycaemic, antihyperglycaemic, and glucose suppressive activities (Table 6.1 to Table 6.5). These effects might be achieved by facilitating insulin release from beta pancreatic cells, inhibiting glucose absorption in gut, stimulating glycogenesis in liver and/or increasing glucose utilization by the body.
The GC-MS analysis of phytochemical constituents of *M. roxburghianus* and *P. fraternus* plant extract were characterized. The gas chromatogram showed that the relative concentration of various compounds getting eluted at different retention times. The GC-MS analysis showed the occurrence of high percentage area of bioactive compounds in both the plant extracts. This study is a step towards understanding the nature of active principles in *M. roxburghianus* and *P. fraternus* (Table 7.1 & Table 7.2 and Figure 7.1 & Figure 7.2). GC-MS analysis of *M. roxburghianus* plant extract showed the presence of twenty nine bioactive compounds that could contribute towards the medicinal properties to the plant. Of these 29 constituents, the major chemical components and their % peak area are di-(2-ethylhexyl)phthalate (44.86 %); 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) Ester (44.86 %);1-Heptadecanamine (7.59%); 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (7.35%); 1-Hexadecanamine (4.69%); 9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)- (4.01%); 4,4a,5,6,7,7aà,8,9-Octahydro-5á-hydroxy-4aá,8à-dim ethylazuleno[6,5-b]furan-3-carboxylic Acid Methyl Ester (2.17%); Cyclopropanetetradecanoic acid, 2-octyl-, methyl ester (2.02%);

2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- (1.97%); Hexanedioic acid, dioctyl ester (1.71%); Benzamide, N,N-diethyl-3-methyl-(1.56%); Pentadecanoic acid, 14-methyl-, methyl ester (1.66%); and Methyl 8,14-Epoxy-15-hydroxy-16-nor-pimarate (1.53 %).

GC-MS analysis of *P. fraternus* plant extract showed the presence of fifty six bioactive compounds that could contribute towards the medicinal properties to the plant. Of these 56 constituents, the major components were 1,2-bis[3',4'-Dimethoxybenzyl]-1,2-bis(methoxymet hyl)ethane (66.40%); Phyllanthin 66.40%); 12,13-seco-1,12-epoxy-16-methoxy norditerpenoid Alkaloid (13.91%); 1-[(4'á)-3'- ethylenedioxy-18'-norkaur-15'-en-17'-yl]pyrrolidine (4.00%); 3á-(Peroxymethyl)-5-vinyl-A,B-bisnor-5á-cholestane (4.00%); 2-Allyloxy-1-(1,1-dimethylpropyl)-4-n-pentadecylbenzene (4.00%); 2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)-à-m ethylstilbene (2.92%); 7-diethylamino-3-heptafluoropropyl-4-methylcoumarin (2.92%); 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (1.54%); Phytol (1.43%); 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]- (1.43%); 5,10-Dihexyl-5,10-diihydroindolo[3,2-b]indole-2,7-dicarbaldehyde (1.12%); 1,5-Dimethoxy-2,4-bis(3-methylphthalidyl)benzol (1.12%); Hexadecanoic acid (1.11%); n-Hexadecanoic acid (1.11%); Lucenin 2 (0.49%); Quercetin 7,3',4'- Trimethoxy (0.28%); and psi, psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy- (0.28%).

The phyto compounds of *M. roxburghianus* and *P. fraternus* plant extract were identified with molecular formula and structure, which may be used for drug development.

A HPLC method was successfully developed for fingerprint analysis of *M. roxburghianus* and *P. fraternus* plant extract. The fingerprint obtained provides a good repeatability in separation pattern which demonstrated that the fingerprint presented is a rapid, reliable and effective method suitable for either qualitatively or quantitatively determination of the constituents present in *M. roxburghianus* and *P. fraternus* plant extract. The result of the study demonstrated confirmed the presence of the potential chemical content of Beta-sitosterol and Gallic acid in *M. roxburghianus* plant extract while the peaks of *P. fraternus* plant extract confirmed the presence of Beta-sitosterol, Gallic acid, Quercitin and Rutin (Table 8.1 to Table 8.5 and Figure 8.1 to Figure 8.15).

The i*n vitro* antioxidant activities of *M. roxburghianus* and *P. fraternus* plant extracts against DPPH, ABTS, lipid peroxidation assay, and nitric oxide. Both *M. roxburghianus*

and *P. fraternus* plant extract exhibited significant antioxidant property. The *M. roxburghianus* plant extract have shown the significant result in DPPH scavenging assay with IC_{50} 64.58 compared to *P. fraternus* plant extract which showed IC_{50} of 116.67. In nitric oxide scavenging assay, *M. roxburghianus* plant extract showed significant result with IC_{50} 208.33 compared to *P. fraternus* plant extract with an IC_{50} of 394.17. *M. roxburghianus* plant extract were found to possess significant radical scavenging and antioxidant property compared to *P. fraternus* plant extract. These observation shows that antidiabetic activity of the plant extract may also be attributed to the antioxidant property of the plant extract (Table 9.1 to Table 9.9 and Figure 9.1 to Figure 9.8).

The *in vitro* ∝ - glycosidase and *in vitro* ∝ - amylase inhibitory activity of *M. roxburghianus* and *P. fraternus* plant extracts respectively was investigated in this study. ∝ - glucosidase and ∝ - amylase inhibitors regulate postprandial hyperglycemia (PPHG) by impeding the rate of carbohydrate digestion in the small intestine and thereby hampering the diet associated acute glucose excursion. PPHG is a major risk factor for diabetic vascular complications leading to disabilities and mortality in diabetics (Shihabudeen *et al.,* 2011). *P. fraternus* and *M. roxburghianus* has been used in traditional medicine for treating diabetes. In this study we have evaluated the ∝-glucosidase inhibitory potential of *P. fraternus* and *M. roxburghianus* extract. The results of the study indicate that *P. fraternus plant* extract exhibited maximum ∝ - glucosidase and ∝ - amylase inhibitory activity. The plants may essentially contain herbal bioactive compounds inhibiting enzyme activity. One of the mechanism of action of the plant extract as anti-diabetic agent could be by inhibiting the activity of intestinal ∝ - glucosidase and ∝ - amylase (Table 10.1, Table 10.2, Table 11.1 and Table 11.2 and Figure 10.1, Figure 10.2, Figure 11.1, Figure 11.2 and Figure 11.3).

The *in vitro* effect of *M. roxburghianus* and *P. fraternus* plant extracts for their effect on glucose uptake in L6 cell line was investigated. While both *the* plant extract have *in vitro* cytotoxicity activity and an effective glucose uptake potential, *P. fraternus* plant extract shows better potential compared to *M. roxburghianus* plant extract. From the present findings, it can be concluded that *P. fraternus* plant extract shows moderate toxicity against L6 cell line. The results obtained in the present study clearly demonstrate that

the *P. fraternus* plant extract enhances glucose uptake under *in vitro* conditions. This may be due to its effect on the number of receptors located in the skeletal muscle cell line. The glucose uptake activity of *P. fraternus* plant extract may be attributed to the GLUT4 translocation (Table 12.1 & Table 12.2 and Figure 12.1 & Figure 12.2).

This was confirmed by the i*n vitro* study of the e*ffect of M. roxburghianus* and *P. fraternus* plant extracts on GLUT-4 gene expression in L-6 myotubes cell line. The CTC_{50} value of *M. roxburghianus* plant extract in L6 cell lines indicate that the maximum cytotoxic effect of *M. roxburghianus* plant extract showed 50% reduction in cell viability upon treatment with highest dose (>1000 µg/ml) while *P. fraternus* plant extract showed 50% reduction in cell viability upon treatment with highest dose (593 µg/ml). The results indicate that *P. fraternus* plant extract showed a potent activity against the *in vitro* L-6 cell line compared to *M. roxburghianus* plant extract. *P. fraternus* plant extract demonstrated the up-regulation of GLUT-4 glucose transporter genes, promoting glucose uptake, improving glucose utilization and disposal in skeletal muscles thereby confirming high potential of *P. fraternus* plant extract in the management of Type 2 diabetes mellitus. *P. fraternus* plant extract can be used to reduce insulin resistance associated with Type 2 diabetes mellitus since the plant possess good insulin sensitizing properties (Table 13.1 & Table 13.2 and Figure 13.1 to Figure 13.3).

The *in vivo* acute oral toxicity study of *M. roxburghianus* and *P. fraternus* plant extracts was investigated. This study was conducted to check the non-toxic nature of the plant extracts. The LD_{50} value of *M. roxburghianus* and *P. fraternus* in female rats after single oral treatment is above 2000 mg/kg body weight and is classified as Category 5. The plant extracts were found to be safe can be further explored for their therapeutic potential in different chronic diseases (Table 14.1 to Table 14.6 and Figure 14.1 to Figure 14.4).

In vivo anti-diabetic activity *of P. fraternus* plant extract against streptozotocin induced diabetes in rats is was investigated. The results suggests that the *P. fraternus* plant extract has beneficial effect on blood glucose level and ameliorative effect on regeneration of pancreatic islets and may be used as a therapeutic agent in the management of diabetes mellitus. The results proved that the *P. fraternus* plant extract resulted in the control over muscle wasting as a result of the glycemic control. *P. fraternus* plant extract

exhibited significant hypoglycemic effect, significant increase in the activities of SOD, GSH and CAT in STZ induced diabetic rats. significant decrease in LPO (TBARS), significantly lower levels of SGOT, SGPT and ALP (Table 15.1 to Table 15.4 and Figure 15.1 to Figure 15.18).

P. fraternus plant extract possess potent antidiabetic property as evident from these studies performed. Further elaborate studies needs to be performed to throw light on the mechanism and confirmation and isolation of active components responsible for the antidiabetic effect of the drug.

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BIODATA

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REPRINTS

Traditional Medicinal Plants and their Prospects for New Drug Development

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Abstract

Herbal drugs have been the basis of treatment of human diseases. Indigenous people derived therapeutic materials from thousands of plants; however discovering medicines or poisons remains a vital question. Ayurveda is a traditional Indian medicinal system practiced for thousands of years. Substantial research activities have been conducted in the field of pharmacognosy, chemistry, pharmacology and clinical therapeutics of ayurvedic medicinal plants. Medicinal plants play a vital role for the development of new drugs. Plants continue to provide us new chemical entities for the development of drugs against various pharmacological targets. New approaches to improve and accelerate the joint drug discovery and development process are expected to take place mainly from innovation in drug target elucidation and lead structure discovery.

Keywords: Drug discovery, Herbal medicine, Ayurveda, Natural products, Drug development

Introduction

The pharmaceutical industry plays an important role in the economic development worldwide. While to serendipitous discovery attributes to development of synthetic drugs, more and more companies are focusing on drug development from plants [1]. Natural products have been the basis of treatment of various human diseases and will remain an important source of drug development [2]. Plants have been the basis of many traditional medicine systems throughout the world for thousands of years. They continue to provide humankind with new remedies [3]. Use of herbal drugs can be dated since the existence of human civilization and has been the pillar of modern medicine [2]. Herbal drugs have beensince ages used in ayurvedic, homeopathic, naturopathic and other medicine systems for the prevention and treatment of diseases and ailments and to support healing and health [4]. Before the introduction of modern medicines, herbal remedieswere utilised entirely for the treatment of diseases [5].

There has been an increased interest in natural product research in recent times. Medicinal plants accounts for a critical role in the development of potent therapeutic agent [4,6]. The diversity of natural products and development of new novel and sensitive techniques to isolate, purify, and structurally characterize these active constituents of complex natural products forms the basis and interest of traditional medicine and therapies development and research [6]. Ancient and modern therapeutic skills and knowledge can be utilisedfor the natural products drug discovery [2].

Importance of Plants as A Source of Newdrugs

The use of traditional medicine has increased in developed countries, mainly due to the failure of modern medicine to provide effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites. The adverse effects of chemical drugs, questioning of the approaches and assumptions of allopathic medicine, their increasing costs and greater public access to information on traditional medicine has also led to an increase in interest in alternative treatments. Plant extracts have become a source of hope as a wide group of medicinal plant preparations are available that have been used over the centuries, almost exclusively basedonempirical evidence. Hence, it has become necessary to revisit the importance of herbal medicines [5].

Ayurveda remains one of the most ancient medical systems widely practiced in the Indian subcontinent and has a sound philosophical, experiential and experimental basis. CharakSamhita and SushrutSamhita (100–500 B.C.) are main Ayurvedic classics, which describe over 700 plants along with their classification, pharmacological and therapeutic properties. Numerous molecules have come out of

Ayurvedic experiential base, including *Rauwolfia* alkaloids for hypertension, psoralens for vitiligo, Holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, Mucunapruriens for Parkinson's disease, piperidines as bioavailability enhancers, baccosides for mental retention, picrosides for hepatic protection, phyllanthis as antivirals, curcumines for inflammation, withanolides and many other steroidal lactones and their glycosides as immunomodulators [7].

Terrestrial plants, especially higher plants, have a long history of use in the treatment of human diseases. Several species, likelicorice (*Glycyrrhiza glabra*), myrrh (*Commiphora* species), and poppy capsule latex (*Papaver somniferum*), were referred to by the first known written record on clay tablets from Mesopotamia in 2600 BC. Theseplants are still in use today for the treatment of various diseases as ingredients of official drugs or herbal preparations used in systems of traditional medicine [3]. Furthermore, drugs like morphine, codeine, noscapine (narcotine), and papaverine isolated from *P. somniferum,* developed as single chemical drugs, are still clinically used. Hemisuccinatecarbenoxolone sodium, a semi-synthetic derivative of glycyrrhetic acid found in licorice, areprescribed for the treatment of gastric and duodenal ulcers in various countries [8].

A significant segment of natural product, based pharmaceuticals, are Plant-derived drugs. According to the World Health Organization (WHO), plants are used as medicinal agents by almost 65% of the world's population. Around 25% of the drugs prescribed are of herbal origin example, anticholinergics (atropine), analgesics (opium alkaloids), antiparasitics (quinine), anticholinesterases (galantamine), antineoplastics (vinblastine/vincristine) [4].

Drug Discovery

Aresearch process is complicated, time-consuming, and costly and the end results are never guaranteed [9]. Drug discovery and development has a long history and dates back to the early days of human civilization.In ancientperiod,drugs were used not just for physical remedies but were also associated with religious and spiritual healing. Sages or religious leaders were often the administrators of drugs. The early drugs or folk medicines were derived from plant products, and supplemented by animal materials and minerals. These drugs were probably discovered through a combination of trial and error experimentation and observation of human and animal reactions because of ingesting such products.

In the early days, until the late 1800s, most drugs were based on herbs or extraction of ingredients from botanical sources. The synthetic drugs using chemical methods were heralded at the beginning of the 1900s, and the pharmaceutical industry was founded. Many drugs were researched and manufactured, but mostly they were used for therapeutic purposes rather than completely curing the diseases. From the early 1930s, drug discovery concentrated on screening natural products and isolating the active ingredients for treating diseases. The active ingredients are normally the synthetic version of the natural products. These synthetic versions, called new chemical entities (NCEs) have to go through many iterations and tests to ensure they are safe, potent and effective.

In the late 1970s, development of recombinant DNA products utilizing knowledge of cellular and molecular biology commenced. The biotechnology industry became a reality. The pharmaceutical industry, together with the advances in gene therapy and understanding of mechanisms of causes of diseases, and the research results from the Human Genome Project, have opened up a plethora of opportunities and made possible the development and use of drugs specifically targeting the sites where diseases are caused [10]. The key factors that necessitated change in biomedical knowledge and technology were the substantial increase in the number of therapeutic targets and discovery of high levels of complexity [11].

Literally, hundreds and sometimes thousands of chemical compounds are made and tested in an effort to finally findone compound, which can achieve a desirable result. There is no standard route for drugs development. A pharmaceutical company may decide to develop a new drug aimed at a specific disease or medical condition. Sometimes, scientists choose to pursue an interesting or promising line of research. In other cases, new findings from university, government, or other laboratories may point the way for drug companies to follow with their own research [9].

A typical herbal medicines drug development process includes differential aspects such as isolation or artificial synthesis of bioactive ingredient(s) in herbal medicines, evaluation of safety and efficacy using systems pharmacological methods, evaluation of safety and efficacy by means of conventional pharmacological methods, regulatory approval of the therapeutic agent to be used and post marketing monitoring [1]. Examples of drugs derived from plants, with their ethno medical correlations and sources are depicted in Table-1.

Table-1: Drugs derived from plants, with their ethno medicinal correlations and sources

Drug	Action or Clinical Use	Plant Source and family
Acetyldigoxin	Cardiotonic	*Digitalis lanata* Ehrh. (Plantaginaceae)
Adoniside	Cardiotonic	*Adonis vernalis* L. (Ranunculaceae)
Aescin	Anti-inflammatory	*Aesculus hippocastanum* L. (Sapindaceae)
Aesculetin	Antidysentery	*Fraxinus rhynchophylla* Hance (Oleaceae)
Agrimophol	Anthelmintic	*Agrimonia eupatori* a L. (Rosaceae)
Ajmalicine	Circulatory disorders	*Rauvolfia serpentina* (L.) Benth ex. Kurz (Apocynaceae)
Allylisothiocyanate	Rubefacient	*Brassica nigra* (L.) Koch (Brassicaceae)
Andrographolide	Bacillary dysentery	*Andrographis paniculata* Nees (Acanthaceae)
Anisodamine	Anticholinergic	*Anisodus tanguticus* (Maxim.) Pascher (Solanaceae)
Anisodine	Anticholinergic	*Anisodus tanguticus* (Maxim.) Pascher (Solanaceae)
Arecoline	Anthelmintic	*Areca catechu* L. (Arecaceae)
Asiaticoside	Vulnerary	*Centella asiatica* (L.) Urban (Apiaceae)
Atropine	Anticholinergic	*Atropa belladonna* L. (Solanaceae)
Berberine	Bacillary dysentery	*Berberis vulgaris* L. (Berberidaceae)
Bergenin	Antitussive	*Ardisia japonica* Bl. (Myrsinaceae)
Bromelain	Anti-inflammatory; proteolytic agent	*Ananas comosus* (L.) Merrill (Bromeliaceae)
Caffeine	CNS stimulant	*Camellia sinensis* (L.)Kuntze (Theaceae)
(+)-Catechin	Haemostatic	*Potentilla fragaroides* L. (Rosaceae)
Chymopapain	Proteolytic; mucolytic	*Carica papaya* L. (Caricaceae)
Cocaine	Local anaesthetic	*Erythroxylum coca* Lamk.(Erythroxylaceae.)
Codeine	Analgesic; antitussive	*Papaver somniferum* L. (Papaveraceae)
Colchicine	Antitumor agent; antigout	*Colchicum autumnale* L. (Colchicaceae)
Convallotoxin	Cardiotonic	*Convallaria majalis* L. (Asparagaceae)

Curcumin	Choleretic	*Curcuma longa* L. (Zingiberaceae)
Cynarin	Choleretic	*Cynaras colymus* L. (Asteraceae)
Danthron	Laxative	*Cassia* spp. (Fabaceae)
Deserpidine	Antihypertensive; tranqulizer	*Rauwolfia canescens* L. (Apocynaceae)
Deslanoside	Cardiotonic	*Digitalis lanata* Ehrh. (Plantaginaceae)
Digitalin	Cardiotonic	*Digitalis purpurea* L. (Plantaginaceae)
Digitoxin	Cardiotonic	*Digitalis purpurea* L. (Plantaginaceae)
Digoxin	Cardiotonic	*Digitalis lanata* Ehrh. (Plantaginaceae)
Emetine	Amoebicide; emetic	*Cephaelis ipecacuanha* (Brotero) A. Richard (Rubiaceae)
Ephedrine	Sympathomimetic	*Ephedra sinica* Stapf. (Ephedraceae)
Etoposide	Antitumour agent	*Podophyllum peltatum* L. (Berberidaceae)
Gitalin	Cardiotonic	*Digitalis purpurea* L. (Scrophulariaceae)
Glaucaroubin	Amoebicide	*Simarouba glauca* DC. (Simaroubaceae)
Glycyrrhizin	Sweetener	*Glycyrrhiza glabra* L. (Leguminosae)
Gossypol	Male contraceptive	*Gossypium* spp. (Malvaceae)
Hemsleyadin	Bacillary dysentery	*Helmsleyaa mabilis* Diels (Apocynaceae)
Hydrastine	Hemostatic; astringent	*Hydrastis canadensis* L. (Ranunculacea)
Hyoscamine	Anticholinergic	*Hyoscyamus niger* L. (Solanaceae)
Kainic Acid	Ascaricide	*Digenea simplex* (Wulf.) Agardh (Rhodomelaceae)
Kawain	Tranquilizer	*Piper methysicum* Forst. f. (Alpheidae)
Khellin	Bronchodilator	*Ammi visnaga* (L.) Lamk. (Apiaceae)
Lanatosides A, B, C	Cardiotonic	*Digitalis lanata* Ehrh. (Plantaginaceae)
Lobeline	Smoking deterrent; respiratory stimulant	*Lobelia inflata* L. (Campanulaceae)
Monocrotaline	Antitumor agent	*Crotolaria sessiliflora* L. (Fabaceae)
Morphine	Analgesic	*Papaver somniferum* L. (Papaveraceae)

Neoandrographolide	Bacillary dysentery	*Andrographis paniculata* Nees (Acanthaceae)
Noscapine	Antitussive	*Papaver somniferum* L. (Papaveraceae)
Ouabain	Cardiotonic	*Strophanthus gratus* Baill. (Apocynacea)
Papain	Proteolytic; mucolytic	*Carica papaya* L. (Caricaceae)
Phyllodulcin	Sweetener	*Hydrangea macrophylla* (Thunb.) DC (Hydrangeaceae)
Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum* Balf. (Fabaceae)
Picrotoxin	Analeptic	*Anamirta cocculus* (L.) W.&A. (Menispermaceae)
Pilocarpine	Parasympathomimetic	*Pilocarpus jaborandi* Holmes (Rutaceae)
Podophyllotoxin	Condylomataacuminata	*Podophyllum peltatum* L. (Berberidaceae)
Protoveratrines A & B	Antihypertensive	*Veratrum album* L. (Liliaceae)
Pseudoephedrine	Sympathomimetic	*Ephedra sinica* Stapf.(Ephedraceae)
Pseudoephedrine, nor-	Sympathomimetic	*Ephedra sinica* Stapf. (Ephedraceae)
Quinine	Antimalarial	*Cinchona ledgeriana* Moens ex. Trimen (Rubiaceae)
Quisqualic Acid	Anthelmintic	*Quisqualis indica* L. (Combretaceae)
Rescinnamine	Antihypertensive; tranqulizer	*Rauwolfia serpentina* (L.) Benth ex. Kurz (Apocynaceae)
Reserpine	Antihypertensive; tranqulizer	*Rauwolfia serpentina* (L.) Benth ex. Kurz (Apocynaceae)
Rhomitoxin	Antihypertensive	*Rhododendron molle* G. Don (Ericaceae)
Rorifone	Antitussive	*Rorippa indica* (L.) Hochr. (Brassicaceae)
Rotenone	Piscicide	*Lonchocarpus nicou* (Aubl.) DC. (Fabaceae)
Rotundine	Analgesic; sedative	*Stephania sinica* Diels (Menispermaceae)
Salicin	Analgesic	*Salix alba* L. (Salicaceae)
Santonin	Ascaricide	*Artemisia maritima* L. (Asteraceae)
Scillarin A	Cardiotonic	*Urginea maritima* (L.) Baker (Hyacinthaceae)
Scopolamine	Sedative	*Datura metel* L. (Solanaceae)
Sennosides A & B	Laxative	*Cassia* spp. (Leguminosae)

Silymarin	Antihepatotoxic	*Silybum marianum* (L.) Gaertn. (Asteraceae)
Stevioside	Sweetener	*Stevia rebaudiana* Bertoni (Asteraceae)
Strychnine	CNS stimulant	*Strychnosnux-vomica* L. (Loganiaceae)
Teniposide	Antitumor agent	*Podophyllum peltatum* L. (Berberidaceae)
Tetrahydropalmatine	Analgesic; sedative	*Corydalis ambigua* (Pallas) Cham.&Schltdl (Papaveraceae)
Theobromine	Diuretic; bronchodilator	*Theobroma cacao* L. (Malvaceae)
Theophylline	Diuretic; bronchodilator	*Camellia sinensis* (L.)Kuntze (Theaceae)
Trichosanthin	Abortifacient	*Thymus vulgaris* L. (Lamiaceae)
Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum* Ruiz. & Pav. (Menispermaceae)
Valepotriates	Sedative	*Valeriana officinalis* L. (Valerianaceae)
Vincamine	Cerebral stimulant	*Vinca minor* L. (Apocynaceae)
Xanthotoxin	Leukoderma; vitiligo	*Ammi majus* L. (Apiaceae
Yohimbine	Aphrodisiac	*Pausinystalia yohimbe* (K.Schum.) Pierre (Rubiaceae)
Yuanhuacine	Abortifacient	*Daphne genkwa* Sieb.&Zucc. (Picornaviridae)

Drug Development

The complex process of drug development includes product development, non clinical studies and clinical studies. A new chemical entity emerges as a candidate to undergo safety test, toxicity tests, pharmacokinetic and metabolism evaluations in in vivo pre- clinical models if it shows promising in vitro pharmacodynamic activity on particular biological targets [12]. Pure active compounds, obtained by bioassay guided isolation from extracts of medicinal plants, are subjected to structure–activity relationship studies (SAR). Toxicity and safety studies as well as clinical tests are carried out, active compounds have to be prepared on an industrial scale, and an appropriate pharmaceutical formulation has to be developed before the compound can be approved as a drug. In a "traditional" medicine system, however, pharmacological evaluation of extracts from medicinal plants may lead to the establishment of standardised extracts. In this case, the industrial production of these standardized extracts can start immediately after toxicity and safety studies. After formulation of the standardised extracts clinical tests can be carried out, which may lead to approval as drugs [13].

The isolation and natural products research in the drug development is depicted in Fig.1.

Clinical trials represent the ultimate premarket testing ground for unapproved drugs. An investigational compound is administered to humans and is evaluated for its safety and effectiveness in treating, preventing, or diagnosing a specific disease or condition. The results of this testing will comprise the single most important factor in the approval or disapproval of a new drug [9].

Challenges

In spite of the success of drug discovery programmes from plants in the past 2–3 decades, future endeavours face many challenges. Natural products scientists and pharmaceutical industries will need continuous improvement of the quality and quantity of

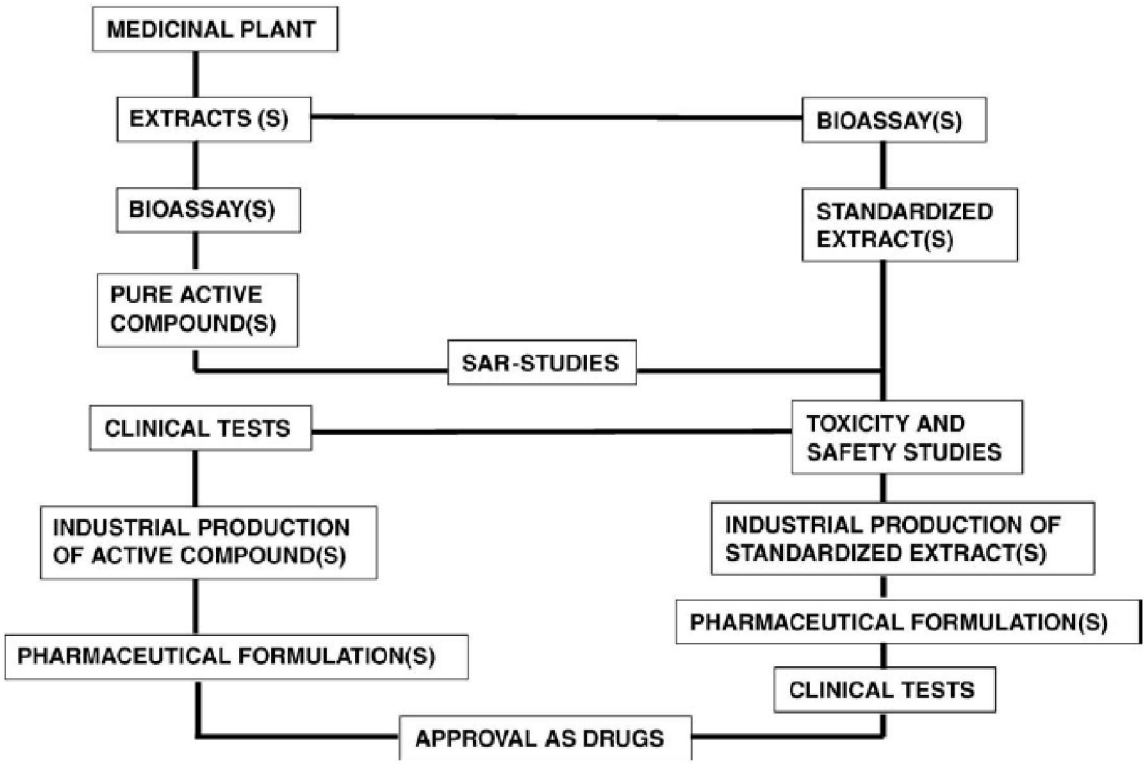

Fig.1: Flow chart for the isolation and natural products research in the drug development

compounds that enter the drug development phase to keep pace with other drug discovery efforts. The process of drug discovery takes an average period of 10 years and cost more than 800 million dollars. Much of this time and money spent on the numerous leads that are discarded during the drug discovery process [3].

Several pitfalls can emerge when deciding to use plants, through either random selection or ethno medical claims involving the targeted disease [14].

As drug discovery from plants has traditionally been time-consuming, faster and better methodologies for plant collection, bioassay screening, compound isolation and compound development needs to be employed.

Crude herbs/plants are generally formulated as tablet and capsule and to some extent as oral liquid preparations. These dosage forms are not successful due to problems encountered in absorption, therapeutic efficacy and poor compliance. Tablet or capsule dosage form requires powdering of crude herbs and particle size affects the process of blending, compression and filling. In addition, homogeneity is difficult to achieve due to the handling of large bulk quantities, high moisture content and inherent nature of raw materials (crude drug)[3].

There are advantages and disadvantages of using plants as the starting point in any drug development program. If one elects to use information suggesting that specific plants may yield useful drugs based on long-term use by humans (ethnomedicine) one can rationalize that any isolated active compounds from the plants are likely to be safer than active compounds from plants with no history of human use. Also, plants are a renewable source of starting material in many but not all cases. It is universally believed that plants provide an unlimited source of novel and complex chemical structures that most likely would never be the subject of a beginning synthetic program, e.g., vinblastine, vincristine, taxol, d-tubocurarine, digoxin. If the active principles derived from plants have novel structures and useful biologic activity, patent protection can be assured. Further, the trend today, especially in an industrial setting, is to seek bioactive compounds from plants that will serve as lead compounds for synthetic or semi synthetic development, to assure patent protection. Thus, this diminishes the need to isolate novel bioactive structures from plants, since the ultimate goal is to use the active compounds to produce synthetic derivatives with lower toxicity and higher efficacy [14].

Conclusions

Till now, natural products compounds discovered from medicinal plants (and their analogues thereof) have provided numerous clinically useful drugs. In spite of the various challenges encountered in the medicinal plant-based drug discovery, natural products isolated from plants will remain an essential component in the search for new medicines. The fact that only about one-tenth of the flowering species occurring globally are investigated for their pharmaceutical potential can be the obvious advantage to begin with plant/ medicinal plant-based drug discovery programmes.

Globally, there is a positive trend in favour of traditional and integrative health sciences both in research and in practice. There are common approaches to drug discovery including use of chemical biology, serendipity, chemical synthesis, combinatorial chemistry and genomics. However, the innovative approaches involve ethno pharmacology, reverse pharmacology, holistic, systems biology and personalized medicine.

With the tremendous increase in the global use of medicinal plants, several concerns regarding the efficacy and safety of the herbal medicines have also been raised. Hence it has become necessary to standardize the efficacy and safety measures so as to ensure supply of medicinal plant materials with good quality.

Herbal medicines and its scientific investigation can be utilized to attain a significant role in herbal drug development for improving health status and developing efficacious drug treatment of diseases.

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Botanical Antimicrobials: an Approach from Traditional to Modern System of Drug Development

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Abstract

Turmeric (*Curcuma* spp), Family- *Zingiberaceae,* has been an age-old panacea for many ills but believe it or not this humble rhizome has proved an incredible success in curing drug addicts/ ailments all over the world. In the past 20 years, modern medicine and the pharmacological industry have turned towards *Curcuma* with the hope of finding remedies for serious diseases as well as natural remedies for common maladies. Thus, there are studies on the chemical contents, structure, and composition of curcuminoids and essential oils of various species, and they are being extensively tested for their medicinal properties against the pathogenic organism. Similarly, in the present investigations, besides compiling the traditional information on *Curcuma*; antifungal evaluation with the essential oils of *Curcuma* spp (*C. amada, C. aromatica, C. caesia, C. domestica* and *C. zedoaria*), against three common pathogenic spp of *Aspergillus* (*Aspergillus flavus* Link Fr. *Aspergillus parasiticus* and *Aspergillus niger* Tiegh.) were also investigated. The efficacy of *Curcuma amada* was found to be the most effective. The minimum effective concentration (MEC) of the oil was ranges from 1.2µl/ml to 2.0µl/ml, at which it also contains heavy doses of inoculums density. The oil's toxicity was thermo stable up to 80°C and did not expire even up to 36 months of storage, the maximum unit taken into consideration. The pure oil kills the test fungi just within a minute; however, its MCC takes 5.30 to 7.00 hrs to kill all the test fungi. While, comparing the MECs of the oil with some synthetics, the oil showed an edge over the synthetic antifungal-Dactrine, Nizaral, Tenaderm. Further, the clinical trial of the oil in the form of ointment (at 1% v/v conc) to topical testing on patients, attending outpatient department (OPD) of MLN Medical College, Allahabad is still in progress.

Keywords: Ethno botanical plants, Curcuma spp, Antimycotic, *Aspergillus* spp.

Introduction

Curcuma is an important genus family Zingiberaceae. Various species have been used medicinally, as a yellow dye for cloth, and for flavoring and coloring food since time immemorial. Its generic name originated from the Arabic word *kurkum* meaning "yellow," and most likely refers to the deep yellow rhizome color of the true turmeric (*Curcuma longa* L.). Besides *C. longa,* there are several species of economic importance as medicine, such as *C. aromatica* Salisb., *C. amada* Roxb., *C. caesia* Roxb., *C. aeruginosa* Roxb., and *C. zanthorrhiza* Roxb. Others are beautiful and splendid plants of great ornamental value, such as *C. alismatifolia* Gagnep., *C. elata* Roxb., and *C. roscoeana* Wall. Locals and tribal people in most Asian countries use *Curcuma* species in religious rituals, as a foodstuff, and as medicinal plants.

Turmeric has been valued as a source of medicine and color in the whole of South Asia, from ancient times. Probably man would have been attracted to this plant due to its attractive color and in due course, it acquired many religious and socio cultural associations. For the ancient people of India, turmeric was the *"Oushadhi* — the medicinal herb," and possibly it might have played a great role in the day-to-day life of ancient Indian's as a wound healer, as a medicine for stomach ache, flatulence, poison, etc., for dyeing clothes and yarns, and for worshipping their gods and goddesses [1]. This plant has acquired great importance in the present-day world with its anti-aging, anti-cancer, anti-Alzheimer's, antioxidant, and a variety of other medicinal properties.

Keeping these views in mind, an attempt has been made to compile the traditional information on *Curcuma* spp and explore their efficacy against *Aspergillus* spp.- causing aspergillopsis.

History and Traditional Uses of Turmeric

The earliest reference about turmeric can be seen in *Atharvaveda* (Ca. 6000 yr B.C.), in which turmeric is prescribed to charm away jaundice. It was also prescribed in the treatment of leprosy. References to turmeric have also been made in the *Yajnavalkyasamhita* (composed, Ca. 4000 yr B.C.) at the time of the epic Ramayana. Turmeric was listed as a colouring plant in an Assyrian herbal dating about 2600 yr B.C. Marco Polo, in 1280 A.D., mentioned turmeric as growing in the Fokien region of China [2]. Evidences indicate that turmeric was under cultivation in India from ancient times. Garcia de Orta (1563) [3] described turmeric under the name *Crocus indicus*. Fluckiger and Hanbury (1879) [4] wrote "several varieties of turmeric, distinguished by the names of the countries or districts in which they are produced are found in the English market; although they present differences that are sufficiently appreciable to the eye of the experienced dealer, the characters of each sort are scarcely so marked or so constant as to be recognizable by mere verbal descriptions [5], while describing with the utmost details the trade in Cochin makes no mention of turmeric.

The Hindus, both tribal and civilized, consider turmeric as sacred and auspicious. It is associated with several rituals from ancient period and the tradition still goes on. In some tribal communities in Tamil Nadu, Andhra Pradesh, and in the Northeast regions of India, a piece of turmeric tied to a thread, dyed yellow with turmeric powder, is used as the nuptial string (*mangalsutra*). Even now, in the village and urban Hindu community, this practice is very much prevalent; rich people use gold chain also along with the natural yellow rhizome, but the poor depend on turmeric alone. Turmeric is also used as an amulet and a piece of turmeric tied on the hand is believed to keep away evil spirits.

Traditional uses of turmeric

As a medicine, turmeric is used in different fields of health care. In tribal medicine, it is used as single or as a compound drug for several ailments. In Ayurveda, it forms a major or important ingredient in many traditional recipes. It is used alone as a single drug remedy also. As a folk medicine or home remedy, it occupies the foremost place. Other traditional systems of medicine such as Chinese, Arabian, etc. also use turmeric as a potent drug. Some of the therapeutic uses are listed below.

1. Cold: Milk boiled with turmeric rhizome or powder, sweetened with sugar, is a popular remedy for cold and other allergic affections.
2. Conjunctivitis: A decoction of turmeric as external wash is effective in relieving pain of purulent conjunctivitis.
3. Coryza: The fumes of burning turmeric, if inhaled, cause a copious mucous discharge and relieve the congestion in coryza.
4. Scorpion sting: The smoke produced by sprinkling turmeric powder over burnt charcoal will relieve scorpion sting if the affected part is exposed to the smoke for a few minutes.
5. Vertigo: A paste of fresh turmeric rhizome, if applied on the head, is good in cases of vertigo.
6. Joint inflammations: A paste of turmeric powder along with lime is a good remedy for joint inflammations.
7. Diabetes: Half ounce (15ml) of fresh turmeric juice with equal quantity of fresh goose-berry juice *(Emblica officinalis)* in empty stomach can control diabetes. Turmeric powder, juice of gooseberry, and honey, if taken together, cure diabetes.
8. Fever: A mixture of a quarter teaspoon of turmeric powder, a small piece of ginger, and 10 black peppercorns boiled in one-and-a-half glasses of water (approx. 300ml) and concentrated to half a glass. To this, honey or sugar candy is added and given three or four times to cure fever.
9. Stomachache: Fresh turmeric juice of about 2Oz, with a pinch of salt, cures stom-achache. Turmeric powder is ground with honey to make pills weighing 10 to 12g, and, if administered one pill daily for one month, can cure frequently recurring stomach pain.
10. Flatulence: A glass of milk boiled with turmeric powder and garlic will relieve flatulence.
11. Constipation: A paste of two chebulic myrobalan *(Terminalia chebula)* and a piece of dried turmeric, daily morning and evening, is effective in chronic constipation.
12. Irregular menstruation: The juice of a large piece of fresh turmeric along with a little salt and cumin seed powder daily in the morning is effective in treating irregular menstruation.

13. Ringworm, itching, or eczema: A paste of fresh turmeric rhizome and fresh neem leaves can be applied.
14. Wounds: The fine powder of dried turmeric should be applied and bandaged to arrest bleeding in wounds.
15. Scars of chicken pox: A paste of fresh turmeric rhizome and red sandalwood *(Pterocarpus santalinus)* can be applied for scars of chicken and smallpox.
16. Cough: 1 to 2g of turmeric powder with honey or ghee.
17. Menorrhagia: Turmeric powder with guggulu (*Longifera guggle*) (powdered gum resin of *Commiphora mukul)* can be used to treat menorrhagia.
18. Purulent eye infections: Boil one part of turmeric powder in 20 parts of water. The strained liquid can be used frequently as eye drops.
19. Filariasis: Turmeric mixed with treacle in cow's urine cures elephantiasis.
20. Piles: An ointment of turmeric, hemp leaves, onion, and warm mustard oil gives great relief when the piles are painful and protruding.
21. Otorrhoea: Turmeric powder and alum powder in a proportion of 1:20, if blown into the ear, will cure chronic otorrhoea.
22. Diarrhea and dysentery: Buttermilk is boiled with turmeric powder, curry leaves, dried ginger, and some salt. It is used as a curry and for drinking to arrest diarrhea and correct digestion.
23. Skin diseases: A decoction made up of curry leaves and turmeric, taken daily, is effective in skin afflictions.
24. Allergic reactions: A pinch of turmeric powder mixed with a small quantity of fresh cow's milk early in the morning on an empty stomach is too much effective in allergy.
25. Insect bites: Turmeric powder mixed with holy basil leaf juice is applied over to the affected area to relieve pain, itching, and inflammation.
26. Inflammation and pain: Fresh turmeric rhizome and the whole plant of *Cyathula pros-trata (Apamarga)* are made into a paste and applied over sprain to relieve inflammation and pain.
27. Common cold: Turmeric powder is mixed with bruised black pepper and spread over a small piece of cotton cloth. It is then rolled to make a wick. Soak it in sesame oil and burn one end of it. The smoke that comes out is good for inhalation for relieving nasal congestion in common cold.
28. Worm infections: A paste of fresh turmeric rhizome and garlic is an effective remedy in worm infections of the foot, especially around the toes. Instead of garlic, henna leaf can be used for better results.
29. Mouth ulcers: Turmeric powder with honey, as external application, is an effective remedy for ulcers in the mouth.
30. Lice on head: Apply turmeric and neem leaves paste along with kerosene oil over the scalp and wash after half an hour using traditional herbal shampoo. A paste of turmeric and custard apple leaves is also said to have similar effect.

Ayurvedic Properties of Turmeric

According to *Ayurveda,* turmeric has the following properties:

- *Rasa* (taste) — *Thikta* (Bitter) and *Katu* (pungent)
- *Guna* (property) — *Rooksha* (irritant, to make dry, rough)
- *Veerya* (potency) — *Ushna* (hot)
- *Vipaka* (metabolic property) — *Katu* (pungent)

Turmeric is bitter in taste and its action is "pungent-like" after digestion and metabolism. Being hot, light, acrid, and irritant, it is able to reduce corpulence; stimulate all functions, and clear channels. Bhavamisra (an *Ayurvedic* scholar, the author of the ancient lexicon *Bhavaprakasa Nighantu)* denotes turmeric as a curing agent for *Kapha* (phlegmatic disorders) and *Pitta* (digestive, metabolic, and related diseases). It is very good for skin afflictions and acts as an enhancer of complexion. It is effective in all types of skin diseases, diabetes, bleeding, and other blood-related diseases, inflammations, anemia, and abscess. In *Rajanighantu* (another ancient lexicon by Nara-hari), *Haridra* (turmeric) is stated to be an effective remedy for rheumatoid arthritis and itching, in addition to the above. *Nighanturatnakara* (yet another ancient lexicon of *Ayurveda)* points out more actions such as anthelmintic property, antipoisonous effects, and curative property in catarrhal affections, anorexia

(absence of appetite), and enlargement of neck glands. Indications for the use of turmeric as a specific single drug are available in *Charaka samhita, Susruta samhita, Ashtanga sangraha,* and the lexicons of Chakradatta and Vangasena (all of which are ancient treatises of Ayurveda), for diabetes, leprosy, extreme thirst, elephantiasis, and calculus.

Therapeutic usage of Turmeric

Jager (1997) [6] compiled 114 biological properties; some important of them are- Abscess, Adenoma, Adenosis, Allergy, Alzheiner's, Amenorrhea, Anorexia, Arthrosis, Asthma, Ath-erosclerosis, Athlete's foot, *Bacillus,* Bacteria, Bite, Bleeding, Boil, Bowen's disease, Bronchosis, Bruise, Bursitis, Cancer, Cancer - abdomen, Cancer - bladder, Cancer - breast, Cancer - cervix, Cancer - colon, Cancer - duodenum, Cancer - esophagus, Cancer - joint, Cancer - liver, Cancer - mouth, Cancer - skin, Cancer - stomach, Cancer - uterus, Cardiopathy, Cataract, Catarrh, Chest ache, Child-birth, Cholecystosis, Circulosis, Cold, Colic, Coma, Congestion, Conjuctivosis, Constipation, Coryza, Cramp, Cystosis, Dermatosis, Diabetes, Diarrhea, Dropsy, Duodenosis, Dysgeusia, Dysmenorrhea, Dyspepsia, Dysuria, Eczemia, Edema, Elephantiasis, Enterosis, Epilepsy, Epistaxis, Esophagosis, Fever, Fibrosis, Fungus, Gallstone, Gastrosis, Gonorrhea, Gray hair, Headache, Hematemesis, Hematuria, Hemorroid, Hepatosis, High blood pressure, High cholesterol, High triglycerides, Hyper lipidemia, Hysteria, Immunodepression, Infection, Inflammation, Itch, Jaundice, Laryngosis, Leprosy, Leukemia, Leishmania, Leukoderma, Leukoplakia, Lymphoma, Malaria, Mania, Morning sickness, Mucososis, Mycosis, Nematode, Nephrosis, Nervousness, Ophthalmia, Osteoarthrosis, Ozena, Pain, Parasite, Polyp, Psoriasis, Puerperium, Radiation injury, Restenosis, Rheumatism, Rhinosis, Ring worm, Scabies, Small pox, Sore, Sore throat, Sprain, Stone, Staphylococcus, Stroke, Swelling, Syphilis, Trauma, Ulcer, Uveosis, Vertigo, Vomiting, Wart, Water retention, Whitelow, Worm, Wound, Yeast.

Pradhan (1999) [7] evaluated the utility of an herbal topical gel containing turmeric in mastitis control and udder health improvement. It was found to be an effective prophylactic in preventing mastitis by maintaining udder health. Curcumin inhibited human HIV-1 integrase *in vitro* and it is a modest inhibitor of HIV-1 and HIV-2 proteases. Curcumin also has anti-Epstein-Barr virus activity. Many turmeric components exert various biological effects, which are summarized in Table-1.

Table-1: Turmeric and their Biological Activities

Component(s)	Biological Activities
Curcumin	Anti-HIV., anti-EBV, antiadenoma - carcinogenic., antiaflatoxin, antiatherosclerotic, antiaggregant, antiangiogenic, antiarachidonate, anticancer, antiedemic, anti-ischemic, anti-inflammatory, antileukemic, antileukotrene, antilymphomic, antimelanomic, antimetastatic, antimutagenic, antinitrososaminic, antioxidant, antiperoxidant, antiprostaglandin, antisarcomic, metal chelator, antithromboxane, antitumor agent, antiviral, apoptotic, cox-2 inhibitor, fibrinolytic, hepatoprotective, immunostimulant, ornithine decarboxylase inhibitor, protease inhibitor, protein kinase inhibitor.
Bis-desmethoxycurcumin	Antiangiogenic, anti-inflammatory, cytotoxic, anticancer
Demethoxy curcumin	Antiangiogenic, anti-inflammatory, anticancer
Tetrahydro curcumin:	Antioxidant, anti-inflammatory
Alpha curcumene	Antioxidant, anti-inflammatory
Ar-turmerone	Anti-inflammatory, antitumour, cox-2 inhibitor, choleretic, hepatotonic
Turmerine	Antimutagenic, antioxidant, DNA protectant Anticancer, antisarcomic.
Curcumol	antitumour (cervix) Antileukopenic, antisarcomic, antitumor,
Curdione	anti-X-radiation
Dehydrocurdione	Analgesic, antiarthritic, antiedemic, anti-inflammatory, antioxidant, antipyretic, calcium channel blocker Antirhinoviral, antiulcer, carminative

***Source**: Duke (2003); Pradhan (1999); Jager (1997) & other MAPs databases.*

Duke (2003) [8] has made an exhaustive list of the known and reported uses of turmeric in the treatment of illnesses. Turmeric is indicated against a variety of health problems and pathological conditions and used traditionally by a large number of ethnic communities in a variety of conditions. Some of the properties are well documented and validated by pharmacological and clinical trials, while many remain to be validated (Table-1).

Curcuma amada Roxb: The most Potent Plant

The name of this species comes from the peculiar smell of the rhizome, which resembles that of an unripe mango. The leaves are plain green and glabrous on both sides. It is a late-flowering species; its greenish-white (or sometimes slightly tinged with rose) inflorescence invariably appears from the center of leaves around mid-October to the beginning of December. Its origin was probably in Bengal, and it is nowadays found cultivated in many parts of India. Bengali people use it for making pickles and curries. It is cultivated in Bengal and Bihar, but cultivation declined drastically due to low market prizes in recent years. Its common names (recently employed) are *amada* in Bengali, and *amadi bihari* or *ama haldi* in Hindi.

Vernacular names of *Curcuma amada*

Arabic -Daruhaldi; **Bengali** – Amada; **English**- Mango ginger; **Gujarati**- Ambahaldi; **Konkani**- Ambahaldi; **Malayalam**- Mangayincchi; **Marathi** –Ambehaldi, Naguri, Bundu sasang; **Persian**-Darchula; **Sanskrit** Ameshta, bahula, bhadra, dhirgharaja, gandaplashika, gauri, gharshani, haldi, haridra, harita, hemaragi, hemaragini, hridvilasini, jayanti, jwarantika, kanchani, kaveri, krimighna, kshamada, kshapa, lakshmi, mangalaprada, mangalya, mehagni, nisha, nishakhya, nishawa, pavitra, pinga, pinja, pita, patavaluka, pitika, rabhangavasa, ranjani, ratrimanika, shifa, shiva, shobhana, shyama, soughagouhaya, suvarna, suvarnavarna, tamasini, umavara, vauragi, varavarnini, varnadatri, varnini, vishagni, yamini, yohitapriya, yuvati; **Tamil**- Mangaincchi; **Telugu**- Mamidiallam; **Urdu**- Ambahaldi.

The Bio-efficacy

The rhizomes are considered cooling; they are used as carminative and to promote digestion. They are supposed to be useful against prurigo. Watt (1889) [9] mentions several medicinal uses for external application, including rheumatism, sprains, and bruises. The ether extract of *Curcuma amada* lowered the cholesterol level of experimentally induced hypercholesterolemic rabbits [10]. The crude extract from rhizomes of *C. amada* showed strong antifungal activity against *Trichophyton rubrum,* common cause of skin infections in West Bengal and Eastern India, and also against *Aspergillus niger* [11]. However, the antifungal efficacy of the essential oils of *Curcumma* spp against pathogens causing aspergillopsis *(Aspergillus* spp.) were investigated as follows:

In vitro efficacy of *Curcuma* spp against *Aspergills* spp.

Extraction and Isolation of Essential oil

The essential oil(s) were extracted separately from the fresh leaves of *Curcuma amada, C. aromatica, C. caesia, C. domestica* and *C. zedoaria* (Family-Zingiberaceae) by hydro distillation using Clevenger's apparatus [12]. A clear light yellow colored oily layer was obtained on the top of the aqueous distillate, later which was separated and dried over anhydrous sodium sulphate. Further, after antifungal screening (Table-2), the most effective oil (i.e. *C. amada*) was analyzed through GC-MS.

GC-MS analysis

Gas Chromatography analysis of the oil was performed on a Perkin- Elmer GC 8500, using a fused capillary column (25m×0.55mm i.d., film thickness 0.25um), coated with dimethtyl siloxane (BP-1). The oven temperature was programmed at 60°C to 220°C at 5°C/min then held isothermal at 220°C for 15min injector temperature, 250°C, detector temperature, 300°C, carrier gas, nitrogen at a linear velocity of 10 psi: split, 1:80 GC-MS data were obtained on a Shimazdu QP-2000 mass spectrometer at 70ev and 250°C. GC column: Ulbon HR-1 equivalent to OV-1, fused silica capillary column 0.25mm×50m, film thickness 0.25µm. The initial temperature was 100°C for 7min. and heated at 5°C/min to 250°C. Carrier gas was helium at a flow rate of 2ml/min. The identified compounds from the leaf oil of *Curcuma amada* are given in Table-3.

Antifungal Investigation of the Essential oil(s)

The minimum effective concentration (MEC) of the oil against the test fungi viz., *Aspergillus flavus, A. parasiticus* and *A. niger* was determined by using the technique of Shukla et al (2001) [13], with a slight modification. Two sets were maintained; one for the treatment set and another for the control. The treatment

set at different concentration of the oil was prepared by mixing the required quantity of the oil samples in acetone (2% of the total quantity of the medium) and then added in pre-sterilized potato dextrose agar medium (PDA). In control set, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. The fungi-static/ fungicidal (MSC/ MCC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes containing potato dextrose broth (Table-2).

The data recorded was the mean of triplicates, repeated twice. The percentage of fungal growth inhibition (FGI) was calculated as per formula

$$FGI(\%)=\frac{Dc-Dt}{Dc}\times 100$$

Where,

Dc- indicates colony diameter in control set, &

Dt- indicates colony diameter in treatment set.

Effect of Inoculums Density

The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was also determined using the method of Shukla et al (2001) [13]. Mycelial discs of 5mm diam of 7-day oil cultures were inoculated in culture tubes containing oil at their respective MCCs. In controls, sterilized water were used in place of the oil and run simultaneously. The numbers of mycelial discs in the treatment as well as control sets were increased progressively up to 25 discs, in multiply of five. Observations were recorded up to seventh day of incubation. Absence of mycelial growth in treatment sets up to 7th day exhibited the oil potential against heavy doses of inoculums (Table-2&4).

Effect of some Physical Factors

Effect of some physical factors viz., temperature (40, 60 and 80^{0}C respectively) and autoclaving (up to 15lb/sq inch pressure for 30min) on efficacy of the oil, at minimum cidal concentration, was also determined. It was determined following the method of Shukla *et al.,* (2001) [13]. Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^{0}C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table-4).

Fungi-toxic Spectrum

The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0µl/ml and 4.0µl/ml respectively) was determined against some common pathogenic fungi viz., *Alternaria alternata* (Fr)Keissler, *Cladosporium cladosporioides* (Fresenius) de Vries, *Curvularia lunata* (Wakker) Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F. udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx; by using the poisoned food technique of Grover and Moore (1962) [14] with slight modification of Shukla *et al.*,(2001) [13] (Table-5).

Minimum Killing Time

The MKT of the pure oil and their respective MCCs of *C. amada* against the test fungi was determined by using the method of Shukla *et.al.,* (2001) [13] (Table-6).

Comparison with some Synthetic Antifungals

The comparative efficacy of the oil of *C. amada* with the synthetic antifungal-Dactrine, Nizaral, Tenaderm was carried out by comparing their MECs [13&15] (Table-7).

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance (P<0.05) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

Results and Discussion

Plants are the basis of life on earth and are central to people's livelihoods. Tribal people are the ecosystem people who live in harmony with the nature and maintain a close link between man and environment. Indian subcontinent is being inhabited by over 53.8 million tribal people in 5000 forest dominated villages of tribal community and comprising 15% of the total geographical area of Indian landmasses, representing one of the greatest emporia of ethno-botanical wealth. The North-eastern states of India that comprises of eight sister states viz. Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura harbors more than 130 major tribal communities of the total 427 tribal communities found in India (2001 census).

Table-2: Minimum Effective Concentration (MEC) of Curcuma spp against test fungi

Curcuma spp	Tested Pathogenic Fungi		
	Aspergillus flavus	*Aspergillus parasiticus*	*Aspergillus niger*
Curcuma amada	2.0 µl/ml	2.2 µl/ml	1.8 µl/ml
C. aromatica	2.8 µl/ml	2.6 µl/ml	2.6 µl/ml
C. caesia	2.8 µl/ml	3.0 µl/ml	2.8 µl/ml
C. domestica	2.6 µl/ml	2.8 µl/ml	2.6 µl/ml
C. zedoaria	3.0 µl/ml	3.0 µl/ml	2.8 µl/ml

Table -3: Chemical composition of essential oil from the leaves of *Curcuma amada* Roxb.

S.No.	Isolated Compounds
1.	Bis-demethoxycurcumin
2.	Curcumin
3.	Demethoxycurcumin
4.	Calarene
5.	β-Caryophyllene
6.	1,8-Cineole
7.	α-Copaene
8.	β-Curcumene
9.	Curzerenone
10.	α-Humulene
11.	Limonene
12.	Myrcene
13.	β-Ocimene
14.	Perillene
15.	α-Pinene
16.	β−Pinene
17.	Terpinen-4-ol
18.	ar-Turmerone

Table-4: Detailed in vitro activity of the oil of Curcuma amada against the test fungi

Properties Studied	Tested Pathogenic Fungi		
	A. flavus	*A. parasiticus*	*A. niger*
MEC	2.0 µl/ml	2.2 µl/ml	1.8 µl/ml
MCC	3.0 µl/ml	3.0 µl/ml	2.0 µl/ml
Inoculum Density at MCC (25discs, 5mm each)	No growth	No growth	No growth
Thermostability at MCC (up to 80°C)	No growth	No growth	No growth
Effect of Autoclaving (15 lb/sq inch pressure for 30 min)	No growth	No growth	No growth

MEC = Minimum Effective Concentration; MCC = Minimum Cidal Concentration

Table-5*: Fungitoxic spectrum of the oil of* Curcuma amada

Fungi Tested	**Lethal Conc** (2.0 µl/ml)	**Hyper Lethal Conc** (4.0 µl/ml
Alternaria alternate(Fr)Keissler	100[c]	100[c]
Cladosporium cladosporioides (Fresenius) de Vries	100[c]	100[c]
Curvularia lunata (Wakker) Boedijin	100[c]	100[c]
Colletotrichum capsici (Syd.) Butler & Bisby	100[c]	100[c]
C. falcatum Went	100[c]	100[c]
Fusarium oxysporum Schlecht	100[c]	100[c]
F. udum de vries	100[c]	100[c]
Helminthosporium maydis Nisikado & Miyakel	100[c]	100[c]
H. oryzae Breda de Haan,	100[c]	100[c]
Penicillium implicatum Biourge	100[s]	100[c]
P. minio-luteum Dierckx	100[c]	100[c]

[s] indicates static; [c] indicates cidal in nature

Table-6: Minimum Killing Time of the oil of *Curcuma amada* against test fungi

Mycelial Growth Inhibition (%)						
Minimum Killing Time (MKT)	**Tested Pathogenic Fungi**					
	A. flavus		***A. parasiticus***		***A. niger***	
	P.O.	MFC	P.O.	MFC	P.O.	MFC
7.0 hrs	100	100	100	100	100	100
6.30	100	80	100	100	100	100
6.00	100	----	100	80	100	100
5.30	100	---	100	-----	100	100
5.00	100	----	100	-----	100	80
2.30	100	----	100	-----	100	-----
2.00	100		100		100	
1.30	100		100		100	
1.00	100		100		100	
30 min	100		100		100	
15	100		100		100	
05	100		100		100	
60 sec	100		80		100	
30	60		60		100	
20	----		---		80	
10	-----	---	----	---	40	---

**P.O. indicates Pure Oil; MFC indicates Minimum Fungicidal Concentration*

The state of Mizoram receives little attention as far as the survey of medicinal plants is concerned. Like other tribes, the tribes in Mizoram {viz., Mizo (Lusei), Maras (Lakher), Lai (Pawi), Chakama, Bru (Riang), Pand, Bawn and Magh (Mog/ Rakhai)} practice traditional herbal medicines, the local knowledge of which has been descending through generations since time immemorial [16]. Ethno medicinal plants are still widely used for curing different disease both in urban and rural areas. There is a need for documentation of such valuable indigenous knowledge and domestication of economically important medicinal plants to decrease pressure over natural resources and to fulfil the requirements of not only the normal and local needs but also up to the global level, hence proposed in the present study.

Table-7: Comparative efficacy of the oil of *Curcuma amada* with some Synthetic antifungals

Oil & Trade Name of Synthetic Antifungals	Active Ingredients	Characteristics features	**Tested Pathogenic Fungi (MECs) µl/ml**		
			A. flavus	*A. parasiticus*	*A. niger*
Curcuma amada	Essential oil	Renewable, biodegradable, ecofriendly.	2.0	2.2	1.8
Dactrine	Miconazole nitrate	Non-renewable, non-biodegradable and hazardous effects	6.0	6.0	6.0
Nizaral	Ketoconazole	Non-renewable, non-biodegradable and hazardous effects	6.0	5.5	5.0
Tenaderm	Tolnaftate	Non-renewable, non-biodegradable and hazardous effects	2.5	3.0	2.0

Further, on comparing the minimum effective concentration (MEC) of the oil of *Curcuma amada, C. aromatica, C. caesia, C. domestica* and *C. Zedoaria*; the MEC of of *C. amada* was found to be most effective against all the test fungi (Table-2). The MEC of of *C. amada* was found to be 2.0µl/ml against *Aspergillus flavus,* 2.2µl/ml against *A. parasiticus* and 1.8µl/ml against *A. niger* but, it was fungicidal at 3.0µl/ml against *A. flavus & A. parasiticus,* and 2.0µl/ml against *A. niger*, respectively (Table- 4).

The efficacy of the oil contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), and it still persisted even up to 80˚C as well as after autoclaving (15lbs/sq inch pressure for 30min), the maximum units taken into consideration. Furthermore, the fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0µl/ml and 4.0µl/ml respectively), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table-5).

The pure oil kills the test fungi just within a minute; however, its MCC ranges 5.30 to 7.00hrs to kill all the fungi [13] (Table-6). On comparing the MECs of the oil with some synthetic antifungals, the MECs of the oil showed an edge over the synthetic antifungals-Dactrine, Nizaral, Tenaderm (Table-7).

Conclusions

The traditional knowledge of tribal is valuable not merely as an indicator of plant to be screened but also equally important to evolve new methodological approach and strategies towards different 'bio-prospects' research. The information presented can be useful for further researches on traditional medicinal plants and their therapeutic aspects.

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HOMALOMENA AROMATICA: AN ETHNOMEDICINAL PLANT, CAN BE A POTENTIAL SOURCE OF ANTIMICROBIAL DRUG DEVELOPMENT

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ABSTRACT

The genera *Homalomena* belongs to the family Araceae of the group Monocotyledon. There are about 140 spices in tropical Asia and South America; two species in India; one in Mizoram, i.e., *Homalomena aromatica* Schott. The plant is very popular among the Mizo-tribal communities. The boiled petiole is used as vegetable, rhizome as aromatic stimulant, powdered rhizome as gun-powder, burnt smoke of rhizome as mosquito repellent and infusion of the plant for easy labor. The juice of whole plant is used as lotion in skin diseases. Besides these, the plant contains strong antimicrobial activity. The minimum cidal concentration (MCC) of the oil against some common human pathogenic fungi was found to be 1.2 to 1.8 µl/ml, which contains heavy inoculums density. The oils toxicity persists up to 80^{o}C and also autoclavable, with a broad fungi toxic spectrum. The pure oil kills the test pathogenic fungi just within a minute; however, its MCC takes 5.30 to 6.30 hrs to kill all the test fungi. Besides this, while comparing the MECs of the oil with some synthetic antifungal drugs, the toxicity of the oil shows an edge over the synthetics- Dactrine, Nizaral, Tenaderm and Batrafine. Relationship of the dermatophytes to the toxicity of the oil vis-a vis phylogeny using molecular data of the pathogens have also been discussed. Further, during pre-clinical investigations, the efficacy of oil contains 60-80% cure of the skin diseases. Based on these findings as well as after detailed *in vitro, in vivo*, clinical as well as multi-central clinical investigations; formulations can be transferred to the pharmaceutical companies.

Keywords: Ethno medicinal plant(s), pathogenic fungi, essential oil, antimicrobial activity.

INTRODUCTION

In India, out of two species of *Homalomena;* the one *Homalomena pendula* (Bl.) Hakh. f. syn. *H. rubescens* (Roxb.) Kunth have been reported to occur in Sikkim and Meghalaya [1]; and the other *Homalomena aromatica* Schott is very frequent to occur in tropical evergreen forests (Kanhmun – Bairabi ranges) of Mizoram, especially in Kolasib, Kawrthah and Mamit forest divisions [2]. Besides this, *H. aromatica* is also reported from Assam and Chittagong hill tracks of Bangladesh [3-7]. Ethno botanical studies, with an aim in search for new drugs, food and other economic plants, have gained worldwide interest in recent years. The conservation and sustainable utilization of the bio-

resources are the main foci of the ethno botanical studies. Keeping these views in mind, in the present investigation, an attempt have been made to explore various scientific investigations of *Homalomena aromatica* - a potential ethno medicinal plant of Mizoram; so that, it can not only become an effective source of herbal medicine for pharmaceutical companies but also by contributing to the preservation and enrichment of the gene bank of such economically important species before they are lost forever.

METHODOLOGY

Ethno botanical investigations

Ethno botanical survey

One of the authors has made frequent field visits in different seasons, during the year 1995-2000 in Kawrthah and Kolasib forest divisions of Mizoram [2]. The local knowledgeable persons of Zamuang, Zawlnuam and Kanhmun villages in Kawrthah forest division, and Kolasib, Medium and Bairabi villages in Kolasib Forest Division were taken to the forests to collect 'Anchiri' plant. The collected plant materials were given necessary treatment in the herbarium of the Environment & Forest Department, Aizawl (Mizoram), and the voucher specimens were deposited in the said herbarium. A few living plants were also planted near Forest Training School, Aizawl as germplasm conversion. Further, their identification was also confirmed with the experts available at the Botanical Survey of India, Allahabad; before the current investigations.

Botanical description

H. aromatica is a perennial, aromatic and rhizomatous herb. The rhizome is covered with dark-brown leafy scales. The mature rhizome is sometimes curved and running over the ground. Numerous white roots were borne on every part of the rhizome. The stem is short and slow growing. The leaves are radical, lucid, 26-27 cm x 10-12 cm, acuminate; base cordate or sagittate; lobes rounded; petiole up to 100 cm long; spadix sub-cylindric; spathe pale green-yellow, erect or pendant; flower arising form the axils and centre of the leaves on the green scape; berries white, oblong, 1-seeded.

Flowering / Fruiting time : June – August.

Habitat

Anchiri is usually found in moist tropical evergreen forests. They grow on humus sandy-loam soil and / or brown loamy-clay soil in moist shady places on river banks and valleys in association with other plants like ferns, *Hedychium* sp. etc. as an undergrowth and shade demander. Usually, they are distributed in moist and warm climates below 1000 m asl.

Status

Locally, *Homalomena aromatica* is a conservation dependant (cd) in the lower risk of the IUCN category [2].

Threats

The greatest threat to 'Anchiri' plant is surface clearance of forests for slash-and burn method of agriculture. The unsustainable harvest and massive collection of the rhizomes for trade is equally dangerous to the local extinction and over exploitation of the genetic resources. Hunting for food and medicine also affect the degree of population reduction.

Conservation measures

The plant being a shade demander can be best conserved in natural habitats, such as protected forests and riverine reserved forests where they grow. Translocation of scattered growing stocks in suitable places through re-introduction programmes by the involvement of local people would be fruitful.

Uses

The rhizome is aromatic and stimulant and the powdered rhizome is used in the preparation of snuff and tobacco [3-5]. The essential oil and the whole plant parts are being used in skin diseases [5-6]. The essential oil (0.5 %) yielded on hydro/ steam distillation is used in perfumery ingredients [5]. The aroma of burnt smoke of dried rhizome is inhaled against influenza and the roots are used for jaundice [7]. The ethno-medical uses included powdered rhizome as gun-powder, boiled petiole as vegetable, burnt smoke of rhizome as mosquito repellant and infusion of the plant for easy labour.

In vitro Investigations

Extraction and Isolation of Essential oil

The essential oil was extracted from the rhizome of *H. aromatica* (1 Kg.) by hydro distillation using Clevenger's apparatus [8]. A clear light yellow colored oily layer was obtained on the top of the aqueous distillate, later which was separated and dried over anhydrous sodium sulphate. The oil thus obtained was subjected for GC-MS analysis as well as for various fungi-toxic investigations.

Antimicrobial Investigation of the Essential oil

The minimum effective concentration (MEC) of the oil against some human pathogenic fungi (*Epidermophyton floccosum* Hartz, *Microsporum gypseum* (Bodin) Guiart et Grigorakis and *Trichophyton rubrum* Castellani) was determined by using the technique of Shahi et al., [9]. Two sets were maintained; one for the treatment set and another for the control. The treatment set at different concentration of the oil was prepared by mixing the required quantity of the oil samples in acetone (2% of the total quantity of the

medium) and then added in pre-sterilized potato dextrose agar medium. In control set, sterilized water (in place of the oil) and acetone were used in the medium in appropriate amount. The fungi-static/ fungicidal (MSC/ MCC) action of the oil was tested by aseptically re-inoculating the fungi in culture tubes containing potato dextrose broth [10] (Table-1).

The data recorded was the mean of triplicates, repeated twice. The percentage of fungal growth inhibition (FGI) was calculated as per formula:

$$FGI(\%) = \frac{Dc - Dt}{Dc} \times 100$$

Where,

Dc indicates colony diameter in control set, &
Dt indicates colony diameter in treatment set.

Effect of Inoculums Density

The effect of inoculums density on the minimum cidal concentration (MCCs) of the oil against the test fungi was also determined [11]. Mycelial discs of 5mm diam of 7-day old cultures were inoculated in culture tubes containing oil at their respective MCCs. In controls, sterilized water were used in place of the oil and run simultaneously. The numbers of mycelial discs in the treatment as well as control sets were increased progressively up to 25 discs, in multiply of five. Observations were recorded up to seventh day of incubation. Absence of mycelial growth in treatment sets up to 7^{th} day exhibited the oil potential against heavy doses of inoculums (Table-1).

Effect of some Physical Factors

Effect of some physical factors viz., temperature (40, 60 and 80^0C respectively) and autoclaving on efficacy of the oil, at minimum cidal concentration, was also determined [12]. Samples of oil in small vials, each contains 1ml, were exposed at 40, 60 and 80^0C in hot water bath, respectively. Further, the oil's efficacy was tested against the test fungi at their respective MCCs (Table-1).

Fungi-toxic Spectrum

The fungi-toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 2.0 µl/ml and 4.0 µl/ml respectively) was determined against some common human pathogenic fungi viz., *Microsporum auddouinii*, *M. canis*, *M. nanum*, *Trichophyton mentagrophytes*, *T. tonsurans* and *T. violaceum* [9 & 13] (Table-3).

Besides these, it was also determined against some common plant pathogenic fungi viz., *Aspergillus parasiticus* Speare, *Cladosporium cladosporioides* Boedijin, *Colletotrichum capsici* (Syd.) Butler & Bisby, *C. falcatum* Went, *Fusarium oxysporum* Schlecht, *F.* (Fresenius) de Vries, *Curvularia lunata* (Wakker) *udum* de vries, *Helminthosporium maydis* Nisikado & Miyakel, *H. oryzae* Breda de Haan, *Penicillium implicatum* Biourge and *P. minio-luteum* Dierckx [12], (Table-3).

Minimum Killing Time

The MKT of the pure oil and their respective MCCs of *H. aromatica* against the test fungi was determined by using the method of Shahi et.al. [9], (Table-3).

Comparison with some Synthetic Fungicides / Drugs

The comparative efficacy of the oil of *H. aromatica* with some synthetic antifungal fungicides/ drugs was carried out by comparing MECs [9] (Table-4 and 5).

All the experiments were repeated twice and each contained three replicates; the data presented in the tables are the mean values.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance ($P \leq 0.05$) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

Phylogenetic study of dermatophytes

To find out the reason why the *H. aromatica* is more effective against certain pathogenic fungi, phylogenetic relationship of the dermatophytes were studied including the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* and identified the species using the base pair sequences of ITS1 [14] (Fig. 1). The ITS1 sequences of the standard strains used in this study and of members of the *Trichophyton* spp complex (*Trichophyton mentagrophytes* isolates from humans, accession no. AB011463; *T. rubrum*, accession no. AB011453; *T. violaceum*, accession no. AB017174; *Microsporum gypsem*, accession no. AB017177 and *Epidermatophyton floccosum*, accession no. AB017181), as reported by Makimura *et al*. [15] were aligned by using the Clustal W computer program [16] and GENETYX-MAC 10.1 software (Software Development Co., Ltd., Tokyo, Japan). Phylogenetic trees were then constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package), version 3.5c [17], and the neighbor-joining (NJ) [18] method in the NJPLOT program [19]. Bootstrap [17] analysis with the Clustal W program was performed by taking 1,000 random samples from the multiple alignments.

RESULTS

The GC-MS analysis of the essential oil of *H. aromatica* shows 'linalool (32.45%), followed by lerpinen-4-ol (14.10%) as major active constituents (Fig-1).

The minimum effective concentration (MEC) of the oil was found to be 1.6 µl/ml, 1.2 µl/ml and 1.4 µl/ml conc against the test fungi *Epidermophyton floccosum, Microsporum gypseum* and *Trichophyton rubrum* respectively; however it was cidal at 1.8 µl/ml against *E. floccosum* and 1.6 µl/ml against *M. gypseum* and *T. rubrum* (Table-1).

The efficacy of the oil contains heavy doses of inoculums (i.e. up to 25 discs, each of 5mm), and it still persisted even up to 80^0 C as well as after autoclaving, the maximum units taken into consideration. Furthermore, the fungi toxic spectrum of the oil at lethal and hyper lethal concentration (i.e. 1.8 µl/ml and 3.6 µl/ml respectively), against some common pathogenic fungi reveals that the oil contains a broad fungicidal spectrum (Table- 3).

The pure oil kills the tested human pathogenic fungi, pure oil kills the tested fungi just within a minute; but, its MCC takes 6.30 to 5.30 hrs to kill all the test fungi (Table- 2). On comparing the MECs of the oil with some synthetic antifungal drugs, the MECs of the oil's toxicity showed edges than the tested synthetic fungicides- Dactrine, Nizaral, Tenaderm and Batrafine (Table- 4).

The phylogenetic relationship of dermatophytic genera *Trichophyton*, *Microsporum*, and *Epidermophyton* was determined on the basis of their ITS1 sequences. The NJ tree was constructed with data for standard strains of dermatophytes [14] demonstrated by using internal transcribed spacer 1 (ITS1) region ribosomal DNA sequences. *Trichophyton* spp. and *Microsporum* spp. form a cluster in the phylogenetic tree with *Epidermophyton floccosum* as an out-group, and within this cluster, all *Trichophyton* spp. except *Trichophyton terrestre* form a nested cluster (100 % bootstrap support). Dermatophytes in the cluster of *Trichophyton* spp. were classified into three groups with ITS1 homologies, each being a monophyletic cluster (100 % bootstrap support). The ITS1 sequences of 6 clinical isolates (Fig- 2) were also determined to identify the species. All strains were successfully identified by comparison of their base sequences with those in the ITS1 DNA sequence database [14]. NJ tree of dermatophytes is shown in Fig- 3.

The relationship of the toxicity of the essential oil vis-à-vis phylogeny was analyzed using molecular data. The effectiveness of the oil was equal in dermatophytes that are close in phylogenetic tree (Fig-3).

Table 1. Antimicrobial activity of the oil of *Homalomena aromatica* against pathogenic fungi

Properties Studied	Human Pathogenic Fungi		
	Epidermophyton floccosum	***Microsporum gypseum***	***Trichophyton rubrum***
MEC	1.6 µl/ml	1.2 µl/ml	1.4 µl/ml
MCC	1.8 µl/ml	1.6 µl/ml	1.6 µl/ml
Inoculum Density at MCC (25discs, 5mm each)	No growth	No growth	No growth
Thermostability at MCC (up to 80^0C)	No growth	No growth	No growth
Effect of Autoclaving (120lbs for 30 min)	No growth	No growth	No growth

MEC = Minimum Effective Concentration; MCC = Minimum Cidal Concentration

Table 2. Minimum killing time of the oil of *H. aromatica* against test fungi

Minimum Killing Time (MKT)	Mycelial Growth Inhibition (%)					
	Epidermophyton floccosum		***Microsporum gypseum***		***Trichophyton rubrum***	
	P.O.	MCC	P.O.	MCC	P.O.	MCC
7.00	100	100	100	100	100	100
6.30	100	100	100	100	100	100
6.00	100	60	100	80	100	100
5.30	100	---	100	---	100	100
5.00	100		100		100	80
2.30	100		100		100	---
2.00	100		100		100	
1.30	100		100		100	
1.00	100		100		100	
30 min	100		100		100	
15 min	100		100		100	
5 min	100		100		100	
60 sec	100		100		100	

30 sec	100		100		100	
20 sec	90		80		100	
10 sec	60	---	70	---	88	---

*P.O. indicates Pure Oil; MCC indicates Minimum Cidal Concentration.

Table 3. Fungi toxic spectrum of the oil of *H. aromatica* against some pathogenic fungi

Fungi Tested	Lethal Concentration (1.8 µl/ml)	Hyper Lethal Concentration (3.6 µl/ml
Human Pathogens		
Microsporum auddouinii (E. Bodin) Guiart & Grigoraki		
M. canis Bodin ex Guég.	100[s]	100[c]
M. nanum C.A. Fuentes	100[c]	100[c]
Trichophyton mentagrophytes Priestley	100[c]	100[c]
T. tonsurans Malmsten	100[c]	100[c]
T. violaceum Sabour.	100[c]	100[c]
Plant Pathogens		
Aspergillus parasiticus Speare	100[s]	100[c]
Cladosporium cladosporioides (Fresenius) de Vries	100[c]	100[c]
Curvularia lunata (Wakker) Boedijin	100[c]	100[c]
Colletotrichum capsici (Syd)Butler& Bisby	100[c]	100[c]
C. falcatum Went	100[c]	100[c]
Fusarium oxysporum Schlecht	100[c]	100[c]
F. udum de vries	100[c]	100[c]
Helminthosporium maydis Nisikado & Miyakel	100[c]	100[c]
H. oryzae Breda de Haan,	100[c]	100[c]
Penicillium implicatum Biourge	100[c]	100[c]
P. minio-luteum Dierckx	100[c]	100[c]

[s] indicates static; [c] indicates cidal in nature

Table 4. Comparative MECs of the oil of *H. aromatica* with some Synthetic Antifungal Drugs

Oil & Trade Name of Antifungal Drugs	Active Ingredients	Minimum Effective Concentration (µl/ml)		
		Epidermophyton floccosum	***Microsporum gypseum***	***Trichophyton rubrum***
H. aromatica	Essential oil	1.6	1.2	1.4
Dactrine	Miconazole Nitrate	6.0	6.0	6.0
Nizaral	Ketoconazole	6.0	0.5	5.0
Tenaderm	Tolnaftate	2.0	1.5	0.8

Table 5. Comparative Efficacy of the oil of *H. aromatica* with some Synthetic Antifungal Drugs

Antimycotic Drugs	Drugs %	Cost (Rs.)		Adverse Effects	Expiry Duration (months)	Environmental impact
		Ointment/g	lotion/ml			
H. aromatica	1%v/v	0.90	0.70	No adverse effects	24-60	Renewable, biodegradable, non-residual toxicity. Non-renewable, non-biodegradable and residual toxicity
Dactrine	2%w/w	2.80	---	Occasionally produced gastrointestinal side effects viz., nausea, vomiting, diarrhea	35	-----do-----

Nizaral	2%w/w	3.75	3.17	Adverse reaction observed were mainly burning, irritation. Drug may block testosterone synthesis	24	-----do-----
Tenaderm	1%w/v	1.06	1.30	Adverse effects were fever, nausea, vomiting, diarrhoea & skin rash, rarely produced irritation	24	----do----
Batrafine	1%w/v	1.50	1.60	----do----	24	

Figure 1. GC-MS analysis of *H. aromatica*: 1.α-pinene + α -thujene (0.60), 2. Sabinene (8.35), 3. (1.25), 4. ρ-cymene (4.75), 5. cis-linalool oxide (0.65), 6. Sabinene hydrat (0.25), 7. trans-linalool oxide (0.65), 8. Linalool (32.45), 9. Lerpinen-4-ol (14.10), 10. α -terpineol (1.85), 11. Piperitone (1.90), 12. Geraniol (0.65), 13. ρ -cymene-8-ol (0.30), 14. Caryophyllene oxide (1.85), 15. Epi-globulol (0.35), 16. Humulene oxide (0.60), 17. Spathulenol (1.85), 18. T-cadinol (0.60), 19. T-muurolol (1.70), 20. α-cadinol (4.00)**.**

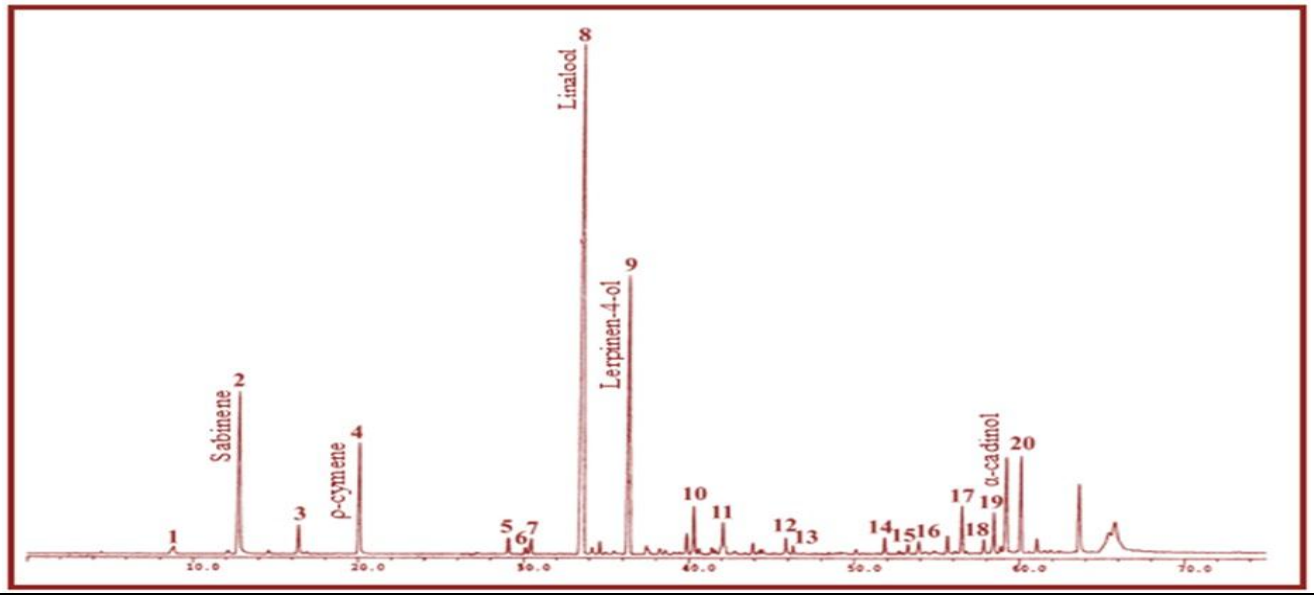


Figure 2. Alignment of ITS1 sequences of standard strains of dermatophytes

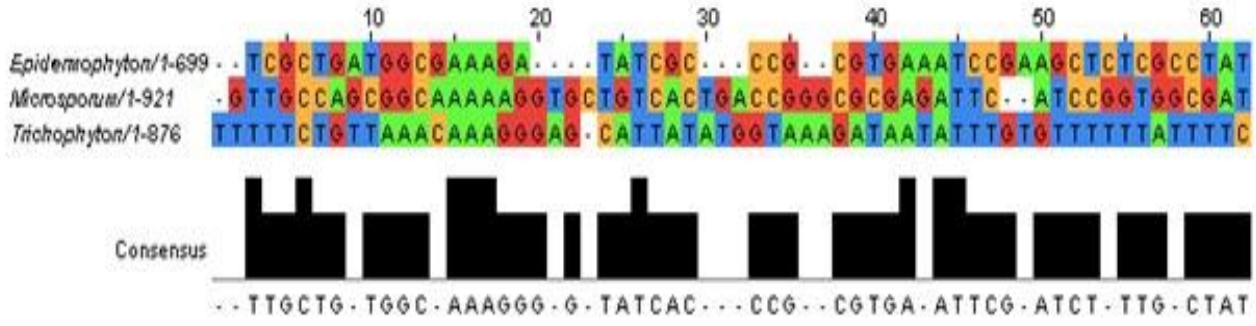
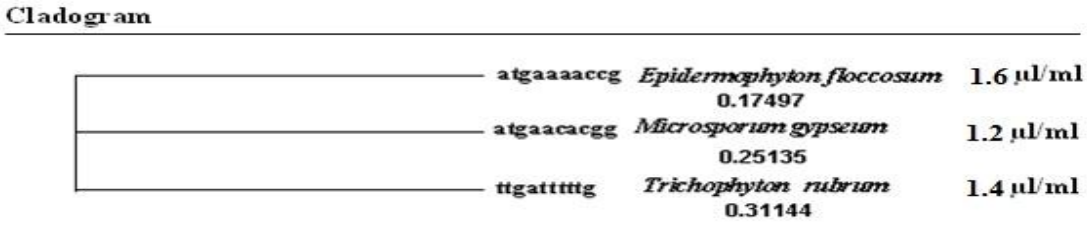


Figure 3. Result of Cladogram (Neigbour Joining Tree plot) standard strains of dermatophytes on the basis of their ITS1 sequences.



DISCUSSION

Essential oils obtained from the leaves of *Cymbopogon martini* var. motia [20], *Hyptis leucodendron* [21]; *Alpinia galangal* [22] was found to contain fungistatic activity. However, some essential oils, *Cymbopogon pendulus* [23]; *Eucalyptus* oil [24]; *Mentha arvensis* [24]; and *Curcuma* spp [25] prove to have fungistatic action at lower concentration and fungicidal action at higher concentration. Similarly, in the present investigation the oil of *H. aromatica* showed fungistatic activity at the lower concentration 1.2 µl/ml against *T. rubrum*, 1.4 µl/ml against *M. gypseum, and* 1.6 µl/ml against *E. floccosum;* and fungicidal at the higher concentration 1.6 µl/ml against *M. gypseum* & *T. rubrum*, and 1.8 µl/ml against *E. floccosum*, respectively. The fungicidal efficacy of the oil persisted heavy inoculums density with quick killing activity as well as having an edge over some synthetic antifungals viz., Dactrine, Nizaral, Tenaderm and Batrafine. A fungicide must not be affected by extreme temperatures. Only a few workers have studied the effect of temperature on antifungal activity of the essential oils. The oil of *Pepromia pellucida* was active up to 80^0C [26]; *Cymbopogon flexuosus* activity up to 100^0C [24], and *Curcuma longa* up to 80^0C [25]. Similarly, in the present investigation the oil of *H. aromatica* was not only thermostable up to 80^0C but also autoclavable up to 15 lb/ sq inch pressure for 30 min.

A substance may behave as a strong fungicidal against certain fungi yet may be ineffective against the other pathogens.Therefore, a clear picture about the toxicity of a fungicide comes only after it is tested against the large number of fungi. The literature showed that essential oils have been found to exhibit narrow or wide range of activity [21,27-29], but in the present study the oil of *H. aromatica* exhibited broad antifungal spectrum. The effectiveness of the oil was equal to those dermatophytes which are close in phylogenetic tree. To understand the relationship of the DNA sequences of the tested fungal strains and their variable response to the different concentrations of active fractions (extracted in the form of essential oil from the rhizome of *H. aromatica*) have been critically analyzed. Further, evaluation of the phylogenetic analysis and identification system, both of which are based on ITS1 rDNA sequences, are continuing in our laboratory with other species and strains. A toxicant should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *H. aromatica* indicate their potentiality to be as ideal antifungal agent against the dermatophytic fungi; hence, the same was further subjected for detailed *in vivo* investigations as well as clinical trials in the form of ointment (at 1% V/V conc.), which is still in progress.

CONCLUSION

Homalomena aromatica is an important 'hidden economy' in the rural Mizoram. In-situ conservation should be enhanced and the micro-environments or ecological niches in which they grow should be protected. Sustainable harvesting techniques need to be adopted and ensure planting of the growing tips on the spot.

Beside this, the preliminary *in vitro* investigations of the oil against some pathogenic fungi (*Epidermophyton floccosum, Microsporum gypseum* and *Trichophyton rubrum*) reveals that after the detailed *in vitro, in vivo* as well as clinical trials, *H. aromatica* can also be an effective antimicrobial agent against the human pathogenic fungi.

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