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ISOLATION AND IDENTIFICATION OF α- GLUCOSIDASE INHIBITOR(S) FROM MEDICINAL PLANTS

A PROJECT REPORT

Submitted by

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in

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ABSTRACT

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Diabetes is a chronic disease characterized by high blood glucose level due to absolute relative deficiency of circulating insulin levels. Different types of oral hypoglycemic agents available for the treatment of diabetes mellitus. There is an increasing demand by patients to use the natural products with anti-diabetic activity as insulin cannot be used orally and continuous use of synthetic oral drugs causes side effects and toxicity. Certain α-glucosidase inhibitors are increasingly being considered for the treatment of diabetes. These are the substances that inhibit α-glucosidase enzyme required to breakdown oligosaccharides, thereby reducing hyperglycemia. The present study was aimed to identify α-glucosidase inhibitors from certain medicinal plants. Among various medicinal plants screened, the aqueous extract of *Psidium guajava* leaf and *Syzygium cumini* Linn seed showed potent inhibitor activity against α-glucosidase enzyme. The inhibitor was found to be non proteinaceous and reversible in nature. The anti-diabetic compound of the extract was then purified by Preparatory TLC & column chromatography and it was finally checked for purity using RP-HPLC.

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CHAPTER 1

INTRODUCTION

1.1 DIABETES

The term diabetes mellitus describes a metabolic disorder of multiple etiologies that is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The causes of type 2 diabetes are either a predominant insulin resistance with a relative insulin deficiency or a predominant insulin secretary defect with or without insulin resistance *(Adolfo et al., 2008).* According to International Diabetes Federation, currently more than 194 million people live with diabetes worldwide and the epidemiological estimates that by 2025 there will be 333 million diabetes sufferers. Almost 25 percent of the population, 60 years and older had diabetes in 2007. The rate of diagnosed diabetes was highest among Native Americans and Alaska Natives (16.5 %). This was followed by blacks (11.8 %) and Hispanics (10.4 %), which includes rates for Puerto Ricans (12.6 %), Mexican Americans (11.9 %), and Cubans (8.2 %). By comparison, the rate for Asian Americans was 7.5 percent with whites at 6.6 percent.

1.2 TYPES

The three main types of diabetes are

- Type 1 diabetes.
- Type 2 diabetes.
- Gestational diabetes.

1.2.1 Type 1 Diabetes

Type 1 diabetes is an autoimmune disease. An autoimmune disease results when the body's system for fighting infection (the immune system) turns against a part of the body. In diabetes, the immune system attacks and destroys the insulin-producing beta cells in the pancreas. The pancreas then produces little or no insulin. At present, scientists do not know exactly what causes the body's immune system to attack the beta cells, but they believe that genetic and environmental factors, possibly viruses, are involved. It develops most often in children and young adults but can appear at any age.

1.2.2 Type 2 Diabetes

Type 2 diabetes mellitus is an increasingly common disorder of carbohydrate and lipid metabolism. Two important characteristics of this disease are insulin resistance, the failure of peripheral tissues; including liver, muscle, and adipose tissue, to respond to physiologic doses of insulin, and failure of pancreatic β-cells to properly secrete insulin in response to elevated blood glucose levels. Obesity is a significant risk factor for the development of type 2 diabetes mellitus. An extremely lean and lipoatrophic models have revealed a similar predisposition to

developing diabetes. Although it may seem paradoxical that both increased adiposity and severely reduced fat mass cause diabetes, a common pathophysiologic process in fat may be responsible for the predisposition to develop hyperglycemia in both conditions *(Nadler et al., 2001).*

Broadhurst proposed the major causative factors for **Non-Insulin Dependent-Diabetes Mellitus (NIDDM)** involving obesity and over fatness; carbohydrate and fat over nutrition; lack of polyunsaturated fatty acids (PUFA) in plasma membranes and unbalanced triglyceride intake; chromium deficiency; and lack of soluble fiber and relevant beneficial Phytochemical *(Broadhurst et al., 1997).*

1.2.3 Gestational Diabetes

Some women develop gestational diabetes late in pregnancy. Although this form of diabetes usually disappears after the birth of the baby, women who have had gestational diabetes have a 20 to 50% chance of developing type II diabetes within 5 to 10 years. Maintaining a reasonable body weight and being physically active may help prevent development of type II diabetes.

As with type II diabetes, gestational diabetes occurs more often in some ethnic groups and among women with a family history of diabetes. Gestational diabetes is caused by the hormones of pregnancy or a shortage of insulin. Women with gestational diabetes may not experience any symptoms *(NIH, 2006).*

1.3 COMPLICATIONS ASSOCIATED WITH DIABETES

1.3.1 SHORT TERM COMPLICATIONS

1.3.1.1 Diabetic Ketoacidosis

Ketoacidosis is chiefly a complication of type 1 diabetes. Diabetic ketoacidosis occur in patients with severe insulin deficiency combined with glucagon excess. Failure to take insulin and exposure to stress are the common precipitating factors. In children, there may be abdominal pain, with or without vomiting. Weakness and drowsiness are commonly present, which may increase, further to unconsciousness or to "Diabetic Coma". Sometimes diabetic coma can occur in elderly patients, with extreme hyperglycemia and dehydration but no ketoacidosis. This is known as Hyperosmolar diabetic coma.

1.3.1.2. Hyperosmolar Non Ketotic Coma

Hyperosmolar nonketotic coma is mainly seen in patients suffering from Type II diabetes. It is caused by severe dehydration due to continuous removal of sugar in urine known as hyperglycemic dieresis. The loss of glucose in urine is so intense that the patient is unable to drink sufficient water to maintain urinary fluid loss.

1.3.1.3 Hypoglycemia

Hypoglycemia is commonly seen in patients suffering from Type 1 diabetes. It may occur when excessive amount of insulin is administered to the patient, leading to fall in blood glucose level. It may also occur if the patient misses a meal. Stress may also lead to Hypoglycemia. Stomach upset, leading to vomiting before the food is digested, also

lowers the blood glucose level. If the patient's low blood glucose level continues then he may pass out and go into coma. If hypoglycemia is not treated, death can occur.

1.3.2 LONG TERM COMPLICATIONS

1.3.2.1 Arteriosclerosis

It is seen in patients of both Type I and Type II Diabetes mellitus. Arteriosclerosis of the extremities is a disease of blood vessels characterized by narrowing and hardening of the arteries that supply the legs and feet. It results in diminished blood flow which can lead to injury of nerves and other tissues. Commonly the effect is seen in the legs and feet. Arteriosclerosis is commonly associated with ulceration, calcification and thrombosis. Calcium deposits in the walls of the arteries leads to narrowing and stiffness of arteries.

1.3.2.2 Diabetic Nephropathy

Kidney damage due to diabetes is called diabetic nephropathy. It is also known as Diabetic glumerulosclerosis. In this a particular type of renal lesion is seen which may be diffuse or nodular. The diffuse lesion occurs mainly due to generalized thickening of the basement membrane of glomerular capillaries. The nodular lesion is in the form of rounded masses of hyaline material which are superimposed upon the diffuse lesion.

1.3.2.3 Diabetic Retinopathy

Retinopathy is the commonest long term complication of diabetes. It is a leading cause of blindness. These are diseased small blood vessels

in the back of the eye which causes the leakage of protein and blood in the retina. Disease in these small blood vessels may also cause the formation of Micro aneurysms. They appear as minute, discrete, circular, dark red spots near to the retinal vessels. They look like tiny hemorrhages.

1.3.2.4 Diabetic Microangiopathy

It is characterized by basement membrane thickening of small blood vessels and capillaries of various organs and tissues such as the skin, eye, skeletal, muscle, kidney, etc. Similar type of basement membrane thickening many also be seen in nonvascular tissues such as peripheral nerves, renal tubules etc. Diabetic Microangiopathy mainly occurs due to recurrent hyperglycemia.

1.3.2.5 Diabetic Neuropathy

It involves temporary or permanent damage to nerve tissue. Nerve tissue gets injured mainly due to decreased blood flow and rise in blood glucose levels. Diabetic neuropathy affects all parts of the nervous system but peripheral nerves are most commonly affected. It affects cranial nerves or the nerves from spinal cord or their branches. Nerve injury normally develops in stage. In earlier stages, tingling sensation or intermittent pain is noted particularly in the extremities such as feet. But in later stage, the pain is continuous and severe.

1.3.2.6 Infections

Diabetics have increased susceptibility to various infections, such as tuberculosis, pneumonias, pyelonephritis, carbuncles and diabetic

ulcers. This may be due to poor blood supply, reduced cellular immunity or hyperglycemia.

1.3.2.7 Heart Disease & Stroke

Patients with diabetes are four times more prone to develop Heart disease than those who do not have diabetes. They may suffer from Heart Attack, Chest Pain or Angina, High Blood Pressure, Stroke, etc. Patient with diabetes may develop silent Heart Attacks - heart attacks that take place without showing any particulars symptoms. It is because in diabetics there is damaged nerve, so the patient does not feel any chest pain, and thus is not aware of the oncoming heart attack.

1.4 TREATMENT FOR TYPE II DIABETES

1.4.1 Sulfonylureas

Sulfonylureas stimulate the β cells of the pancreas to release more insulin. Chlorpropamide (brand name Diabinese) is the only first-generation sulfonylurea still in use today. The second generation sulfonylureas are used in smaller doses than the first-generation drugs. There are three second-generation drugs: glipizide (brand names Glucotrol and Glucotrol XL), glyburide (Micronase, Glynase, and Diabeta), and glimepiride (Amaryl). These drugs are generally taken one to two times a day, before meals. All sulfonylurea drugs have similar effects on blood glucose levels, but they differ in side effects, based on the frequency and interactions with other drugs.

1.4.2 Meglitinides

Meglitinides are drugs that also stimulate the β cells to release insulin. Repaglinide (brand name Prandin) and nateglinide (Starlix) are meglitinides. They are taken before each of three meals.Because sulfonylureas and meglitinides stimulate the release of insulin, it is possible to have hypoglycemia (low blood glucose levels).

1.4.3 Biguanides

Metformin (brand name Glucophage) is a biguanide. Biguanides lower blood glucose levels primarily by decreasing the amount of glucose produced by the liver. Metformin also helps to lower blood glucose levels by making muscle tissue more sensitive to insulin so that glucose can be absorbed. A side effect of metformin may be diarrhea, but this is improved when the drug is taken with food.

1.4.4 Thiazolidinediones

Rosiglitazone (Avandia) and pioglitazone (ACTOS) are in a group of drugs called thiazolidinediones. These drugs help insulin work better in the muscle and fat and also reduce glucose production in the liver. The first drug in this group, troglitazone (Rezulin), was removed from the market because it caused serious liver problems in a small number of people. Both drugs appear to increase the risk for heart failure in some individuals.

1.4.5 α-glucosidase inhibitors

Acarbose (brand name Precose) and meglitol (Glyset) are alpha-glucosidase inhibitors. These drugs help the body to lower blood glucose

levels by blocking the breakdown of starches, such as bread, potatoes, and pasta in the intestine. They also slow the breakdown of some sugars, such as table sugar. Their action slows the rise in blood glucose levels after a meal. They should be taken with the first bite of a meal. In this respect, the inhibitors of α- glucosidase, a typical exo-type amylolytic hydrolase that releases α-glucose from the non-reducing end of a polysaccharide and oligosaccharide, are of particular interest. The potent α-glucosidase inhibitor Acarbose, a polysaccharide synthesized by Actinomyces, has been reported to retard or prevent the absorption of meal derived glucose into circulation and to blunt the postprandial rise in plasma glucose (*O'Dea and Turton, 1985*). However, its use has been limited by the side-effects such as flatulence and diarrhea due to colonic fermentation of non absorbed sugar. Small α-glucosidase inhibitors with a molecular weight below 250, which can be absorbed appreciably from the gut into the bloodstream, are arousing great interest as therapeutic agents. Glycosidases are involved in a wide variety of functions. In order to be useful as an antidiabetic agent, the inhibitory activity should be more specific. That is, the inhibitor should be specific for intestinal brush border α- glucosidases but should have no other effect on the glycoprotein processing glycosidases (*Jacob, 1995*). Most of the presently available α-glucosidase inhibitors such as swainsonine, castanospermine, calystegine and deoxynojirimycin show broad activity spectra against various glycosidases and have been shown to inhibit the processing and maturation of glycoproteins. In addition to the inhibition of α-glucosidase, castanospermine also inhibited β-glucosidase; swainsonine is a potent inhibitor of α-mannosidase; calystegine inhibits β-glucosidase and α-galactosidase. Owing to their toxicities, these compounds were considered unsuitable for therapeutic use. Hence, it was

aimed to develop a potent plant based α-glucosidase inhibitors with the following objectives.

1.5 OBJECTIVES

The present study is performed with the following objectives

- To screen the selected 13 medicinal plants for α-glucosidase inhibitors.
- To isolate the active inhibitor(s) from the rich source.
- To study the chemical characteristics of the inhibitor.

2. LITERATURE REVIEW

CHAPTER 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

Enzyme inhibitors have potential value in many areas of disease control and treatment. The control of kinetics of carbohydrate digestion and monosaccharide absorption could be value in the prevention and control of conditions such as diabetes, obesity, hyperlipoproteinaemia and hyperlipidaemia, and in this respect inhibitors of glucosidase are of particular interest *(Kim and Nho., 2004).*

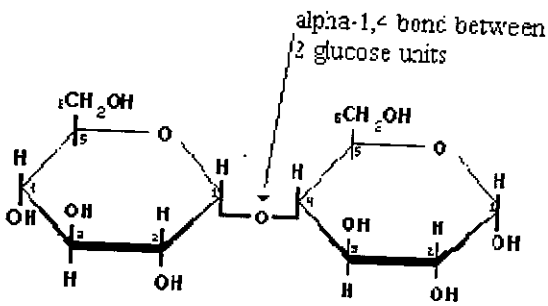
Intestinal α-glucosidase **(EC 3.2.1.20)** plays an important role in carbohydrate digestion and absorption. Therefore an inhibitor of intestinal α-glucosidase could be expected to retard carbohydrate digestion and absorption *(Ohta et al., 2002).*

2.2 ENZYME ACTION

Glucosidases are located in the intestinal brush-border surface of small intestinal cells and they catalyze the cleavage of glycosidic bonds in oligosaccharides or glycoconjugates. Several glucosidases are specific for the cleavage of glycosidic bonds depending on the number, position, or configuration of the hydroxyl groups in the sugar molecule. Thus, α- and β-glucosidases are able to catalyze the cleavage of glycosidic bonds

involving terminal glucose connected at the site of cleavage, respectively, through α- or β-linkages at the anomeric center. The transition state structure for the substrates of these enzymes has a pseudo axial orientation of the C–O bond and a skew conformation, suggesting that the main differences between α- and β-glucosidases are concerned with positioning of the catalytic nucleophile and the catalytic proton donor, represented by two carboxylic acid units *(Melo et al., 2006).*

Fig 2.1 Disaccharide (Maltose) showing α-1,4 glycosidic bond



2.3 CLASSIFICATION

Intestinal α-glucosidase is divided into four enzymes: maltase, glucoamylase, sucrose, and isomaltase. Among them, maltase is the major enzyme which is responsible for the digestion and absorption of dietary starch, whereas sucrase can only hydrolyze sucrose *(Toda et al., 2000).*

2.4 ROLE OF α-GLUCOSIDASE IN BIOCHEMICAL PROCESS

The activity of glucosidases is fundamental to several biochemical processes such as

(i) Degradation of diet polysaccharides to furnish monosaccharide units, which are then able to be metabolically absorbed and used by the organism,

(ii) Lysosomal glycoconjugate catabolism and glycoprotein processing, and

(iii) Biosynthesis of oligosaccharide units in glycoproteins or glycolipids.

2.5 PLANTS IN THE TREATMENT OF DIABETES

A large number of indigenous plants used as foods and medicines, around the world, are known for their ability to lower blood sugar levels. The world health organization estimates that 4 billion people, or 80% of the world population, use herbal medicine for some aspect of primary health care. Herbal medicines are economic as well as effective (*Fatima et al., 2004*).

Medicinal plants used to treat hypoglycemic conditions are of considerable interest to ethnobotanical community as they are recognized to contain valuable medicinal properties in different parts of a plant. In traditional medicine diabetes mellitus is treated with diet, physical exercise and medicinal plants, even though, more than 1200 plants are used around the world in the control of diabetes mellitus and approximately 30% of the traditionally used antidiabetic plants were pharmacologically and chemically investigated. On the other hand, potential hypoglycemic agents have also been detected for more than 100 plants used in diabetic therapy. Traditional treatment may provide the valuable clues for the development of new oral hypoglycemic agents and

simple dietary adjuncts. More than 100 medicinal plants are mentioned in the Indian system of medicine including folk medicines for the management of diabetes, which are effective either separately, are in combinations (*Ayyanar et al., 2008).*

2.5.1 Psidium guajava

The leaves of *P. guajava* inhibit the increase of plasma sugar level in alloxan induced diabetic rats, during glucose tolerance test. In addition, the butanol and water-soluble fractions were found to suppress adrenalin induced lipolysis in fat cells from rats' epididymal adipose tissues. Various parts of *P. guajava* have been used for the treatment of diabetes mellitus. Methanolic extract (51%) of *P. guajava* leaves showed hypoglycaemic effect in type 2 diabetes. *P. guajava* fruits are considered a good source of antioxidants *(Rai et al., 2007)*

The aqueous extract of *Psidium guajava* leaves has a good effect to lower blood glucose. Flavonoid glycosides such as strictinin, isotrictinin and pedunculagin are the effective constituents, which have been used in clinical treatment of diabetes to improve the sensitivity of insulin. A glycoprotein with the molecular weight of 50,000-100,000 was also identified as active component for anti-diabetes. *(Li et al., 2004)*

2.5.2 *Syzygium cumini*

Phytochemicals screening of the ethyl acetate and methanol extracts of *Syzygium cumini* seed used in the study revealed that the crude extracts contained alkaloids, amino acids, steroids and triterpenoids. *Syzygium cumini* seed also have various medicinal values such as anti-inflammatory, anti-diabetic and analgesic activities *(Kumar et al., 2009).*

A compound, Mycaminose was isolated from *Syzygium cumini* seed extract. It was also suggested that the mechanism of action of Mycaminose was similar to that of Glibenclamide, drug that has been used for many years to treat diabetes to stimulate insulin secretion from pancreatic β cells. It was also found that Ethyl acetate and methanol extract of *Syzygium cumini* seed possess antidiabetic effect in STZ-induced diabetic rats. Further comprehensive chemical and pharmacological investigations are needed to elucidate the exact mechanism of the hypoglycemic effect of *Syzygium cumini* seed *(Kumar et al., 2008).*

2.5.3 *Andrographis lineata*

The plant is annual herb found in the hedgerows throughout the plains in India and commonly cultivated in gardens. Leaf is shade dried, powdered and taken orally along with cow's or goat's milk. **Dosage:** 2 teaspoon of powder is taken twice a day after food for 2-3 months *(Ayyanar et al., 2008).*

2.5.4 *Abrus precatorius*

The plant is a climber commonly known as Wild Liquorice and found through the plains of India. Leaf of this plant is mixed with the leaves of *Andrographis paniculata, Gymnema sylvestre* and seeds of *Syzygium cumini.* The mixture is shade dried and ground into powder and taken orally along with cow's milk. **Dosage:** About 50 ml of mixture is taken twice a day before food for 120 days *(Ayyanar et al., 2008).*

The chloroform – methanol extract of *Abrus precatorious* Linn seed was able to reduce alloxan hyperglycemic blood glucose levels. The

extract was seen to be slightly more potent than chlorpropamide -a known antidiabetic drug in the class of sulfonylurea. The potency was measured interms of longer time of action and higher percentage reduction in blood glucose levels. The chloroform – methanol extract comprised most of the lipid – soluble compounds in the seed. The mechanism of blood glucose reduction of this extract may be as a result of the ability of the fat soluble extract to bind to receptor sites especially the peroxisome proliferator – activated receptors. These receptors are the chief regulators of glucose metabolism. Further investigations are needed to elucidate the active ingredients in these extract *(Monago et al., 2005)*

2.5.5 *Costus speciosus*

Hexane, ethyl acetate and methanol crude extracts administered at the dose of 250 mg/kg, 400 mg/kg and 400mg/kg, respectively for 60 days to STZ-induced hypoglycemic and normo-glycemic rats and it was found that the hexane crude extract of *C.Speciosus* rhizome was effective in decreasing the serum glucose level and normalizing the other biochemical parameters in diabetic rats. The hexane extract was more potent than the ethyl acetate and methanol extracts. The evidence suggested that the rhizome of *C.Speciosus* could be beneficial for the protection and alleviation of diabetic complications. Further studies need to be carried out to define the active principle(s) present in the hexane crude extract *(Daisy et al., 2008).*

2.5.6 *Nelumbo nucifera*

Oral administration of the ethanolic extract of rhizomes of *Nelumbo nucifera* markedly reduced the glycaemia of healthy, glucose-fed hyperglycemic and streptozotocin-induced diabetic rats compared to

control. The extract improved glucose tolerance and potentiated the action of exogenously injected insulin in normal rats. The extract exhibited 73% and 67% efficiency than that of tolbutamide in normal and diabetic rat respectively *(Bnouham et al., 2006)*.

The root nodes of lotus are used in China to treat diabetes by folk patients. Oral administration of the ethanolic extract of *Nelumbo nucifera* rhizomes can markedly reduce the blood sugar level of normal, glucose-fed and streptozotocin-induced hyperglycemic rats. The activity-guided isolation resulted in the isolation of tryptophan from the node of lotus rhizome. The pharmacological tests showed that tryptophan could lower the blood glucose level significantly in glucose-fed hyperglycemic mice and exhibited over 44% of activity compared with tolbutamide. The crude protein isolated from lotus seeds, a tonic nourishment and medicine, also caused a significant decrease in the blood glucose level of diabetic albino rats after 2 weeks of treatment *(Li et al., 2004)*.

2.5.7 *Catharanthus roseus*

The ethanol extract of *Catharanthus roseus* lowered the blood glucose level in alloxan induced hypoglycemic rats. The leaves seem to have a promising value for the development of potent phytomedicine for diabetes. The leaves *C.roseus* is also useful in reducing the serum total cholesterol and triglyceride levels. The possible mechanism by which the ethanolic extracts of *C.roseus* lowered blood glucose levels in diabetic rats might be by increasing glycogenesis, inhibiting gluconeogenesis in the liver, or inhibiting the absorption of glucose from the intestine. Further comprehensive pharmacological investigations are needed to

elucidate the exact mechanism of the antihyperglycemic and antihyperlipidemic effect of *C.roseus (Most.Afia Akhtar et al., 2007).*

Ethanolic extract of leaf and flower decreases blood glucose levels. Recent studies showed that aqueous extract decreases blood glucose levels about 20% in diabetic rats, dichlromethane and methanol extract decreases blood glucose levels to 49%-58% significantly better then controls. Rats pretreated with the alcoholic extract were rendered completely immune to the diabetes-inducing effect of streptozotocin, while the aqueous extract had only a minor preventive effect. The hypoglycemic effects appeared to be the result of increased glucose utilization in the liver. No adverse effect were observed except that serum acid and alkaline phosphatase levels were elevated in both untreated diabetic rats and in diabetic rats given Catharanthus compared to healthy controls. The cause of this was not investigated. A 70% ethanol extract of leaves in an oral dose 400mg/kg was shown to be 20% as effective as tolbutamide in diabetic rats, though much safer (*Eric yarnell., 2004*).

2.5.8 *Punica granatum*

Oral administration of the aqueous-ethanolic (50%, v/v) extract of the flowers of *Punica granatum* produced a significant decrease in glycaemia in normo-glycemic, glucose-fed hyperglycaemic and alloxan induced diabetic rats *(Bnouham et al., 2006).*

Male abortive flowers of *Punica granatum* are also used for the treatment of diabetes mellitus in Unani medicine in India. Oral administration of the aqueous-ethanolic (50%, v/v) extract of *Punica granatum* flowers led to a significant blood glucose lowering effect in normal, glucose-fed and alloxan-induced diabetic rats. The extract of

Punica granatum seeds was also reported to have anti-diabetic activity. Ursolic acid may be the active constituent *(Li et al., 2004).*

2.5.9 *Butea frondosa*

Butea frondosa (Syn: monosperma) is traditionally used in Indian system of medicine for the treatment of diabetes. Aqueous extract of bark significantly reduced the blood glucose level in normal and alloxan induced diabetic mice. Preliminary phytochemical screening reveals presence of saponin glycosides, tannins and proteins. Aqueous extract of Butea frondosa bark is an attractive material for the development of good phytomedicine for diabetes *(Deore et al., 2008)*

2.5.10 *Artemisia pallens*

Oral administration of the methanol extract of the aerial parts of Artemisia pallens (used in Indian folk medicine for the treatment of diabetes mellitus) led to significant blood glucose lowering effect in glucose-fed hyperglycaemic and alloxan-induced diabetic rats. In fasted normal rats, the extract caused a moderate hypoglycaemic effect at a higher dose. The water extract (1000 mg/kg) was inactive *(Subramonian et al., 1996).*

2.5.11 *Ocimum sanctum*

The aqueous extract of leaves of *Ocimum sanctum* showed significant reduction in blood sugar level in both normal and alloxan induced diabetic rats. Significant reduction in fasting blood glucose, uronic acid, total amino acid, total cholesterol, triglyceride and total lipid indicated the hypoglycemic and hypolipidemic effects of *Ocimum*

sanctum in diabetic rats. Oral administration of plant extract (200 mg/kg) for 30 days led to decrease in the plasma glucose level by approximately 9.06 and 26.4% on 15 and 30 days of the experiment respectively. Renal glycogen content increased 10 fold while skeletal muscle and hepatic glycogen levels decreased by 68 and 75% respectively in diabetic rats as compared to control. This plant also showed antiasthemitic, antistress, antibacterial, antifungal, antiviral, antitumor, gastric antiulcer activity, antioxidant, antimutagenic and immunostimulant activities (*Manish Modak et al., 2007*)

Dietary supplementation of fresh tulsi leaves in a dose of 2 gm/kg body weight for 30 days led to significant lowering of blood glucose levels in test group. Intake of *Ocimum sanctum* also led to significant increase in levels of superoxide dismutase, reduced glutathione and total thiols, but marked reduction in peroxiodised lipid levels as compared to untreated control group. The leaves were found to possess both superoxide and hydroxyl free radical scavenging action *(Jothi Seth et al., 2004)*

2.5.12 *Cuminum cyminum*

Oral administration of 0.25 g kg(-1) body weight of *C. cyminum* for 6 weeks to diabetic rats resulted in significant reduction in blood glucose and an increase in total haemoglobin and glycosylated haemoglobin. It also prevented a decrease in body weight. *C. cyminum* treatment also resulted in a significant reduction in plasma and tissue cholesterol, phospholipids, free fatty acids and triglycerides. Histological observations demonstrated significant fatty changes and inflammatory cell infiltrates in diabetic rat pancreas. But supplementation with

C. cyminum to diabetic rats significantly reduced the fatty changes and inflammatory cell infiltrates. Moreover, *C. cyminum* supplementation was found to be more effective than glibenclamide in the treatment of diabetes mellitus *(Dhandapani et al., 2002).*

Cuminaldehyde had about 1.8 and 1.6 times less inhibitory activity against α-glucosidase than acarbose and quercitin, respectively *(Lee et al., 2005).*

3. MATERIALS & METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT MATERIALS

Different parts of plant such as flowers ,leaves, seed and peel were collected from botanical garden , TNAU, Coimbatore and some plants were purchased from the medicinal herb market in Coimbatore city and all of them were botanically identified (Table 3.1.1 and Fig 3.1).

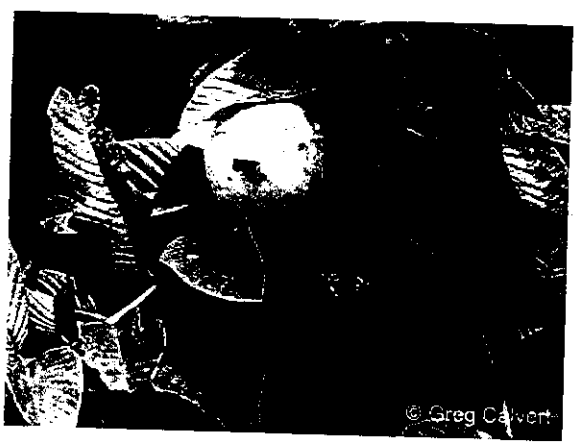
Table 3.1.List of medicinal plants, their common name and part under investigation

Scientific name	Common/ vernacular name	Part(s) under investigation
Psidium gujava	Guava	Leaf
Syzygium cumini	Naval	Seed
Andrographis lineate	Siriyanangai	Leaf
Abrus precatorius	Rosary pea	Leaf
Costus speciosus	Spiral ginger	Rhizome
Nelumbo nucifera	Lotus	Petal and stem
Catharanthus roseus	Madagascar periwinkle	Leaf
Punica granatum	Pomegranate	Peel
Butea frondosa	Flame of forest	Leaf and flower

Artemisia pallens	Marikoluthu	Leaf
Ocimum sanctum	Tulsi	Leaf
Cuminum cyminum	Jeeragam	Seeds

Fig 3.1 Medicinal plants selected for investigation

Psidium guajava



Syzygium cumini

Nelumbo nucifera

Catharanthus roseus

Butea frondosa

Andrographis lineata

punica granatum

Artemisia pallens

Ocimum sanctum

Cuminum cyminum

Abrus precatorius

Costus speciosus (Rhizome)

3.2 PREPARATION OF PLANT EXTRACT

3.2.1 Apparatus and glassware required

- Mortar & pestle.
- Centrifuge tubes, 50 ml.
- Centrifuge – Kubota 3700 model.

3.2.2 Procedure

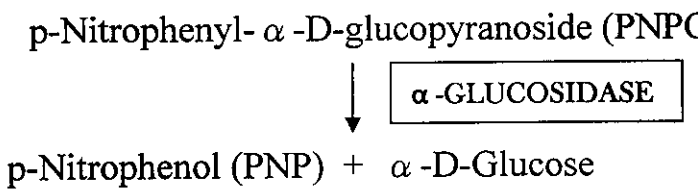
Aqueous extract were prepared from the fresh plant material as follows:

1. Five grams of fresh desired plant part is taken and washed well with distilled water.
2. Then the plants were dried for few minutes to remove water.
3. Using ice cold mortar and pestle, the plant material were ground with 30mL of distilled water.

3. The intestines were minced with a surgical knife and homogenized in a Potter-Elvehjem type of homogenizer, in 50 ml of 0.1M Potassium phosphate Buffer of pH 6.8.
4. After 30 mins, the homogenates were centrifuged for 30 mins at 10,000 RPM at 4°C.
5. The Supernatant was used as a crude enzyme source.

3.4 α-GLUCOSIDASE INHIBITOR ASSAY

3.4.1 Principle



3.4.2 Materials required

- Test tubes.
- Pipettes.
- Spectrophotometer – Beckman coulter DU 530 model

3.4.3 Reagents Required

- p-Nitrophenyl – α- D- Glucopyranosidase (10 mM).
- Crude glucosidase enzyme extract.
- Sodium Carbonate (100 mM).
- Potassium phosphate buffer (0.1M, pH: 6.8)

4. Then the homogenate was centrifuged at 10,000 RPM for 20 mins.
5. The supernatant was collected separately and used as a source if inhibitor for the assay.

3.3 PREPARATION OF SMALL INTESTINE HOMMOGENATE

3.3.1 Chemicals and materials required

- NaCl, 0.9%.
- Potassium phosphate buffer (0.1 M, pH 6.8).
- Rat small intestine.

3.3.2 Apparatus and glassware required

- Surgical knife.
- Potter- Elvehjem type homogenizer.
- Beaker, 100 ml.
- Centrifuge tubes, 50 ml.
- Centrifuge Kubota 3700 model.

3.3.3 Procedure

Rat small intestine homogenate was prepared according to the method described by Lossaw et al.,(1964) with some slight modifications.

1. Rats were exsanguinated by withdrawal of blood from the heart (usually 8-10 cm) under ether anesthesia.
2. Small intestine was removed and washed with 30ml of 0.9% NaCl and placed in ice cold 0.9% NaCl.

3.4.4 Procedure

Four test tubes were taken and marked as Reagent Blank (RB), Reagent Control (RC), Inhibitor Control (IC) and Inhibitor Test (IT). The assay tubes were added with the reaction mixtures as follows and the inhibitor assay carried out.

Reagents (in ml)	RB	RC	IC	IT
Buffer	1.4	1.2	1.2	1.0
Enzyme	-	0.2	-	0.2
Inhibitor Extract	-	-	0.2	0.2
Incubate at room temperature for 20 minutes				
Substrate	0.3	0.3	0.3	0.3
Incubate at room temperature for 30 minutes				
Sodium carbonate	2.0	2.0	2.0	2.0
Measure the absorbance at 400 nm in the spectrophotometer with RB as the blank				

3.4.5 Calculation

$$\% \text{ inhibiton} = 100 * \{(OD_{400}\, IT - OD_{400}\, IC) - (OD_{400}\, RC)\}/\, (OD_{400}\, RC)$$

3.5 AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate precipitation was carried out as described by Sadasivam and Manickam, 2004.

3.5.1 Principle

The solubility of proteins is markedly affected by the ionic strength of the medium. As the ionic strength is increased, protein solubility at

first increases. This is referred to as 'Salting in'. However, beyond a certain point, the solubility begins to decrease and this is known as 'Salting out'. At a low salt concentration, the solubility of a protein is very high. However, as we keep increasing the salt concentration, the proteins begin to precipitate out. It is observed that the hydrophobic proteins precipitate out first, with the hydrophilic proteins precipitating at a much higher salt concentration. In lab scale purification strategies, ammonium sulphate precipitation is often used as a first purification and concentrating procedure. When the protein is in the solution, water molecules surround the protein forming hydrogen bond with the protein. When ammonium sulphate is added, it takes up the water molecules around the protein, exposing the hydrophobic sides on the protein. Because hydrophobic groups tend to be together, the protein will aggregate and thus come out of the solution.

3.5.2 Procedure

1. 10ml of the plant extract was taken in a beaker.
2. To the extract, 7.07 grams of ammonium sulphate was added under constant stirring conditions. The ammonium sulphate quantity was measured with respect to 100% saturation.
3. Extracts were allowed to stand at 0^0C for half an hour to facilitate precipitation.
4. After half an hour, the contents of the beaker were transferred into centrifuge tubes and were centrifuged at 6000rpm for 20 min.
5. After centrifugation, the supernatant of the tubes were collected and checked for inhibitor activity. The pellet in the centrifuge

tube was collected separately, dissolved in buffer and checked for inhibitor activity.

3.6 DIALYSIS (Harris E L V *et al.*, 1989)

3.6.1 Materials

- Dialysis bag
- Beaker, 500ml
- Magnetic stirrer

3.6.2 Procedure

1. The dialysis bag was taken and the one end of the bag was tightly tied and the other end was tied after adding the sample of 2 ml.
2. The sample was made by adding enzyme 200µl, inhibitor 200µl and buffer 1000 µl.
3. The dialysis bag was then immersed in the beaker containing 500ml of phosphate buffer of pH 6.8.
4. The beaker was then kept in the magnetic stirrer at 0-4°C.
5. The buffer used was changed for every 6 hours.
6. After 12 hours one end of the bag is unwinded and a sample volume of 500 µl was taken in the micro centrifuge tube and it was marked as test.
7. Another micro centrifuge tubes was taken and a sample volume of 500 µl was taken and it was marked as control and α-glucosidase inhibitor assay was performed.

3.7 SEPARATION OF COMPOUNDS BY THIN LAYER CHROMATOGRAPHY (TLC) (Roger *et al.*, 1987)

3.7.1 Materials

- Silica gel, G grade with 13% gypsum.
- TLC plate (20x20 cm)
- TLC developing tank

3.7.2 Procedure for preparation of TLC plates

1. Clean 20 x 20 cm glass plates were taken. Before using, the plates were rinsed well with detergent and then with water. After that the plates were dried and wiped well with tissue paper that was soaked in benzene.
2. For one plate about 20 g of silica gel G was weighed and transferred to a wide-mouthed conical flask.
3. To the conical flask about 30-35 ml water was added (Quantity of water may vary with different batches of silica gel) and was shaken thoroughly for 30 sec to get a uniform slurry.
4. The silica gel was uniformly applied over the plates by means of a spreader whose thickness was already adjusted to 0.25 or 0.5 mm.
5. The plates that were coated with the silica gel were dried in air at room temperature. After that plates were activated by keeping it in the oven at 110°C for 2 hr.
6. Without disturbing the silica gel layer, the standard compound and the test samples were spotted with the help of a capillary tube or a microlitre syringe.

7. About 120ml of the solvent mixture of the corresponding ratio was added to the TLC tank. In order to saturate the chamber, a filter paper was wetted and was placed over the inner sides of the tank
8. The TLC plate was placed inside the chamber and tightly covered by means of the lid.
9. When the solvent system reaches the top of the plate, it was removed and air-dried.
10. Finally the plate was sprayed uniformly with the spraying reagent and the spots were noted.
11. The Rf values of the spots were calculated and the corresponding silica gel fraction was scraped.
12. For preparative TLC, the sample was applied as a streak, developed and then a portion of the TLC plate was sprayed to detect the sample. Then the corresponding region of silica gel was scraped.
13. The scraped silica gel was extracted by mixing with methanol and then centrifuged to get the clear filtrate. The filtrate evaporated and was made up with water. This was used as the inhibitor source for the assay.

3.7.3 Solvent system used for separation of compounds

- **Chloroform: ethanol: water, 6:3:1**
- **Ethyl acetate : water : petroleum ether, 3:1:6**
- **Acetic acid : chloroform: water, 9:1:1**
- **Ethyl acetate:Ethanol:Water = 5:1:5**

3.7.4 Spray reagent

Reagent

- 0.1% ethanolic solution of Aluminium chloride.

Procedure

- The TLC plate from its chamber was taken and allowed to air dry.
- Initially the air dried plate was sprayed with Reagent.
- After spraying, the TLC plate was viewed under UV-light for fluorescence.

3.8 COLUMN CHROMATOGRAPHY

3.8.1 Principle

The basis of any form of chromatography is the partition or distribution coefficient (k_d) which describes the way in which a compound distributes itself between two immiscible phases, such as solid/liquid or gas/liquid.

Chromatography columns are considered to consist of a number of adjacent zones in each of which there is sufficient space for the solute to achieve complete equilibrium between the mobile and stationary phases. Each zone is called a theoretical plate and its length in the column is called the plate height. The more efficient the column is the greater the number of theoretical plates involved.

3.8.2 Materials

- Chromatographic column of suitable dimension made up of transparent plastic or glass: Generally, gel filtration is carried out in longer columns (up to 1m) depending upon the type of gel filtration medium used.
- Stationary phase: Silica gel of mesh size 100 -200.

3.8.3 Procedure

1. Suspend the gel (for instance, silica gel) in a large volume of water or preferably in elution buffer. Pour it, into the glass column.
2. Plug the bottom of column tube with glass wool or sintered filter and stand upright the column.
3. Make a good slurry of the gel (stationary phase) in a suitable buffer.
4. Pour a small volume of buffer into the column to avoid trapping of any air bubbles in the plug immediately followed by the slurry to the full of column. The top portion may be carefully, gently stirred prior to pouring additional slurry to the growing column, if necessary. Wait until the gel settle down to the desired height by gravitational force.
5. Equilibrate the column thoroughly by passing the elution buffer.
6. Apply the sample in column buffer onto the top of the bed. The sample volume should preferably limited to 1-3% of the total bed volume. The sample can be applied to the top by careful pipetting or conveniently through the buffer pipeline.

7. Once the sample entered the column, add solvent so as to separate the compounds present in the extract based on their polarity.
8. Compounds were eluted using ethanol, ethanol: water (1:1 V/V) and water.
9. Flavonoids assay was performed.

3.9 FLAVONOIDS ASSAY

Flavonoid assay was performed using the aluminium chloride colorimetric method as described by *Sathish Kumar et al., (2008).*

1. From the fractions collected 0.1ml of sample was taken in different test tubes.
2. Make up the volume to 2.5ml using distilled water and take 2.5ml of distilled water in another test tube to serve as blank.
3. Add 75µl of 5% sodium nitrite to all the test tubes including blank.
4. Incubate at room temperature for 5 minutes.
5. Add 150 µl of 10% aluminium chloride to all the test tubes.
6. Incubate at room temperature for 6 minutes.
7. Add 0.5 ml of 1M sodium hydroxide and measure the red color developed at 510 nm.
8. Pool the fractions based on their OD and perform α-glucosidase inhibitor assay.

3.10 RP-HPLC

The purified fraction obtained from column chromatography was subjected to RP-HPLC to purify the α-glucosidase inhibitor to homogeneity.

3.10.1MATERIALS

- Shimadzu HPLC model RP4a equipped with a variable length UV detector
- C_{18} 5μ column (250*4.6mm)
- Mobile phase: Acetonitrile (A), Water containing 0.1% phosphoric acid (B).

3.10.2 CONDITIONS

Wavelength: Wavelength set between 200-400nm, retention at 285nm and 365nm.

Temperature: 25°c

3.10.3 METHOD

1. Solvents were degassed before use.
2. The column was equilibrated with mobile phase.
3. Twenty microlitre of column chromatography purified sample was filtered through membrane filter (0.22 μm) and then injected into the sample introduction system.

4. The compounds were eluted employing the following time schedule:

Time(min)	Solvent concentration
0-5	5% A, 95% B
5-10	10%A, 90%B
10-25	15%A, 85%B
25-45	40%A, 60%B
45-55	95%A, 5%B
55-65	5%A, 95%B

5. The elution time was set for 65min.
6. Flow rate was adjusted to 1ml/min.
7. The chromatogram of the sample was recorded.

4. RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 α-GLUCOSIDASE INHIBITOR ASSAY

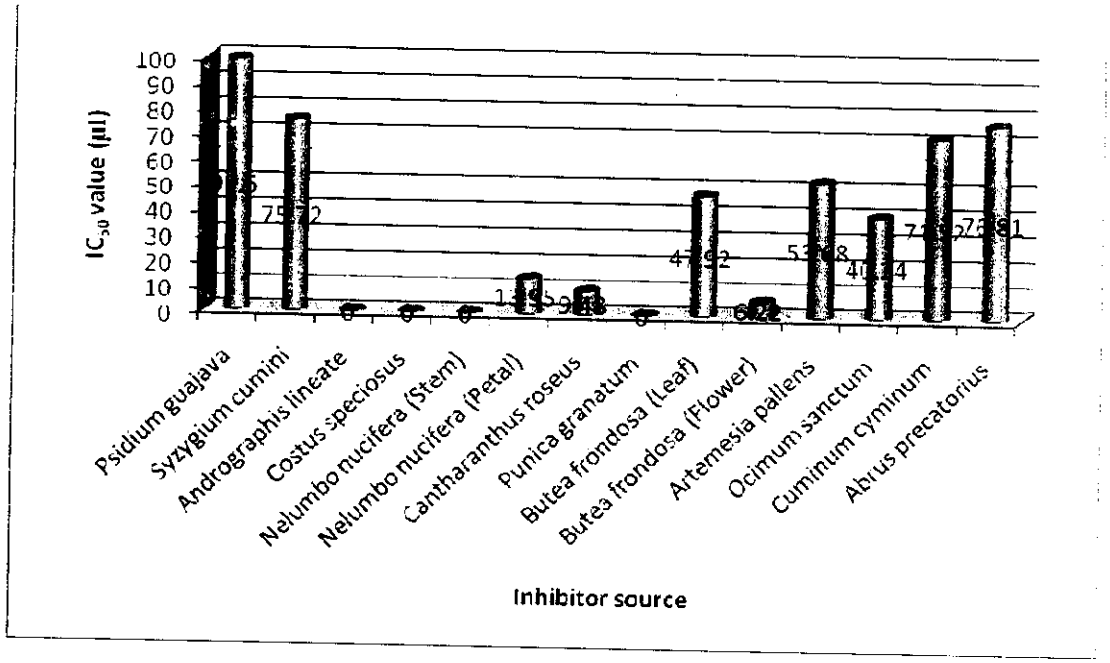
Different medicinal plants were checked for α-glucosidase inhibitory activity by PNPG method. Rat Small Intestinal homogenate was used as source of α-glucosidase. Out of 12 medicinal plants screened 9 plants showed inhibition against α-Glucosidase whereas *Andrographis lineata, Punica granatum, Cantharanthus speciosus* showed no inhibition against α-Glucosidase . In the case of *Nelumbo nucifera,* aqueous extract of stem showed no inhibition whereas aqueous extract of petal showed inhibition of about 13.95% against α-Glucosidase.

The results of the assay implies that the aqueous extract of *Guava (Psidium guajava)* leaves showed the maximum inhibition of α-Glucosidase of about 99.50% and this was followed by *Abrus precatorius* leaf extract which showed inhibition of about 76.81%. The results are shown in Table 4.1 and in Fig 4.1.

Table 4.1. % inhibition showed by various plant extract

PLANT NAME	% INHIBITION
Psidium guajava	99.50
Syzygium cumini	75.72
Andrographis lineate	No inhibition
Costus speciosus	No inhibition
Nelumbo nucifera (Stem)	No inhibition
Nelumbo nucifera (Petal)	13.95
Cantharanthus roseus	9.48
Punica granatum	No inhibition
Butea frondosa (Leaf)	47.92
Butea frondosa (Flower)	6.22
Artemesia pallens	53.68
Ocimum sanctum	40.24
Cuminum cyminum	71.52
Abrus precatorius	76.81

Fig 4.1 Comparison of % inhibition showed by various medicinal plants



Dose dependent study was carried out for all the nine plants which showed inhibition against α-Glucosidase. Dose dependent study (i.e) with varying inhibitor concentration was carried out so as to compare the IC_{50} values of the inhibition (IC_{50} values shown in Table 4.2) [(i.e.) concentration of the inhibitor required to inhibit 50% activity of the enzyme]. The results are presented in the Tables 4.3 to 4.12 and in Fig 4.3 to 4.12.

Mai and Chuyen (2007)., have already reported α-glucosidase inhibition by aqueous leaf extract of *Psidium guajava* (60.8%) and *Nelumbo nucifera* (51.4%). The % inhibition shown by *Psidium guajava* in present study is greater than that reported by Mai and Chuyen, indicating that Phytochemical constituents vary with the geographical location.

Most.Afia Akhtar et al.,(2007) have already reported that the possible mechanism by which the ethanolic extracts of *Catharanthus roseus* lowered blood glucose level in diabetic rats may be due to the inhibition of absorption of glucose from intestine. It was confirmed through our present study that one of the reason for the reduction in blood glucose level in diabetic rats is due to the inhibition of α-glucosidase in small intestine.

Punica granatum flowering parts have been recommended in unnani literature as remedy for diabetes. *Punica granatum* flowering parts extract demonstrated potent inhibitory effect on the α-glucosidase activity which is contradicting to our findings that *Punica granatum* peel extract have no inhibitory effect on α-glucosidase *(Kota et al., Journal unknown).*

Table 4.2 IC_{50} values of α-glucosidase inhibitors from various medicinal plant parts

Inhibitor source	IC50 values (µl)
Psidium guajava	61.0
Artemisia pallens	180
Ocimum sanctum	232.56
Cuminum cyminum	145.012
Syzygium cumini	49.7
Butea frondosa (leaf)	210.02
Butea frondosa (flower)	713.28
Cantharanthus roseus	484.96
Abrus precatorius	127.56

Fig 4.2 Comparison of IC_{50} values

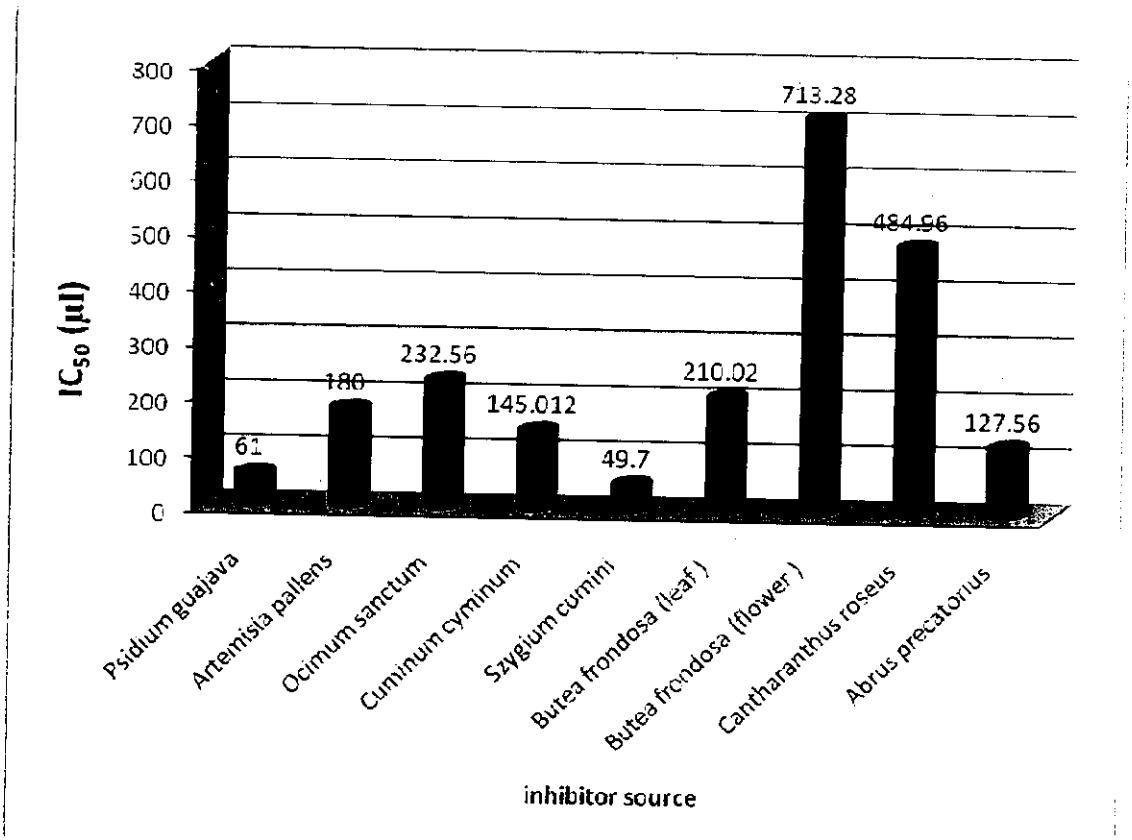


Table 4.3 Inhibitor assay for *Psidium guajava*

Inhibitor Concentration (in µl)	%Inhibition
50	31.94
75	75.03
85	77.86
100	91.23
150	99.50
200	99.50

Fig 4.3 % Inhibition Vs inhibitor volume of *Psidium guajava*

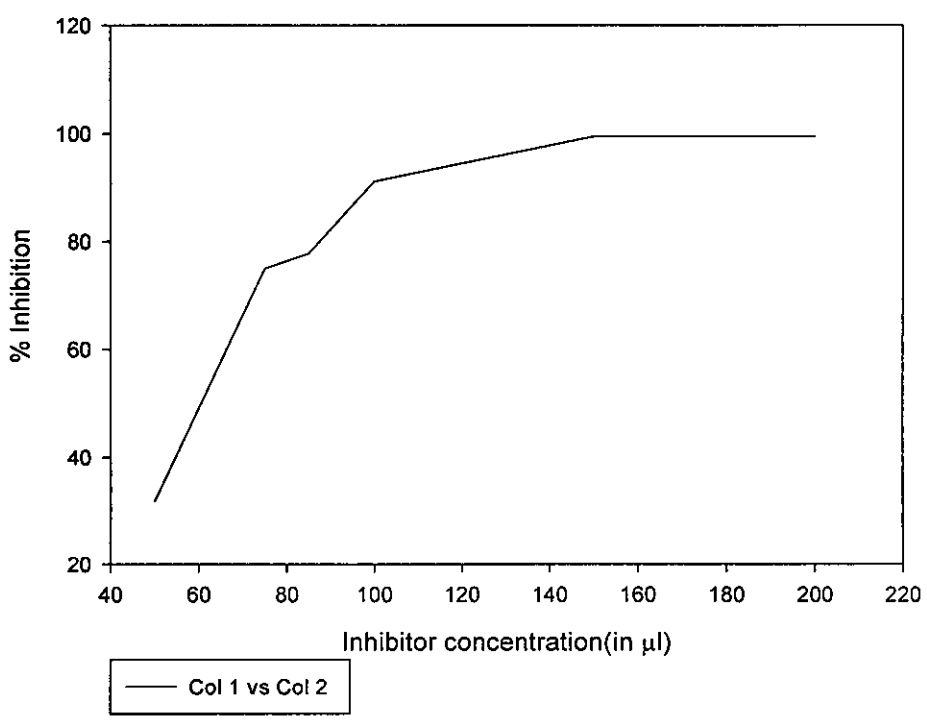


Table 4.4 Inhibitor assay for *Artemisia pallens*

Inhibitor Concentration (in µl)	%Inhibition
50	19.76
200	53.68
300	80.06
400	98.77
500	99.38

Fig 4.4 % Inhibition Vs inhibitor volume of *Artemisia pallens*

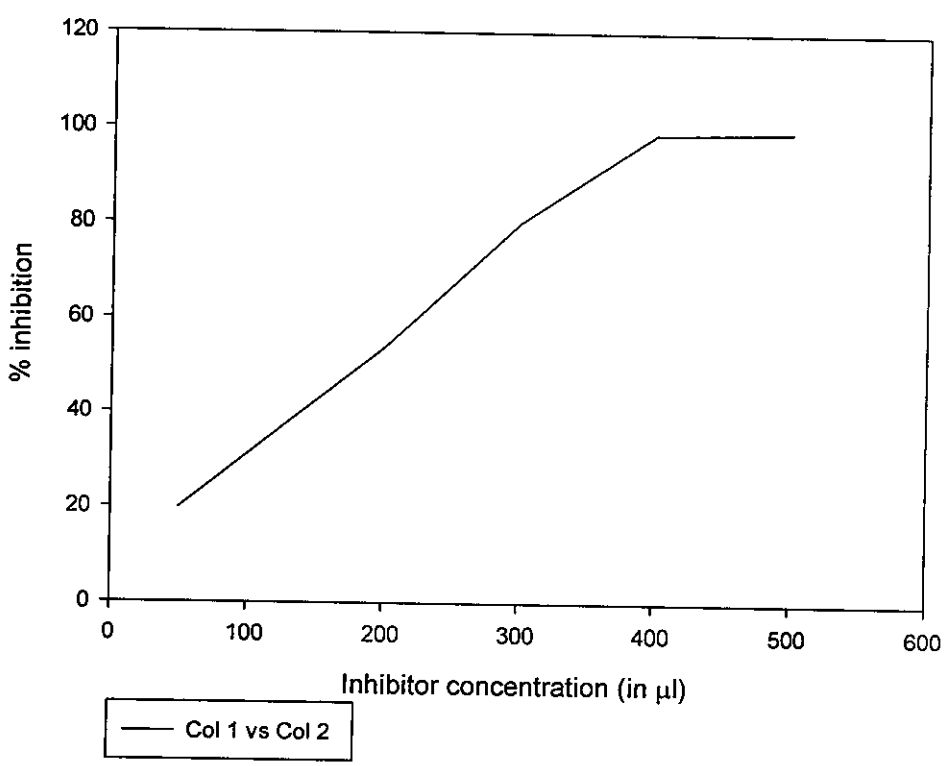


Table 4.5 Inhibitor assay for *Ocimum sanctum*

Inhibitor Concentration (in µl)	%Inhibition
50	2.3
75	7.03
100	11.5
150	26.42
200	40.24
300	70.00
400	90.67
500	91.57

Fig 4.5 % Inhibition Vs inhibitor volume of *Ocimum sanctum*

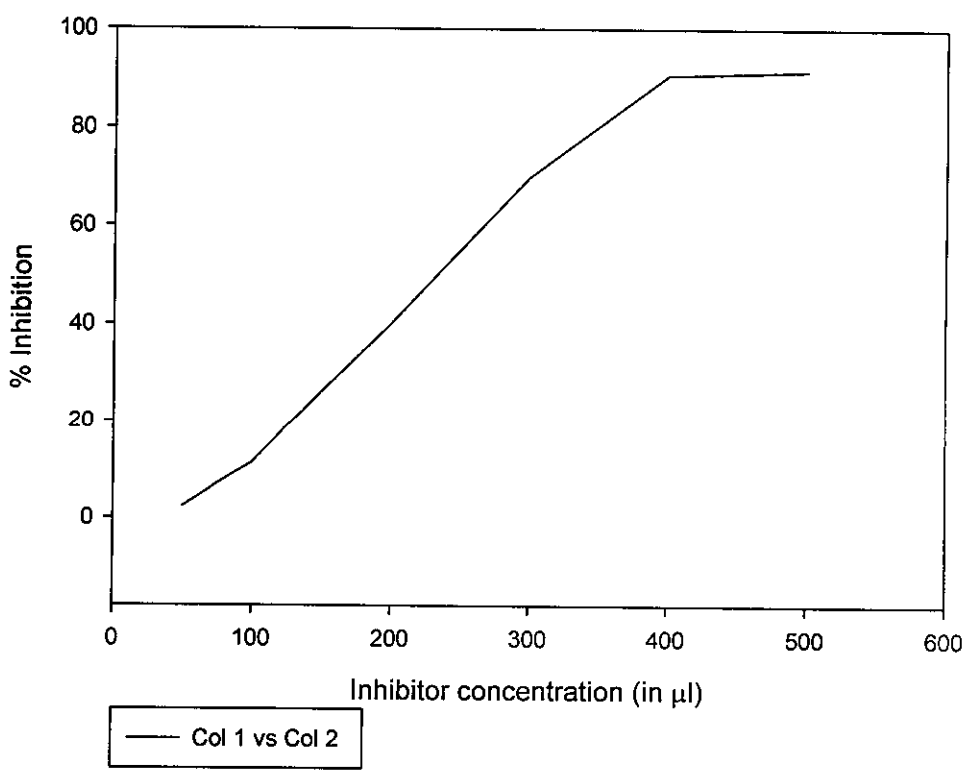


Table 4.6 Inhibitor assay for *Cuminum cyminum*

Inhibitor Concentration (in µl)	%Inhibition
50	No inhibition
75	11.87
100	13.8
150	55.39
200	71.52

Fig4.6 % Inhibition Vs inhibitor volume of *Cuminum cyminum*

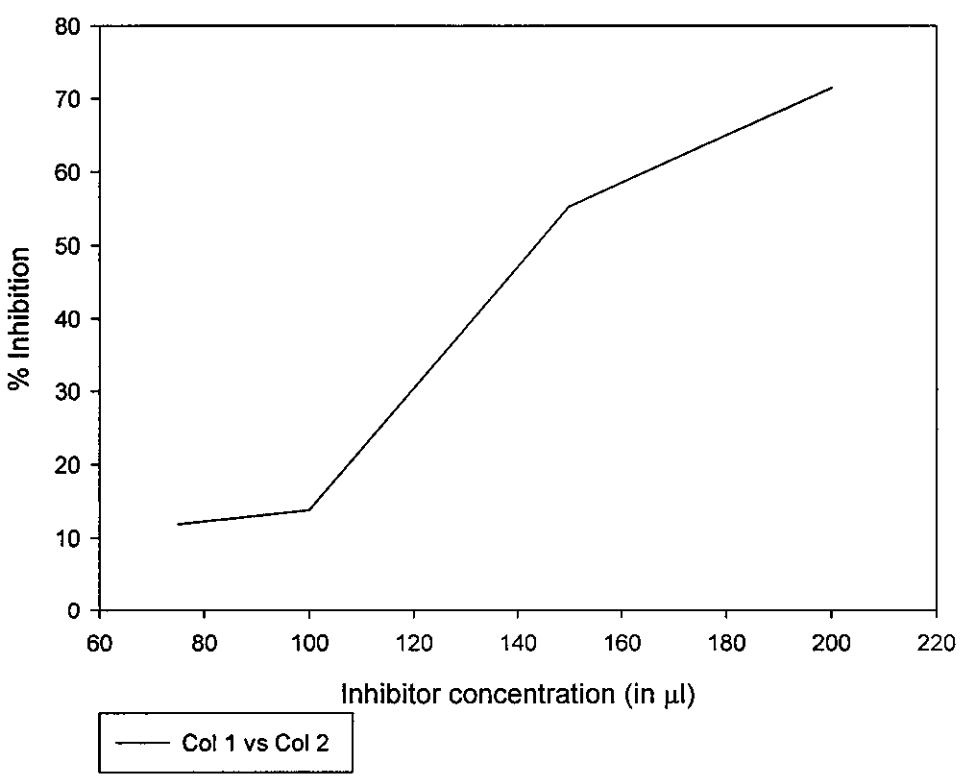


Table 4.7 Inhibitor assay for *Syzygium cumini*

Inhibitor Concentration (in µl)	%Inhibition
50	50.5
100	70.72
200	75.66
300	81.74
400	86.22
500	90.47
600	94.83

Fig 4.7 % Inhibition Vs inhibitor volume of *Syzygium cumini*

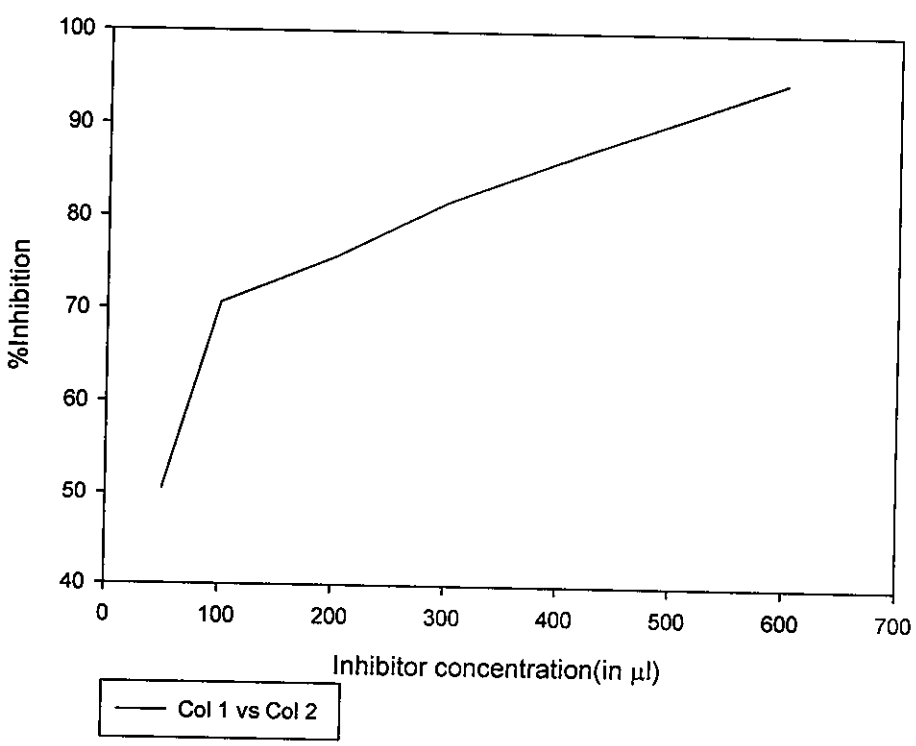


Table 4.8 Inhibitor assay for *Butea frondosa (Leaf)*

Inhibitor Concentration (in µl)	%Inhibition
100	24.95
200	47.92
300	81.95

Fig 4.8 % Inhibition Vs inhibitor volume of *Butea Frondosa(leaf)*

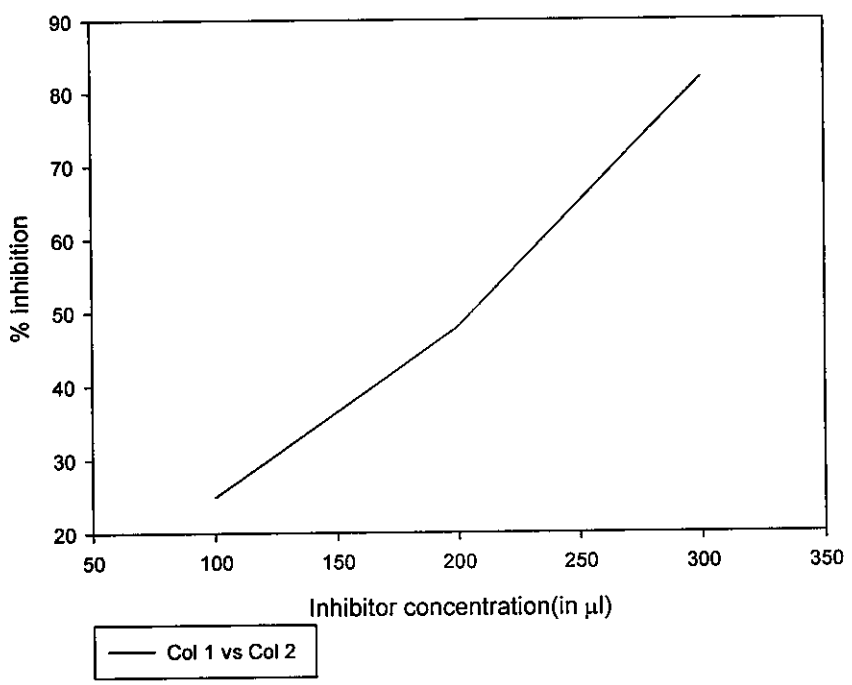


Table 4.9 Inhibitor assay for *Butea frondosa (Flower)*

Inhibitor Concentration (in µl)	%Inhibition
200	6.22
300	18.39
400	22.18
500	34.01
600	39.27
700	46.71
800	74.59

Fig 4.9 % Inhibition Vs inhibitor volume of *Butea Frondosa (Flower)*

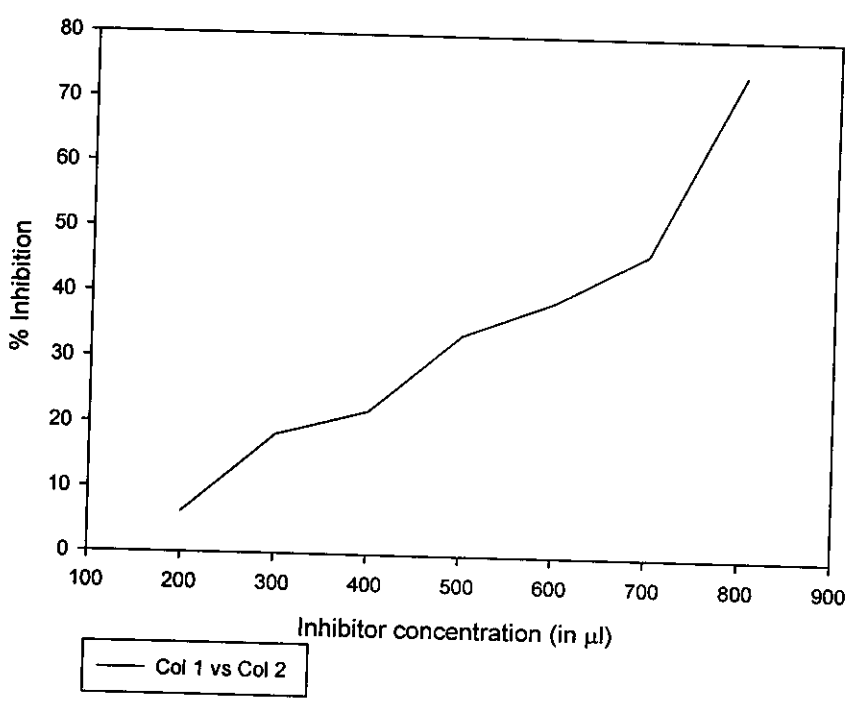


Table 4.10 Inhibitor assay for *Cantharanthus roseus*

Inhibitor Concentration (in µl)	%Inhibition
200	9.48
300	13.28
400	27.00
500	54.16
600	75.91

Fig 4.10 % Inhibition Vs inhibitor volume of *Cantharanthus roseus*

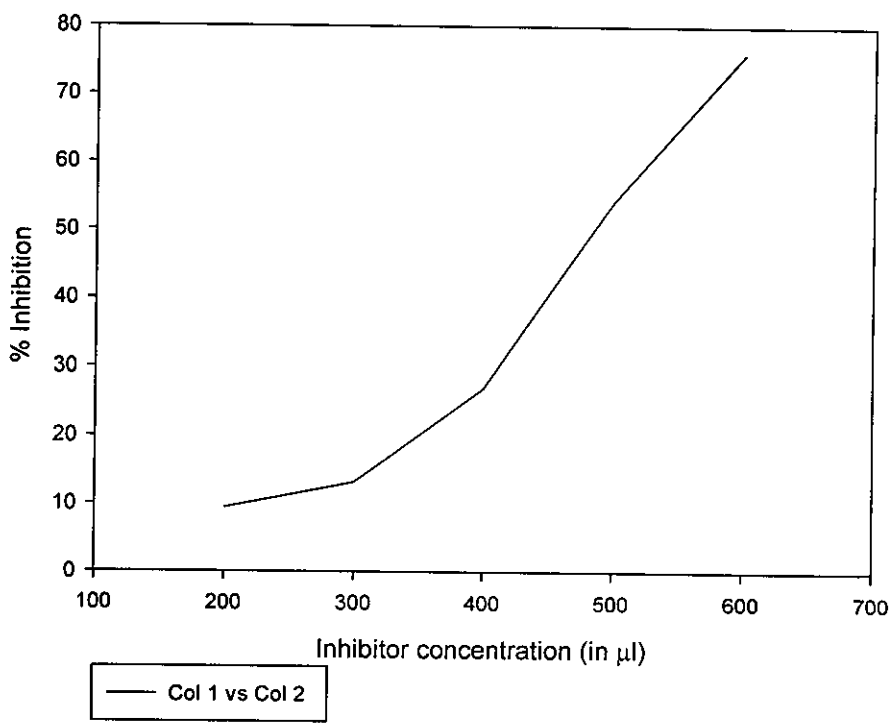


Table 4.11 Inhibitor assay for *Abrus precatorius*

Inhibitor Concentration (in µl)	%Inhibition
100	40.37
200	76.81
300	99.05
400	98.10
500	99.68

Fig 4.11 % Inhibition Vs inhibitor volume of *Abrus Precatorius*

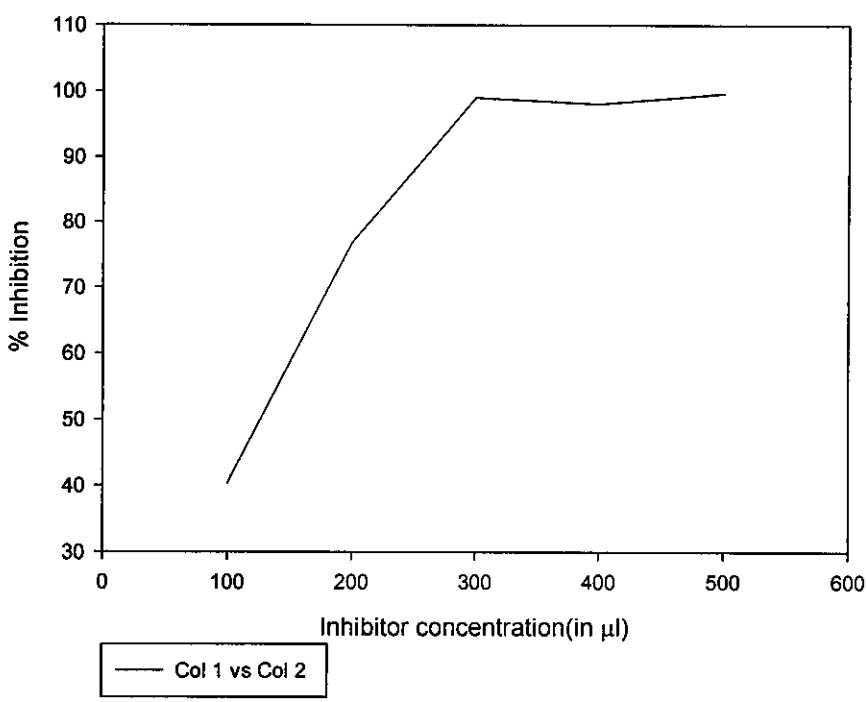
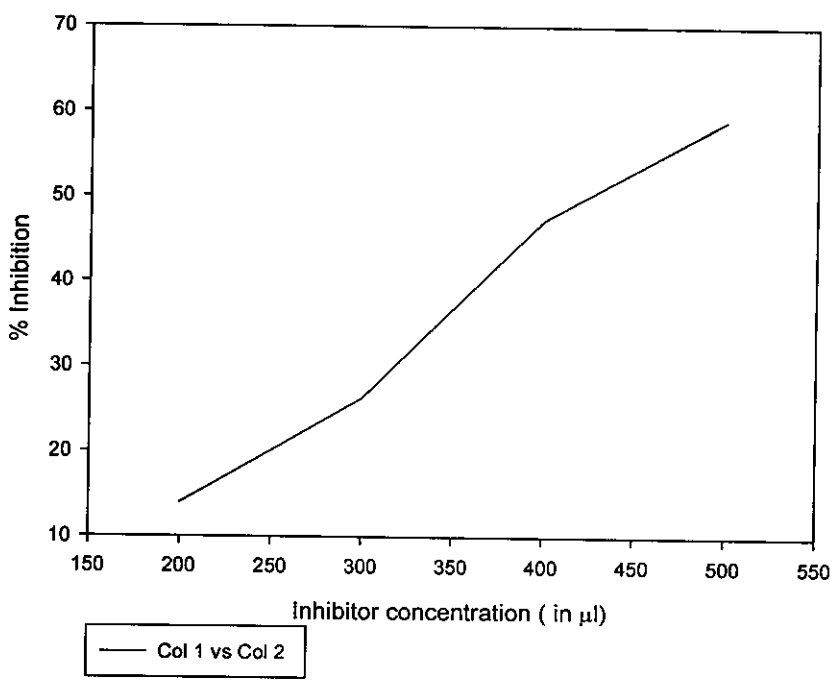


Table 4.12 Inhibitor assay for *Nelumbo nucifera (Petal)*

Inhibitor Concentration (in µl)	%Inhibition
200	13.95
300	26.32
400	47.26
500	59.01

Fig 4.12 % Inhibition Vs inhibitor volume of *Nelumbo nucifera (Petal)*



4.2 AMMONIUM SULPHATE PRECIPITATION

Aqueous extract of the plant which showed inhibition against α-Glucosidase were subject to Ammonium sulphate precipitation to check the nature of inhibitor (i.e.) whether it is proteinaceous or Non-proteinaceous.The extract along with the Ammonium sulphate at 100% saturation were centrifuged for 20min at 10,000 rpm and the supernatant was checked for any α-Glucosidase inhibitor activity. Similarly the pellets obtained due to ammonium sulphate precipitation were dissolved in distilled water and checked for α-Glucosidase inhibitor activity and the results were presented in the Table 4.13.

Table 4.13 %Inhibition of protein and non protein fractions after Ammonium sulphate precipitation

PLANTS	NON PROTEIN %INHIBITION	PROTEIN %INHIBITION
Psidium guajava	82.56	49.31
Artemisia pallens	23.05	70.52
Cuminum cyminum	35.5	-
Ocimum sanctum	29.86	51.11
Nelumbo nucifera (petal)	27.78	-
Abrus precatorius	31.6	70.45
Butea frondosa(leaf)	44.90	29.24
Cantharanthus roseus	24.66	11.66
Syzygium cumini	60.70	27.91
Butea frondosa (flower)	22.26	27.62

It was inferred from the results that the supernatant had %inhibition similar to that of the crude extract in the case of *Psidium guajava, Syzygium cumini, Butea frondosa (leaf), Cuminum cyminum, Nelumbo nucifera (petal) and Cantharanthus roseus* indicating the inhibitor was non protein in nature.

It was found out from the results that the %inhibition of the crude extract is similar to that of the pellets in the case of *Artemisia pallens, Abrus precatorius, Ocmium sanctum and Butea frondosa (flower)* indicating the inhibitor was proteinacious in nature.

4.3 SEPARATION OF COMPOUNDS BY TLC

The aqueous extract of *Psidium guajava, Syzygium cumini, Cantharanthus roseus, Abrus precatorius* was taken and TLC was performed with various solvents. The extract was directly used as a source of inhibitor for α-glucosidase. TLC plates were sprayed with 0.1% ethanolic solution of Aluminium chloride reagent which showed fluorescence in longwave UV light indicating the presence of flavonoids (shown in Fig 4.13 & 4.14). Preparative TLC was done to obtain the purified compounds in adequate concentration. The reagent which showed fluorescence was scrapped out and extracted with HPLC grade ethanol and centrifuged. The supernatant was evaporated and dissolved in a known volume of water and this was used as a source of inhibitor assay. The % inhibition showed by the crude extract is more or less similar to the inhibition showed by the compounds separated by TLC.

Fig 4.13 Separation of compounds by TLC – *Psidium guajava*

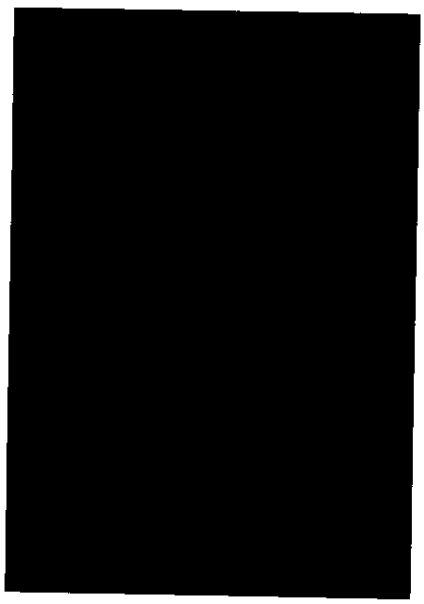
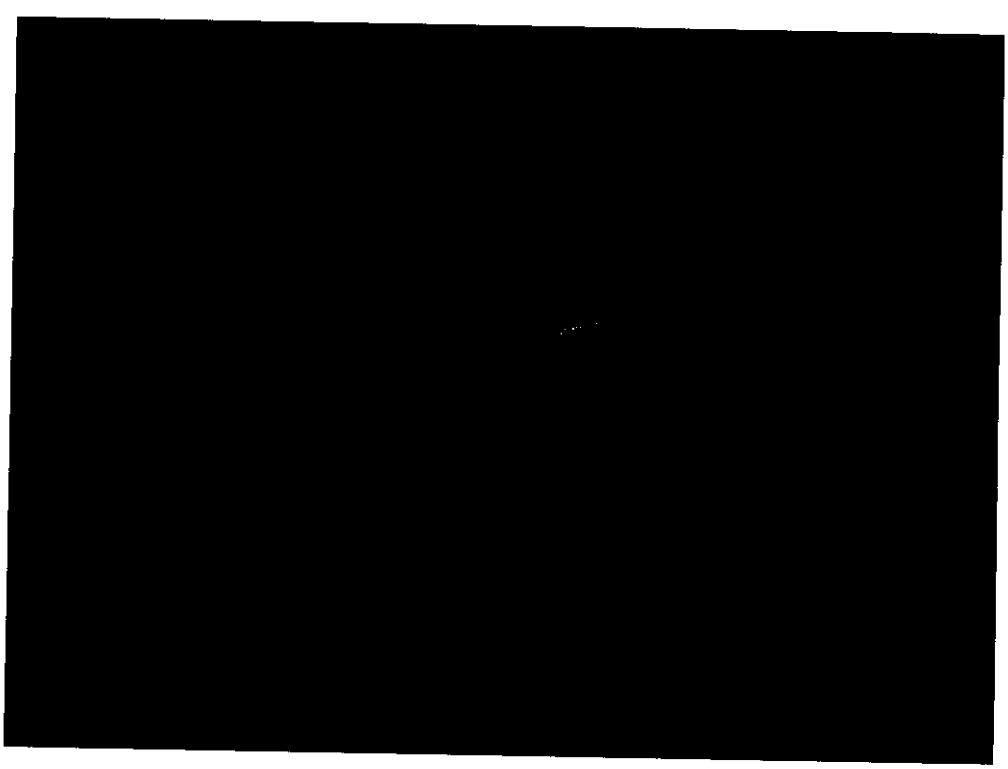


Fig 4.14 Separation of compounds by TLC – *Syzygium cumini*



Osman et al., 1974 and Begum et al., 2002 have reported more than 20 compounds guava extract The major constituent of its leaves were identified to be Tannins, β-sitosterol, maslinic acid, essential oil, triterpenoids and flavonoids *(Liang Q et al., 2005).*

Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit) and contains appreciable amounts of vitamin A as well. Guava fruits are also a good source of pectin - a dietary fiber. The leaves of guava are rich in flavonoids, in particular, quercetin. Much of guava's therapeutic activity is attributed to these flavonoids *(Zhongjun M.A, et al., 2004).*

The flavonoids have demonstrated antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava; it is able to relax intestinal smooth muscle and inhibit bowel contractions *(Koo Hui Miean and Suhaila Mohamed, 2001).* In addition, other flavonoids and triterpenes in guava leaves show anti-spasmodic activity.

Guava's main plant chemicals include: alanine, alpha-humulene, alpha-hydroxyursolic acid, alpha-linolenic acid, alpha-selinene, amritoside, araban, arabinose, arabopyranosides, arjunolic acid, aromadendrene, ascorbic acid, ascorbigen, asiatic acid, aspartic acid, avicularin, benzaldehyde, butanal, carotenoids, caryophyllene, catechol-tannins, crataegolic acid, D-galactose, D-galacturonic acid, ellagic acid, ethyl octanoate, essential oils, flavonoids, gallic acid, glutamic acid, goreishic acid, guafine, guavacoumaric acid, guaijavarin, guajiverine, guajivolic acid, guajavolide, guavenoic acid, guajavanoic acid, histidine,

hyperin, ilelatifol D, isoneriucoumaric acid, isoquercetin, jacoumaric acid, lectins, leucocyanidins, limonene, linoleic acid, linolenic acid, lysine, mecocyanin, myricetin, myristic acid, nerolidiol, obtusinin, octanol, oleanolic acid, oleic acid, oxalic acid, palmitic acid, palmitoleic acid, pectin, polyphenols, psidiolic acid, quercetin, quercitrin, serine, sesquiguavene, tannins, terpenes, and ursolic acid *(Misra and Seshadri, 1968)*. Diatrol II, which contains guava leaf powder, can help diabetes symptoms as well as stomach, dysentery, and other digestion maladies. Several flavonoids, glycosides, terpenoids etc have been shown to possess antidiabetic property *(Rai et al., 2007)*.

Preliminary phytochemical studies of *Syzygium cumini* extract have identified the presence of alkaloids, flavonoids, tannins, triterpenoids, phytosterol, glycosidase, saponins. Among various phytochemicals such as glycosides, flavonoids, tannins and alkaloids posses hypoglycemic activity *(Kumar et al., 2009)*.

HPLC chromatograms of *Eugenia jambolana (S. cumini)* seed powder extracted with dichloromethane showed seven peaks. Tri-terpenoids, tannins, gallic acid and oxalic acid were detected using suitable chemical tests. Alkaloids were present in small amounts *(Sridhar et al., 2005)*

Mycaminose, a compound isolated from *Syzygium cumini* seed extract significantly reduced blood glucose level in diabetic rats. Reduction in blood glucose level is due to the increase in pancreatic secretion of insulin from beta cells of the islets of langerhans *(Kumar et al., 2008)*.

It has been known that hydrolysable tannins are potent enzyme inhibitors, acting by their tendency to precipitate proteins. This precipitation is presumed to occur by the formation of hydrogen bonds between the hydroxyl group of the tannins and the peptide linkage of the proteins *(Cannell et al., 1988)*. Present study of identifying α-glucosidase inhibitor from *Syzygium cumini and Psidium guajava* has indicated that the inhibitor, probably to be a flavonoid.

Polyphenol rich extracts from soft fruits inhibit α-amylase and α-glucosidase. Strawberry and raspberry extracts were effective α-amylase inhibitors than blueberry, Black current or red cabbage. α-glucosidase was more readily inhibited by blueberry and black current extracts. In accordance with this, our plants of interest *Syzygium cumini and Psidium guajava* showed good inhibition against α-glucosidase.

4.4 COLUMN CHROMOTAGRAPHY

In order to isolate the active compounds, column chromatography was carried out for the crude aqueous extract of *Psidium guajava, Syzygium cumini, Cantharanthus roseus, Abrus precatorius, Butea frondosa (flower)*. Compounds were eluted using ethanol, ethanaol: water (1:1 V/V) and water. Fractions were collected and assayed for flavonoids. Fractions showing similar results were pooled together and α-glucosidase inhibitor assay was carried out and the results were presented in the Table 4.14 to 4.18.

Table 4.14 %inhibition of *Psidium guajava* fraction obtained through column chromatography

Fractions	% inhibition
7,8	71.87
11,12	64.18
13,14,15	62.11
5,9,10	61.45
16	35.73
1,4	10.91

Table 4.15 %inhibition of *Sygium cumini (seed)* fraction obtained through column chromatography

Fractions	%inhibition
3,4	99
2,5	89.49
8,11,12	75.93
7,10	64.76
15 – 19,21	64.59

Table 4.16 %inhibition of *Abrus precatorius* fraction obtained through column chromatography

Fractions	% inhibition
16,17,18	54.27
11,12	53.79
13,14,15	44.27
9	38
8,10	35.93
7	0.2

Table 4.17 %inhibition of *Butea frondosa (flower)* fraction obtained through column chromatography

Fractions	%inhibition
10,11,12	42.75
6	35.24
5,7	29.7
4,8	13.72

Table 4.18 %inhibition of *Butea frondosa (leaf)* fraction obtained through column chromatography

Fractions	%inhibition
13,17,23	38.35
11,12	22.08
9,10	19.6

4.5 RP-HPLC

Using RP-HPLC the enriched fractions of column chromatography which showed maximum inhibition against α-glucosidase are checked for the purity. The chromatogram of the two samples (*Psidium guajava and Syzygium cumini*) are shown in the Fig 4.15 to 4.18.

Fig 4.15 RP-HPLC chromatogram of *Psidium guajava* at 360nm.

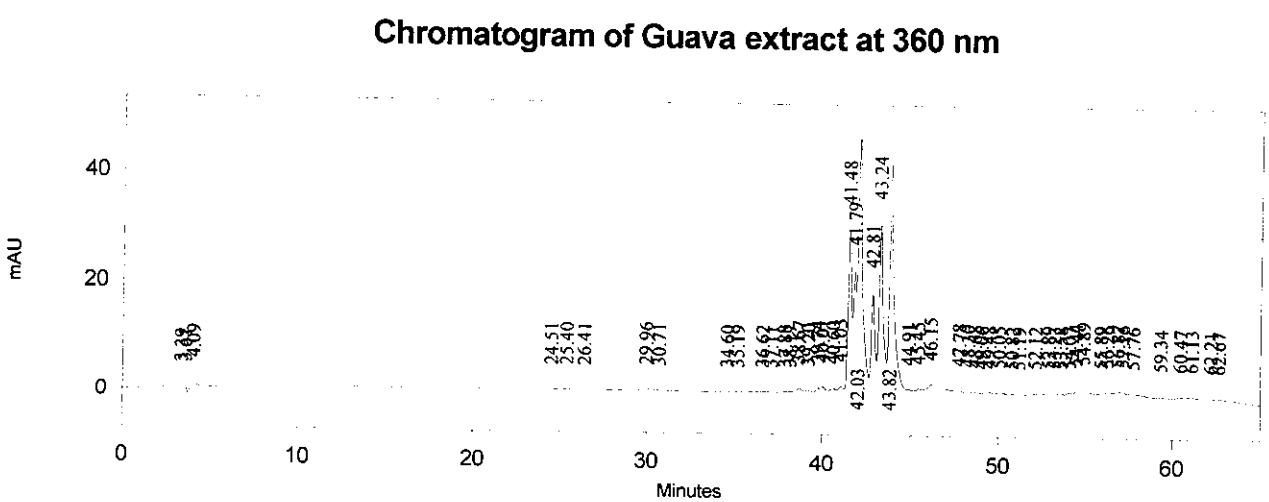


Fig 4.16 RP-HPLC chromatogram of *Psidium guajava* at 285nm.

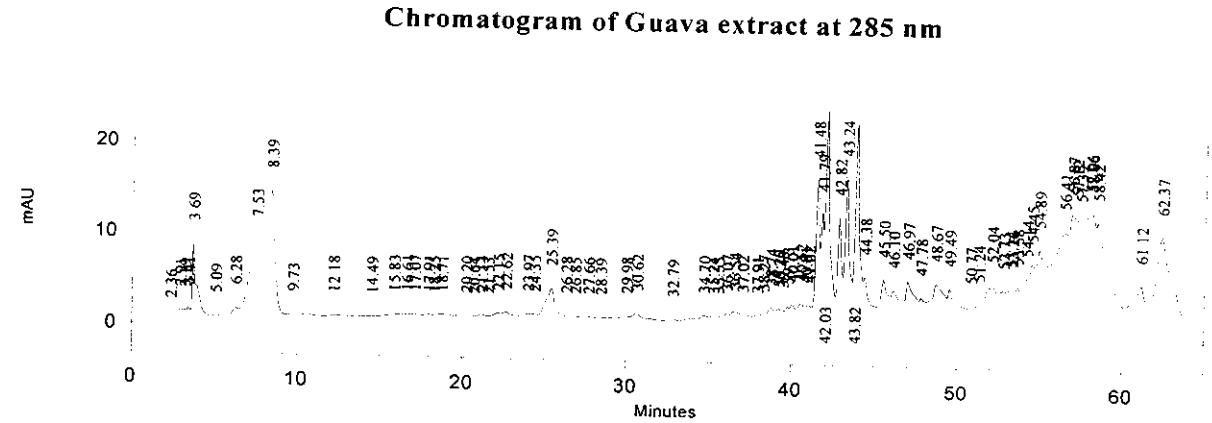


Fig 4.17 RP-HPLC chromatogram of *Syzygium cumini* at 360nm.

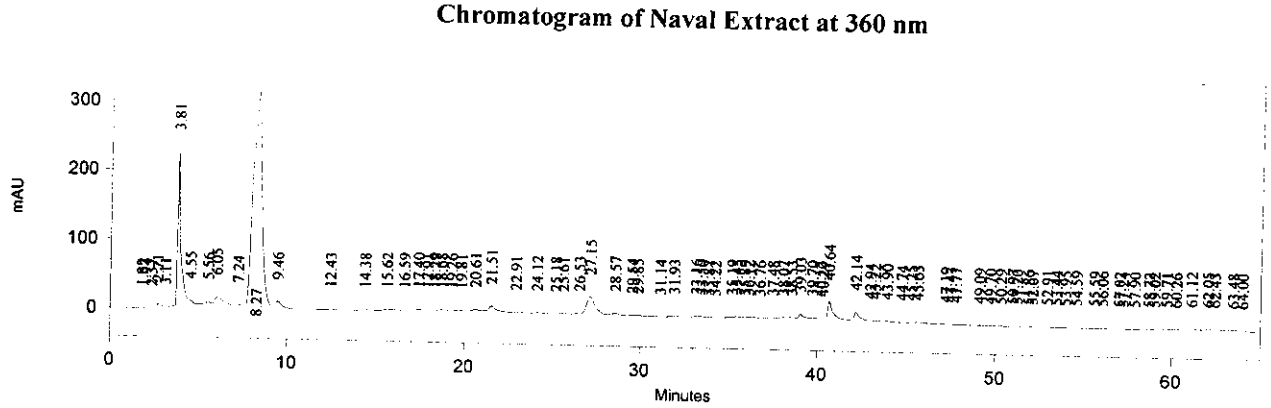
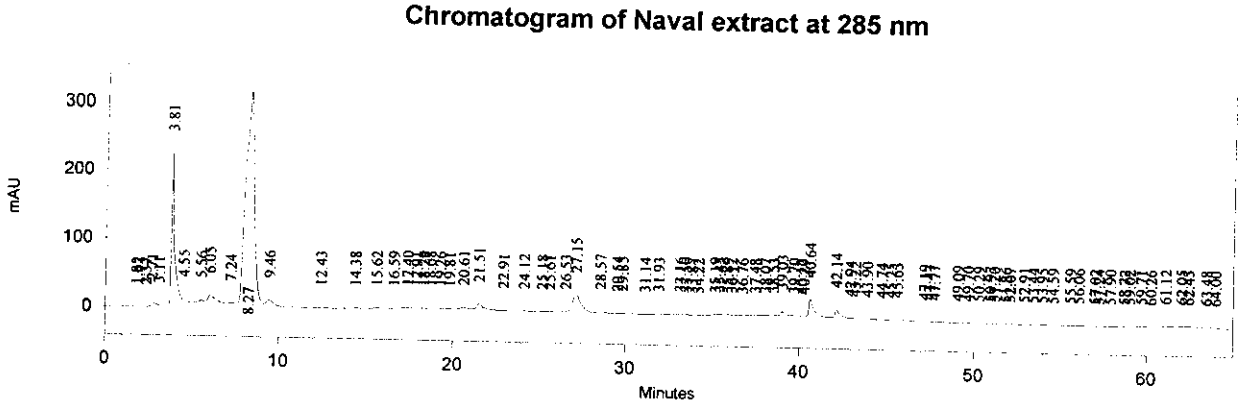


Fig 4.18 RP-HPLC chromatogram of *Syzygium cumini* at 285nm.



4.6 DIALYSIS

Dialysis experiment was performed to find out the type of inhibition shown by the inhibitor and the results showed that the inhibitor from *Psidium guajava and Syzygium cumini* seed extract were reversible in nature. The reversible inhibitor binds to the enzyme in a reversible manner without any formation of covalent bonds.

4.7 TIME DEPENDENT STUDY

The time dependent assay was performed for the aqueous extract of *Psidium guajava and Syzygium cumini*. To an ependroff, 500µl of the inhibitor extract, 800µl of buffer and 200µl of enzyme was added and incubated for 1 hr. Aliquots (300µl) was removed at every 10 min interval and inhibitor assay was performed. About 300µl was removed at 0 min and used as inhibitor control.

Table 4.19 Time dependent assay for *Psidium guajava*

Time(min)	% inhibition
10	68.00
20	65.33
30	57.60

Fig 4.19 Time dependent inhibitor assay for *Psidium guajava*

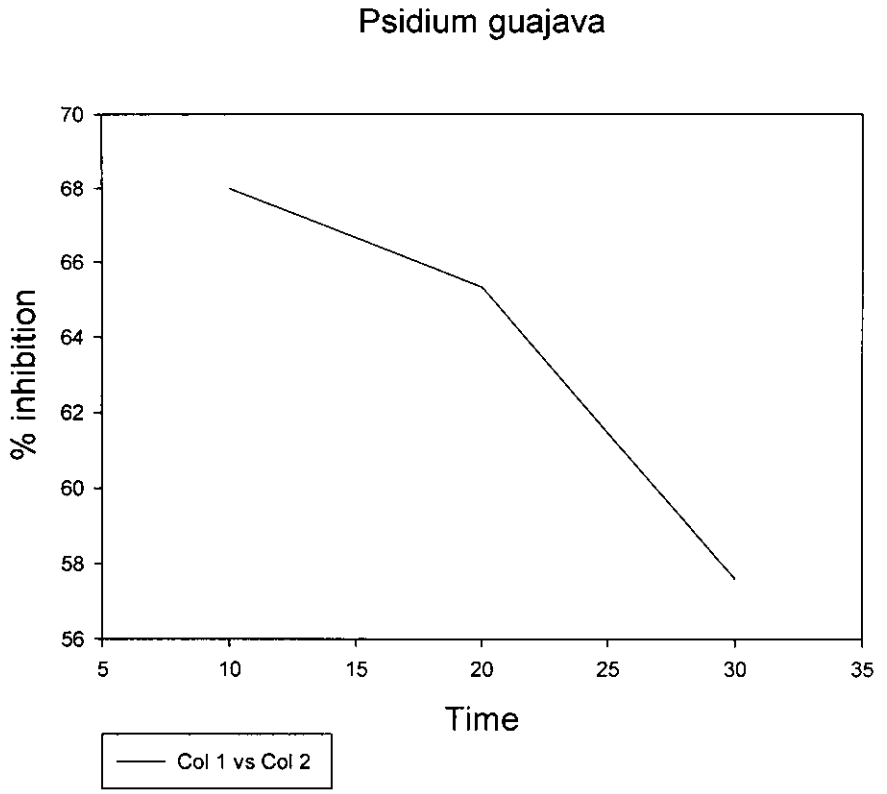
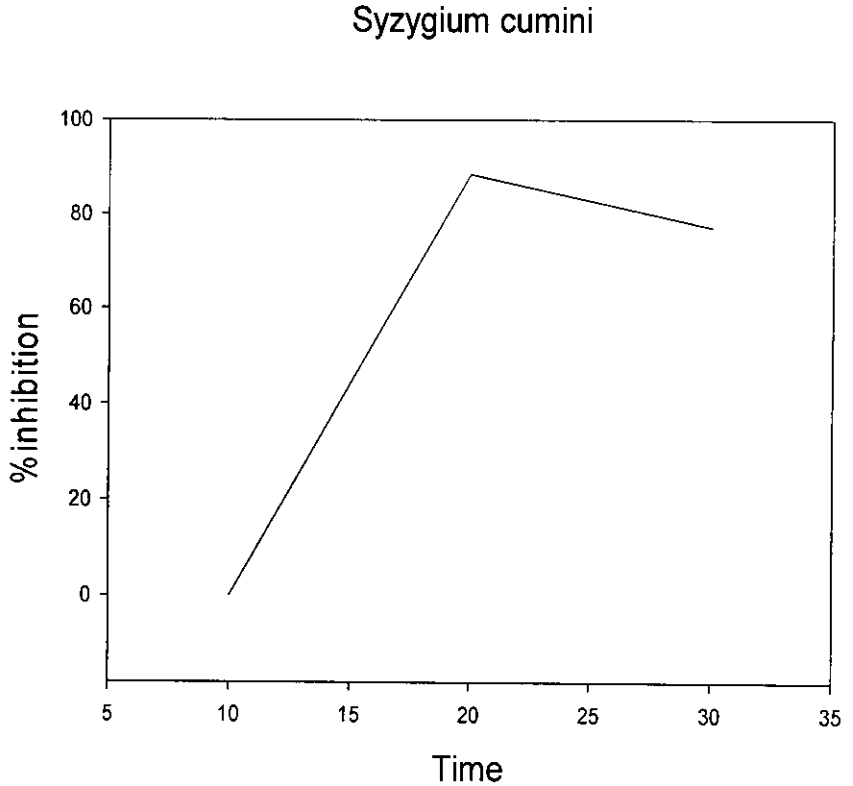


Table 4.20 Time dependent assay for *Syzygium cumini*

Time(min)	% inhibition
10	NO
20	88.53
30	77.33

Fig 4.20 Time dependent inhibitor assay for *Syzygium cumini*



Studies of α-glucosidase inhibition over time shows that the inhibition decreased linearly over time indicating that the inhibitor very probably acted reversibly.

4.8 COMBINATORIAL ASSAY

In an eppendroff, 0.5ml of Psidium guajava leaf extract and 0.5ml of Syzygium cumini seed extract were added and incubated for 1 hr. After incubation 0.5ml was removed from the eppendroff and α-glucosidase inhibitor assay was performed. Inhibition was found to be 86.72%

5. CONCLUSION & FUTURE PERSPECTIVES

CHAPTER 5

CONCLUSION

Natural α-glucosidase inhibitors from medicinal plants offer an attractive therapeutic approach to the treatment of diabetes by decreasing the absorption of glucose from small intestine. Powerful synthetic α-glucosidase inhibitors are available, but cause various side effects. Hence α-glucosidase inhibitor from medicinal plant extract are potentially safer. Out of 13 medicinal plants screened, we have identified a potent reversible α-glucosidase inhibitor from the aqueous extract of *Psidium guajava and Syzygium cumini* linn seed. The antidiabetic compound of the extract was then partially purified by preparative thin layer chromatography & column chromatography and it was finally checked for purity using RP-HPLC.

FUTURE PERSPECTIVE

The column chromatography purified sample was sent to Indian Institute of Technology (IIT), Madras to record FT-IR spectra and sample has also been sent to Central Drug Research Institute (CDRI), Lucknow to record LC-ESI-MS and H^1 NMR. The mass spectra of the separated compounds obtained through LC-ESI-MS will be used to identify the compounds. Further animal studies have to be carried out to check for the toxicity of the active compounds. The result of the present study suggests *Psidium guajava and Syzygium cumini* to have the potential role in the management of diabetes, based on its ability to inhibit α-glucosidase. Thus, there is possibility for the development of plant based medicine for treating diabetic patients in future, since potency of herbal drug is significant and they have negligible side effects than the synthetic anti-diabetic drugs.

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