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IDENTIFICATION OF STARCH BLOCKER FROM MEDICINAL PLANTS

A PROJECT REPORT

Submitted by

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&
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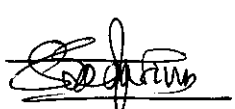
Certified that this project report entitled **"Identification of Starch Blocker from Medicinal Plants"** is the bonafide work of "**Mr. K. Karthic and Mr. K. S. Kirthi Ram**" who carried out the project work under my supervision.



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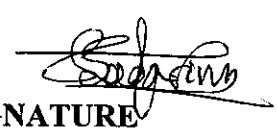

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ABSTRACT

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Diabetes is the chronic disease characterized by high blood glucose levels due to absolute relative deficiency of circulating insulin levels. Different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes mellitus. There is an increasing demand by patients to use the natural product with anti-diabetic activity as insulin cannot be used orally and continuous use of synthetic drugs causes side effects and toxicity. Starch blockers in natural source are considered for control of diabetes. We have identified starch blockers from certain medicinal plants. Starch blockers are substances that inhibit the digestive pancreatic α-amylase enzyme required to break down dietary starches for normal absorption, thereby reducing the usual rise in blood sugar (hyperglycemia) levels of both healthy people and diabetics. Among the medicinal plants the aqueous phase of *Syzygium cumini Linn* seeds showed potent inhibitor activity against the porcine pancreatic α-amylase enzyme. The anti-diabetic activity of the extract was then effectively enriched by preparative thin layer chromatography into fractions with different Rf values and it was finally checked for purity using RP-HPLC.

The dialysis experiment showed that the inhibition was reversible in nature. While characterizing the inhibition kinetics by Dixon plot it was found that the inhibition was non-competitive in nature .

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CHAPTER 1

INTRODUCTION

Diabetes is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by insulin. This results primarily in elevated fasting and postprandial blood glucose levels. If this imbalanced homeostasis does not return to normalcy and continues for a protracted period of time, it leads to hyperglycemia that in due course turns into a syndrome called diabetes mellitus. There are two main categories of this disease. Type 1 diabetes mellitus also called insulin-dependent diabetes mellitus (IDDM) and Type 2, the non insulin dependent diabetes mellitus (NIDDM). IDDM represents a heterogeneous and polygenic disorder, with a number of human leukocyte antigen complex (non-HLA) loci contributing to the disease susceptibility. This form of diabetes accounts for 5 to 10% of all cases and the incidence is rapidly increasing in specific regions. It is estimated that incidence of Type 1 diabetes will be about 40% higher in the year2010 than in 1997, and yet there is no identified agent substantially capable of preventing this type of disease. NIDDM is far more common and results from a combination of defects in insulin secretion and action. This type of disease accounts for 90 to 95% of all diabetic patients. Treatment of Type 2 diabetes is complicated by several factors inherent to the disease process, typically, insulin resistance, hyper insulinemia, impaired insulin secretion and reduced insulin-mediated glucose uptake. Utilization herbal drugs have served as a major source of medicines for the prevention and treatment of diabetes mellitus. It is estimated that more than 200 species of plants exhibit hypoglycemic properties. Synthetic hypoglycemic agents can produce serious side effects and are not suitable for use during pregnancy (Lamer 1985.).Therefore, the search for more effective and safer hypoglycemic compounds has continued to be an important area of active research, and after the recommendations made by WHO on diabetes mellitus, research on hypoglycemic compounds from medicinal plants has become an important aspect .

Recent advances in understanding of the activity of α-amylase and α-glucosidase have led to the development of the anti-diabetic drugs. The enzyme α-amylase (EC 3.2.1.1)

catalyzes the hydrolysis of the (1- 4) - α-D-glycosidic linkages of starch, amylose, amylopectin, glycogen, and various maltodextrins. Alpha-amylases are produced by a diverse variety of organisms: bacteria, fungi, plants, and animals. Two kinds of α-amylases are produced by many mammals, salivary α-amylase from the parotid gland and pancreatic α-amylase from the pancreas. The extracts from herbs are able to significantly inhibit the α-amylase enzyme and researchers are now trying to identify the specific active compounds which are responsible for this inhibition. When the active component has been isolated and characterized the scientist believe it should be possible to evaluate whether the active compound is likely to have advantages in terms of efficacy or side effects over currently marketed anti-diabetic drugs that interfere with starch digestion.

Some medicinal plants have been reported to show the effects of antidiabetic activity (Tiwori *et al.*, 2002). The present investigation was initiated with the following objectives,

1. Screening of medicinal plants for α- amylase inhibitory activity.
2. Isolation and purification of compounds showing α- amylase inhibitor activity.
3. To identify the type of inhibition showed by the α- amylase inhibitor.

REVIEW OF LITERATURE

CHAPTER 2

Review of literature

2.1 Diabetes

Diabetes is an inability to metabolize carbohydrates resulting from inadequate production or utilization of insulin. People with diabetes cannot properly process glucose, a sugar the body uses for energy. As a result, glucose stays in the blood, causing blood glucose to rise. At the same time, however, the cells of the body can be starved for glucose. Diabetes can lead to poor wound healing, higher risk of infections, and many other problems involving the eyes, kidneys, nerves, and heart.

2.2 Types of diabetes

The three main types of diabetes are

- Type 1 diabetes
- Type 2 diabetes
- Gestational diabetes

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. A person who has type 1 diabetes must take insulin daily to live. It develops most often in children and young adults but can appear at any age.

Symptoms of type 1 diabetes usually develop over a short period, although beta cell destruction can begin years earlier. Symptoms may include increased thirst and urination,

constant hunger, weight loss, blurred vision, and extreme fatigue. If not diagnosed and treated with insulin, a person with type 1 diabetes can lapse into a life-threatening diabetic coma, also known as diabetic ketoacidosis.

Type 2 Diabetes

The most common form of diabetes is type 2 diabetes. About 90 to 95 percent of people with diabetes have type 2. This form of diabetes is most often associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity, and certain ethnicities. About 80 percent of people with type 2 diabetes are overweight. Type 2 diabetes is increasingly being diagnosed in children and adolescents. When type 2 diabetes is diagnosed, the pancreas is usually producing enough insulin, but for unknown reasons the body cannot use the insulin effectively, a condition called insulin resistance. After several years, insulin production decreases. The result is the same as for type 1 diabetes—glucose builds up in the blood and the body cannot make efficient use of its main source of fuel.

The symptoms of type 2 diabetes develop gradually. Their onset is not as sudden as in type 1 diabetes. Symptoms may include fatigue, frequent urination, increased thirst and hunger, weight loss, blurred vision, and slow healing of wounds or sores. Some people have no symptoms.

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 percent chance of developing type 2 diabetes within 5 to 10 years. Maintaining a reasonable body weight and being physically active may help prevent development of type 2 diabetes.

As with type 2 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

2.3 Treatments of diabetes

Type 1 diabetes

Almost 100% of Type 1 diabetics use insulin .Insulin is now a widely used modern medicine and is now the main medicine used to treat Type 1 diabetes, usually starting immediately from diagnosis Type 2 diabetics do not usually need insulin at diagnosis.

Insulin pens is a device that looks like a pen with a cartridge. Some pens use replaceable cartridges of insulin; other pen models are totally disposable. A fine, short needle, similar to the needle on an insulin syringe, is on the tip of the pen. Users turn a dial to select the desired dose of insulin and press a plunger on the end to deliver the insulin just under the skin.

Insulin jet injectors send a fine spray of insulin through the skin by a high-pressure air mechanism instead of needles.

External insulin pumps connect to narrow, flexible plastic tubing that ends with a needle inserted just under the skin near the abdomen. The insulin pump is about the size of a deck of cards, weighs about 3 ounces, and can be worn on a belt or in a pocket. Users set the pump to give a steady trickle or "basal" amount of insulin continuously throughout the day. Most pumps today have the option for setting several basal rates. Pumps release "bolus" doses of insulin (several units at a time) at meals and at times when blood sugar is too high based on the users' programming. Frequent blood glucose monitoring is essential to determine insulin dosages and to ensure that insulin is delivered.

Transplantation

In recent years, researchers have focused increasing attention on transplantation for people with type 1 diabetes. Current procedures include:

- **Pancreas transplantation.** Pancreas transplants have been performed since the late 1960s. Most are done in conjunction with or after a kidney transplant. Kidney failure is one

of the most common complications of diabetes, and receiving a new pancreas when we receive a new kidney may actually improve kidney survival. Furthermore, after a successful pancreas transplant, many people with diabetes no longer need to use insulin. Unfortunately, pancreas transplants aren't always successful. Our body may reject the new organ days or even years after the transplant, which means we will need to take immunosuppressive drugs the rest of our life. These drugs are costly and can have serious side effects, including a high risk of infection and organ injury. Because the side effects can be more dangerous to our health than diabetes, On the other hand, pancreas transplantation may be an option if we are age 45 or younger, have type 1 diabetes and need or have had a kidney transplant, or if insulin doesn't control your blood sugar.

- **Islet cell transplantation.** The pancreas contains about 1 million islet cells, 75 percent to 80 percent of which produce insulin. The beta cells that produce insulin reside in the islets. Although still considered an experimental procedure, transplanting these cells may offer a less invasive, less expensive and less risky option than a pancreas transplant for people with diabetes. In islet cell transplantation, doctors infuse fresh pancreas cells into the liver of the person with diabetes. The cells spread throughout the liver and soon begin to produce insulin.

Treatment of type 2 diabetes

Controlling the blood sugar is essential to feeling healthy and avoiding long-term complications of diabetes. Some people are able to control their blood sugar with diet and exercise alone. Others may need to use insulin or other medications in addition to lifestyle changes.

A number of drug options exist for treating type 2 diabetes, including:

- **Sulfonylurea drugs.** These medications stimulate the pancreas to produce and release more insulin. Second-generation sulfonylureas such as glipizide (Glucotrol, Glucotrol XL), glyburide (DiaBeta, Glynase PresTab, Micronase) and glimepiride (Amaryl) are prescribed most often. The most common side effect of sulfonylureas is low blood sugar, especially during the first four months of therapy.
- **Meglitinides.** These medications, such as repaglinide (Prandin), have effects similar to sulfonylureas, but not as likely to develop low blood sugar. Meglitinides work quickly, and the results fade rapidly.

- **Biguanides.** Metformin (Glucophage, Glucophage XR) is the only drug in this class available in the United States. It works by inhibiting the production and release of glucose from the liver, which means we need less insulin to transport blood sugar into our cells. One advantage of metformin is that it tends to cause less weight gain than do other diabetes medications. Possible side effects include a metallic taste in our mouth, loss of appetite, nausea or vomiting, abdominal bloating, or pain, gas and diarrhea.
- **Alpha-glucosidase inhibitors.** These drugs block the action of enzymes in the digestive tract that break down carbohydrates. That means sugar is absorbed into the bloodstream more slowly, which helps prevent the rapid rise in blood sugar that usually occurs right after a meal. Drugs in this class include acarbose (Precose) and miglitol (Glyset). Although safe and effective, alpha-glucosidase inhibitors can cause abdominal bloating, gas and diarrhea. If taken in high doses, they may also cause reversible liver damage.
- **Thiazolidinediones.** These drugs make the body tissues more sensitive to insulin and keep the liver from overproducing glucose. Side effects of thiazolidinediones, such as rosiglitazone (Avandia) and pioglitazone hydrochloride (Actos), include swelling, weight gain and fatigue. A far more serious potential side effect is liver damage.
- **Drug combinations.** By combining drugs from different classes, we may be able to control the blood sugar in several different ways. Each class of oral medication can be combined with drugs from any other class. Most doctors prescribe two drugs in combination, although sometimes three drugs may be prescribed. Newer medications, such as Glucovance, which contains both glyburide and metformin, combine different oral drugs in a single tablet.

Treatment of gestational diabetes

Treatment of gestational diabetes involves eating a balanced diet and getting regular exercise to keep the blood sugar (glucose) levels within an acceptable range. Although a carefully balanced diet and/or insulin injections have been used to control gestational diabetes, there is new evidence that high insulin levels also can be damaging The list of treatments in Gestational diabetes includes the following

- Diet changes
- Exercise - with care and under medical advice; take extra care if using insulin.
- Diabetic blood sugar control

- Self monitored blood glucose testing
- Ketone testing
- Insulin

2.4 Natural Treatments for Diabetes

Most people with this type of diabetes are treated with diet, exercise and oral prescription drugs. Some people require the use of insulin to control blood sugar. Several alternative methods may help when used under medical supervision in conjunction with standard treatments. Once herbs and other measures for controlling blood sugar work, medication needs to be adjusted.

Diet

A balanced diet is recommended. Foods that emphasize vegetables, legumes, whole grains, and fiber can influence the release of sugar into the bloodstream. Foods that should be avoided are refined sugar, processed food, junk food, pastries, and cookies. All foods cause a varying insulin response in our bodies. Foods that have a higher rating on the "glycemic index", a scale developed by nutrition researchers at the University of Toronto, cause a higher insulin spike than those with a lower rating.

Higher glycemic index foods are white bread, bagels, English muffins, packaged flaked cereal, instant hot cereals, frozen desserts, dried fruit, whole milk, hot dogs, and luncheon meat. Lower glycemic index foods are most fresh vegetables, leafy greens, 100% whole grain bread, sweet potato, skim milk, buttermilk, chicken, lean cuts of beef, pork, and veal, white-fleshed fish, and many nuts. There are many factors that can influence glycemic index, such as cooking and preparation method.

Herbs, Nutritional Supplements and Vitamins

Fenugreek is a spice commonly used in India and the Middle East in cooking. Numerous studies suggest that fenugreek can reduce blood sugar and cholesterol levels in people with diabetes. Fenugreek seeds can have a bitter taste, so people sometimes prefer to take it in capsule form. A typical dose range is 5 to 30 g three times per day with meals. Known side effects of high doses include mild digestive distress. Fenugreek should not be used by pregnant or nursing women.

Gymnema is an Ayurvedic herb that is believed to have a marked effect on blood

sugar control. Gymnema is used for mild cases of type 2 diabetes, in conjunction with standard treatments. A typical dose range is 400 to 600 mg per day of an extract standardized to contain 24% gymnemic acids.

Chromium is an essential trace mineral. It plays a major role in sugar metabolism. There is a growing body of evidence that shows that chromium may help bring blood sugar levels under control in type 2 diabetes. A typical dose of chromium for diabetes is 200 to 400 mcg per day.

Vanadium is an essential trace mineral that may mimic insulin to help regulate blood sugar. It is found in black pepper, dill seed, and unsaturated vegetable oil, as well as in vitamin supplements.

Treatments using Diabetic pills

Diabetes pills in tablets form are a more common diabetes treatment than insulin injections, used mainly in the treatment of Type 2 diabetes instead of insulin. The majority of diabetics are Type 2 and are typically diagnosed over forty and often overweight. These pills are widely used in the treatment of Type 2 diabetes. Most Type 2 diabetics can use pills as the sole treatment for many years, though may require some insulin in later years as the diabetes progresses. A few Type 2 diabetics may require insulin at diagnosis and then go off insulin once they learn to control their diabetes with diet and pills. There are a variety of pills that help to control or reduce blood sugar levels. They are called "oral hypoglycemics" which just means pills taken by mouth (oral) that lower (hypo) the blood glucose (glycemia).

Diabetes pills do not contain insulin, but are medications that help in other ways. There are several different types of pills that attempt to control blood sugars different ways. Some of the different ways of controlling blood sugars using pills include:

- Sulfonylureas: Increasing insulin production by helping stimulate the pancreas to produce more insulin to overcome any deficiency or resistance. These were the only pills available prior to the 1990's and are the most widely used.
- Biguanides: Reducing "insulin resistance" to help the body use the insulin it has.
- Metformin: Slowing down the liver's output of extra sugars into the bloodstream
- Acarbose: Slowing down digestion of sugars and carbohydrates in the stomach or intestines.

Different pills or combinations of pills work for different people. Not only is everyone different, but there are also variations in Type 2 diabetes. Some Type 2 diabetics are predominantly "insulin resistant" which means they have plenty of insulin, but their muscles resist it and cannot use the insulin to process sugar properly. Other Type 2 diabetics are predominantly "insulin deficient" which means they do not produce enough insulin. And often there is some combination of insulin resistance and deficiency. Most pills have some side effects and each type of pill has its own specific side effects.

2.5 Anti-diabetic compounds from plants

Three novel sesquiterpene lactones, Lactucain A (1), B (2), and C (3), and a new furofuran lignan, lactucaside (4), were isolated from *Lactuca indica* along with nine known compounds, 11â, 13-dihydrolactucin, cichoriosides B, quercetin, quercetin 3-*O*-glucoside, rutin, apigenin, luteolin, luteolin7-*O*-glucuronide, and chlorogenic acid. Among these compounds, latucain C (3) and lactucaside (4) showed significant antidiabetic activity. Compound 1 was obtained as an amorphous powder. (Hou *et al.*, 2003)

Nelson *et al.*(2005) mentioned in their article aqueous or ethanol infusions of *Azorella compacta* (llareta) in common with many other plants have been used as antidiabetic in the popular medicine in the altiplanic region of Chile. In order to determine if the diterpenic compounds chemically elucidated and isolated from this plant are responsible for this effect, streptozotocin diabetic rats (507 ± 67 mg/mL glucose) were injected with two injections of diterpenic compounds mulinolic acid, azorellanol, and mulin-11,13-dien-20-oic acid at 180 mg/mL. Glycemia of animals treated with mulinolic acid and azorellanol was decreased to 243 ± 2 and 247 ± 14 mg/mL respectively, values very close to those reached by chlorpropamide injection used in controls. After 3 h treatment with mulin-11,13- dien-20-oic acid no effect was detected. The blood serum insulin in diabetic rats (146 ± 58 pg/mL) was lower than in control rats. After injection of azorellanol, insulin was elevated to 247 ± 23 pg/mL but with mulinolic acid, insulin was not changed. The antihyperglycemic effect of these compounds may explain the effectiveness of llareta in popular medicine. Because of the similarity to the hypoglycemic medication chlorpropamide, azorellanol could be acting on the â cells of pancreatic islets, while mulinolic acid may act upon glucose utilization or production in the liver.

The antidiabetic activity-guided fractionation and isolation of the 80% EtOH extracts from Peucedani Radix (*Peucedanum japonicum*, Umbelliferae) was carried out. The dried material (3.2 kg) was extracted with 80% aqueous EtOH (10 L×3) at room temperature for one week and at 60-70°C for two days. The resultant 80% EtOH extract (1.2 kg) was subjected to successive solvent partitioning to give $CHCl_3$ (PE-HC, 10 g), *n*-BuOH (PE-B, 20 g) and H2O (PE-W, 850 g) soluble fractions. The *n*- BuOH fraction showed significant antihyperglycemic activity. Thus, the *n*-BuOH extract (20 g) was chromatographed on a silica gel column using a gradient solvent system of EtOAc: MeOH: H_2O (9:2:0.5~0:1:0) to give four fractions (P1~P4). The P2 fraction (4 g) showed significant antidiabetic activity and was chromatographed on a silica gel column eluted with EtOAc:MeOH:H_2O (9:2:0.3) to give three subfractions (P2-1~P2-3). The hypoglycemic active P2-1 and P2-2 fractions were further chromatographed. The subfraction P2-1 (230 mg) was purified with RP Lobar®-A column (50% MeOH) and HPLC (reverse phase; RP, 45% MeOH) to yield 1 (3 mg). The subfraction P2-2 (1.6 g) was purified with Sephadex LH- 20 (MeOH) to give four subfractions (P2-21-P2-24). The second subfraction P2-22 (910 mg) was purified with RP Lobar®-A column (45% MeOH) to yield 2 (7 mg). (Lee *et al.*, 2004) In this results showed that coumarin and a cyclitol as active principles, that is, peucedanol 7-*O*-â-D-glucopyranoside (1) and *myo*-inositol (2). Their structures were identified by spectroscopic methods. Compound 1 showed 39% inhibition of postprandial hyperglycemia at 5.8 mg/kg dose, and compound 2 also significantly inhibited postprandial hyperglycemia by 34% ($P<0.05$)

Liu *et al.* (2005) investigated the effects of garlic oil and diallyl trisulfide on glycemic control in rats with streptozotocin-induced diabetes. Diabetic rats received by garlic oil (100 mg/kg body weight), diallyl trisulfide (40 mg/kg body weight), or corn oil every other day for 3 weeks. Control rats received corn oil only. Both garlic compounds significantly raised the basal insulin concentration. The insulin resistance index as assessed by homeostasis model assessment and the first-order rate constant for glucose disappearance were significantly improved by both garlic compounds ($P<0.05$). Oral glucose tolerance was also improved by both garlic compounds and was accompanied by a significantly increased rate of insulin secretion ($P<0.05$). Glycogen formation (but not that of lactate or carbon dioxide) from glucose by the soleus muscle in the presence of 10 or 100 AU/ml of insulin was

significantly better after treatment with both garlic compounds. Both garlic oil and diallyl trisulfide improve glycemic control in diabetic rats through increased insulin secretion and increased insulin sensitivity.

The actions of bis-benzylisoquinoline alkaloids isolated from Stephania tetrandrine were investigated in the hyperglycemia of STZ-diabetic mice. A main bis-benzylisoquinoline alkaloid, fangchinoline (0.3—3 mg/kg) significantly fell the blood glucose level of the diabetic mice in a dose-dependent manner. (Tsutsumi *et al.*, 2003) The effect of fangchinoline was 3.9-fold greater than that of water extract of Stephania. However, another main compound, tetrandrine (1—100 mg/kg) did not have any effect. The water extract of Astragali did not affect singly but potentiated the anti-hyperglycemic action of fangchinoline (0.3 mg/kg). Out of used compounds (1 mg/kg) isolated from Stephania, fangchinoline, fangchinoline 2_-*N*- a-oxide and 2_-*N*-norfangchinoline, which are substituted with 7-hydroxy side chain for 7-*O*-methyl side chain, decreased to near 50% of high blood glucose level. In addition, tetrandrine 2_-*N*- b-oxide, tetrandrine 2_-*N*- a-oxide, tetrandrine 2-*N*- b-oxide, fangchinoline 2_-*N*- a-oxide, which are added to 2- or 2_-*N*-oxide side chain, also decreased to near 50% of the high blood glucose level. In conclusion, fangchinoline but not tetrandrine from Stephania shows the anti-hyperglycemic action in the STZ-diabetic mice. The demethylation of 7-O-position and/or addition of 2- or 2_-*N*-oxide side chain in bis-benzylisoquinoline compounds in Stephania have a role for the induction of the anti-hyperglycemic actions.

Extracts of herbs with promising antidiabetic capacity, used in traditional medicine, have been analyzed by high-performance liquid chromatography (HPLC) coupled with diode-array\detection (DAD) *(Wang et al., 2005).* On the basis of the structures, solubility,and acid–base properties of the two compounds, the method was established with a C18 column and a mobile phase containing acetonitrile and aqueous phosphate buffer. The first mobile phase was a 10:90 binary mixture of aceto nitrile and buffer. When the same binary mixture in different proportions(15:85, 10:90, 5:95) was tested it was found that as the proportion of acetonitrile in the mobile phase was reduced the retention times of two compounds gradually increased. As a result, a mobilephase comprising a 10:90 (v/v) acetonitrile–0.05 M phosphate buffer (pH 3.2) binary mixture was finally adopted to achieve reasonable retention. Resolution, RS, of 3.29 for neomangiferin and 4.27for mangiferin was

achieved with retention factors, k, of 0.91 ± 0.05 for neomangiferin and 2.20 ± 0.04 for mangiferin, results which met the analytical criteria discussed above.The detection wavelength was chosen as a compromise between theand those of other interfering components of the mobile phase. Taking all things into consideration, a wavelength of 317 nm was finally selected for detection; this proved reasonable for simultaneous determination of neomangiferin and mangiferin in *A. asphodeloides* rhizome. With these chromatographic conditions baseline resolution was achieved with reasonable retention times and symmetrical peaks for the two compounds. Typical chromatograms obtained from a standard mixture of the two compounds Neomangiferin and Mangiferin.

2.6 Proteinaceous alpha-amylase inhibitor

Studies of the structures of the numerous enzyme inhibitors found in cereal grains have led to the recognition of a super family of homologous proteins which includes inhibitors of alpha-amylase, proteinase and bifunctional inhibitors active against two or more classes of enzymes (*Alam and Gourinath, 2001; Octavio and Rigden, 2002; Richardson, 1991*). The first alpha-amylase inhibitor determined was that of the monomeric 13 kD known as 0.31 form, from wheat *(Kashlan et al., 1981)*. Other dimeric 0.19, 0.23, 0.28, 0.53 forms of wheat inhibitors of exogenous alpha-amylase was later shown *(Oneda et al., 2004; Kondo and Ida, 1995; Roy and Gupta, 2000; Richardson, 1991; Octavio and Rigden, 2002)*.

The inhibitory effect of 0.19 á-amylase inhibitor (0.19 AI) from wheat kernel on the porcine pancreas á-amylase (PPA)–catalyzed hydrolysis of *p*-nitrophenyl--D-maltoside (*p*NP-G2) was examined. 0.19 AI is a homodimer of 26.6 kDa with 13.3-kDa subunits under the conditions used. The elution behaviors in gel filtration HPLC of PPA and 0.19 AI indicated that a PPA molecule bound with a 0.19 AI molecule (homodimer) at a molar ratio of 1:1. 0.19 AI inhibited PPA activity in a competitive manner with an inhibitor constant, K_i, of 57.3 nM at pH 6.9, 30°C, and the binding between them was found to be endothermic and entropy-driven. The activation energy for the thermal inactivation of 0.19 AI was determined to be 87.0 kJ/mol, and the temperature, T_{50}, giving 50% inactivation in a 30-min incubation at pH 6.9 was 88.1°C. The high inhibitory activity of 0.19 AI against PPA and its high thermal stability suggest its potential for use in the prevention and therapy of obesity and diabetes (Hiroshi *et al.*,2004).

Kidney beans, Phaseolus vulgaris, contain a proteinaceous inhibitor of alpha-amylase, which we have named phaseolamin. The inhibitor has been purified to homogeneity by conventional protein fractionation methods involving heat treatment, dialysis, and chromatography on DEAE-cellulose, Sephadex G-100, and CM-cellulose. Phaseolamin is specific for animal alpha-amylases, having no activity towards the corresponding plant, bacterial, and fungal enzymes, or any other hydrolytic enzyme tested. Optimal inhibitory activity is expressed during preincubation of enzyme and inhibitor at pH 5.5 and 37 degrees. Substrate prevents inhibition. Measurement of the stoichiometry on inhibition showed that a 1:1 complex of alpha-amylase and inhibitor is formed. Complex formation was demonstrated by chromatography on Sephadex G-100. The phaseolamin-amylase complex is dissociated at low pH values, apparently as a result of destruction of the enzyme; the complex cannot be dissociated by other conditions unfavorable for inhibition (low temperature or high pH). Phaseolamin inhibits hog pancreatic alpha-amylase in a noncompetitive manner. (Marshall *et al.*,1975).

Wiegand *et al.*(1995) determined the three-dimensional structure of PPA and its complexes with the pseudo-octasaccharide inhibitor V-1532 of the so called trestatin family from Streptomyces galbus and the proteinaceous inhibitor Tendamistat from Streptomyces tendae, and revealed the nature of their interactions responsible for their very high inhibition constants of 7.10-10 and 3.6.10-10 M respectively. PPA, a polypetide chain of 496 amino acid residues, exhibits a central A-domain a (beta/alpha)8 barrel, a B-domain between beta-stand 3 and alpha-helix 3, and a C-terminal domain C. In the Tendamistat complexed form about 30 % of the water accessible surface of Tendamistat is in contact with PPA. Four segments of the Tendamistat polypeptide chain (a total of 15 amino acid residues) are involved in binding. One segment containing the staggered side chains of the triplet Trp18, Arg19, Tyr20, typical for this class of inhibitors, binds to the catalytic site. The other segments fill out the groove in the PPA molecule; this groove is the substrate binding region and can also be occupied by the carbohydrate inhibitor V-1532. This region contains at least five subsites, which bind one glucose moiety each. This pseudo-oligosaccharide contain acarviosine (an N-linked pseudo-disaccharide) and a varying number of glucose residues. The arcaviosine binds to subsite 3 and 4, where the catalytic cleavage of the alpha-1,4-glycosidic bond of the natural substrate occurs. The comparison of the structures of free PPA,

PPA/V-1532 and PPA/Tendamistat showed the characteristic conformational changes, which accompany inhibitor binding and distinguish pseudo-oligosaccharide inhibitors from proteinaceous inhibitors. Although both classes of inhibitors block the sugar binding subsites in the active site region, the extreme specificity and binding affinity of the proteinaceous inhibitors is supposed to be due to an intricate interaction pattern involving areas farther apart from the catalytic center.

2.7 Non-Proteinaceous alpha-amylase inhibitor

The yield of methanolic extract and total phenol and non polar content of flowered parts from *Achillea ligustica* ALL. were reported. GC-MS analysis of the non polar fraction showed that the triterpene moretenol was the major constituent (17.228%) followed by stigmast-6-en-3 β-ol, veridiflorol and β-amyrin (7.524%, 5.078% 4.470%, respectively). The antioxidant activities of the methanolic extract and its fractions from *A. ligustica* were carried out using two different *in vitro* assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and lipid peroxidation of liposomes assay. Methanolic extract showed higher radical scavenging activity on DPPH (IC50 of 50 mg/ml). This activity is probably due to the phenolic fraction which shown an IC50 value of 22 mg/ml. A different result was obtained from the methanolic extract on the lipid peroxidation of liposomes (IC50 of 416 mg/ml). The α-Amylase inhibition assay was applied to evaluate antidiabetic activity. The methanolic extract showed weak activity (28.18% at 1 mg/ml) while the *n*-hexane fraction showed 74.96% inhibition at 250μg/ml. (Conforti *et al.*, 2005)

The extract of *Persea american* mill contained pharmacologically active compounds such as saponins, tannins, phlobatannins, flavonoids, alkaloids, and polysaccharides. The result of evaluation of antidiabetic activity of the extract of *p. american* 100–200 mg/kg) to alloxan-diabetic rats produced significant reduction ($P < 0.01$) in BGL in a dose-dependent manner. The extract of *Persea american* mill contained pharmacologically active compounds such as saponins, tannins, phlobatannins, flavonoids, alkaloids, and polysaccharides. The result of evaluation of antidiabetic activity of the extract of *p. american* 100–200 mg/kg) to alloxan-diabetic rats produced significant reduction ($P < 0.01$) in BGL in a dose-dependent manner.

Trivedi *et al.* (2004) induced diabetes in albino rats by administration of a single dose of alloxan monohydrate 5% (125 mg/kg). Effects of three different doses of shilajit (50, 100 and 200 mg/kg/day, orally), alone for 4 weeks and a combination of shilajit (100 mg/kg/day, orally) with either glibenclamide (5 mg/kg/day, orally) or metformin (0.5 g/kg/day, orally) for 4 weeks were studied on blood glucose and lipid profile.In the diabetic rats, all the three doses of shilajit produced a significant reduction in blood glucose levels and also produced beneficial effects on the lipid profile. The maximum effect was observed with the 100 mg/kg/day dose of shilajit. Combination of shilajit (100 mg/kg) with glibenclamide (5 mg/kg/day) or metformin (0.5 gm/kg/day) significantly enhanced the glucose lowering ability and improvement in lipid profile than any of these drugs given alone.

Saseha *et al.*(2002) investigated the selected enzymes (α- amylase, trypsin, and lysozyme) were allowed to react with some simple phenolic and related compounds (caffeic acid, chlorogenic acid, ferulic acid, gallic acid, m-, o-, and p-dihydroxybenzenes, quinic acid, and p-benzoquinone). The derivatized enzymes obtained were characterized in terms of their activity. In vitro experiments showed that the enzymatic activity of the derivatives was adversely affected. This enzyme inhibition depended on the reactivity of the phenolic and related substances tested as well as on the kind of substrate applied. The decrease in the activity was accompanied by a reduction in the amount of free amino and thiol groups, as well as tryptophan residues, which resulted from the covalent attachment of the phenolic and related compounds to these reactive nucleophilic sites in the enzymes. The enzyme inhibition correlates well with the blocking of the mentioned amino acid side chains.

Lili *et al.* (2004) studied the inhibition of tannic acid on human salivary amylase (HSA). Kinetic constants(K_{EI} K_{ESI}) show that tannin is as potent inhibitor of HSA as acarbose. For this reason , tannin is suggested to be tested for the prevention of dental caries.

Youn and Robyt examined the inhibition kinetics of acarbose analogues., Aspergillus oryzal, Barillus for the inhibition of four different α- amylase :human salivary and porcine pancreatic α- amylase. *Aspergillus oryzae, Bacillus amyloliquefaciens* , human salivary, and porcine pancreatic a-amylases. The three inhibitors showed mixed, noncompetitive inhibition, for all four a-amylases. The acarbose inhibition constants, Ki, for the four a-amylases were 270, 13, 1.27, and 0.80 mM, respectively; the Ki values for G6-Aca were 33, 37, 14, and 7 nM, respectively; and the G12-Aca Ki constants were 59, 81, 18, and 11 nM,

respectively. The G6-Aca and G12- Aca analogues are the most potent a-amylase inhibitors observed, with Ki values one to three orders of magnitude more potent than acarbose, which itself was one to three orders of magnitude more potent than other known α- amylase inhibitors.

MATERIALS & METHODS

CHAPTER-3

Materials and methods

3.1 Medicinal plants used in the study

Medicinal plants selected for the present study are given in table 1:

Table 1. List of medicinal plants used in the present study.

S.No	Common Name	Scienticfic Name	Family
1.	Ilanthapazham(tam)	*Zizyphus mauritiana Lam*	Rhamnaceae
2.	Vilvam(tam)	*Aegle marmelos*	Rutaceae
3.	Curry leaf	*Murraya koenigii*	Rutaceae
4.	Sirukurinchana(tam)	*Gymnema sylvestre*	Asclepiadaceae
5	Naval pazham(tam)	*Syzygium cumini Linn*	Mirtaceae
6.	Vilampazham(tam)	*Limonia acidissimia (new)* *Feronia elephantum (old)*	Rutaceae
7.	Avaram poo (tam)	*Cassia auriculata*	Cesalpinaceae
8.	Seenthil (tam)	*Tinospora cordifolia*	Menispermaceae
9.	Kuppaimeni (tam)	Acalypha indica	Euphorbaiaceae
10.	Fenugreek	*Trigonella foenum graecum*	Fabaceae
11	Drum stick	*Moringa oleifera*	Moringaceae
12.	Guava(tam)	*Psidium guajava Var. pomiferum*	Mirtaceae

3.2 Extraction of the leaf pigments

Fig-a: Extraction of *Syzygium cumini Linn* seeds

Chemicals and materials

- Petroleum ether
- Acetone
- NaCl, 10%
- Fresh leaves

Apparatus and glass wares

- Separating funnel, 500 mL
- Beaker, 100 mL
- Erlenmeyer flask
- Mortar & pestle
- Glass rod

Syzygium cumini Linn

Procedure

1. Using a pestle and mortar five grams of fresh leaves of the medicinal plants listed in table were ground with 22 mL acetone and 3 mL petroleum ether.

2. The extract was filtered using filter paper.

3. The filtrate was poured into a separating funnel and mixed with 20 mL of petroleum ether and 20 mL of 10% aqueous sodium chloride solution.

4. The separating funnel was shaken carefully. The layers were allowed to separate and the lower one was allowed to drain into a beaker. This phase was named as the aqueous extract. The upper layer was evaporated at room temperature and the same volume was made up with water. This upper layer was named as organic extract. The aqueous extract is again treated with the equal volume of ethyl acetate. Again the phases are allowed to separate using separating funnel. The aqueous extract is named as 2^{nd} aqueous phase. The organic extract is evaporated and is made up with the equal volume of water. It is named as 2^{nd} organic phase.

3.3 Preparation of Enzyme Working Standard

The porcine pancreatic α-amylase powder (Sigma A-3176) was used as the source of enzyme. The enzyme stock concentration of 1mg/mL was prepared initially using 2M-

phosphate buffer at pH-6.9. The working standard was prepared by mixing 3mL of the enzyme stock (1mg/mL) with 2mL of the phosphate buffer pH-6.9.

3.4 Assay of α-Amylase Inhibitor Activity

Materials

- Micro centrifuge tubes
- Pipettes
- Spectrophotometer
- Water bath

Reagents

- Starch solution, 1%..
- Dinitrosalicylic acid reagent (DNS reagent): One gram of dinitrosalicylic acid was dissolved in 20 ml of 2N NaOH and 50 ml water. Then 30 g potassium sodium tartarate was added and made up to 100mL with water.

Principle

This method was used to test the presence of free carbonyl group (C=O) which was present in the reducing sugars. This involves the oxidation of the aldehyde functional group; for example, aldehyde group in glucose and the ketone functional group in fructose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) was reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions:

Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself was not necessary for the color reaction, was added in the reagent to absorb the dissolved oxygen. However, it is was suspected that there were many side reactions, and the actual stoichiometry of the reaction was more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it was necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reaction such as the decomposition of sugar also competes for the availability of 3, 5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color. Although this was a convenient and relatively inexpensive

method, due to the relatively low specificity, one must run blanks diligently if the colorimetric results are to be interpreted correctly and accurately. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds were not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar was added to this sample. The increase in the absorbance upon the second color development was equivalent to the incremental amount of sugar added.

Procedure

1. Two micro centrifuge tubes were taken, one was labelled as control (C) and the other was labelled as test (T).
2. To the test 200 µL of the crude enzyme was added.
3. Next to both the micro centrifuge tubes 100 µL of the buffer (pH-7) was added.
4. Freshly prepared substrate (starch 1%) of 25 µL was added to both the tubes.
5. Then distilled water was added to all the tubes making the final volume to 600µL.

3.5 Optimization of Inhibitor Concentration

1. A series of ten micro centrifuge tubes were taken, five tubes were labeled as control (C1-C5), and other five test tubes were labeled as test (T1-T5)
2. Apart from this three other micro centrifuge tubes were taken and labeled as $C_{substrate}$, C_{enzyme}, $C_{activity}$ respectively.
3. To all the micro centrifuge tubes labeled from T1-T5, C_{enzyme} and $C_{activity}$.200 µL of the enzyme was added using micropipette.
4. To all the micro centrifuge tubes 100uL of the buffer was added
5. Next, to all the tests the crude inhibitor was added in the varying concentrations of 25 µL ie 25, 50, 75 µL etc and was also added to all control tubes.
6. Phosphate buffer at pH-6.9 was added to all the tubes making the total volume to 500 µL.
7. Then all the tubes were incubated in room temperature for 20mins.
8. Next 100 µL of 1% starch was added to all tubes except C_{enzyme} and the tubes were

incubated at room temperature for 5min.

9. After incubation 500 µL of freshly prepared DNS was added to all micro centrifuge tubes and the tubes were boiled in the water bath for 5min.
10. After heating in water bath the micro centrifuge tubes were vortex mixed and finally the absorbance was measured

3.6 Separation of Compounds by Thin Layer Chromatography (TLC)

Materials

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

Procedure

Preparation of TLC plates

1. A Clean 20 x 20 cm glass plates was taken. Before using it was rinsed well with detergent and then with water. After that the plates were dried and wiped well with tissue paper that was soaked with benzene.
2. For one plate about 20 g of silica gel G (or H) weighed and was transferred to a wide-mouthed conical flask.
3. To the conical flask about 30-35 mL water was added (Quantity of water may very 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 to 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 (diazotized sulphanic acid in better) and the spots were noted.
11. The Rf values of the spots were calculated and the corresponding silica gel fraction was scraped.
12. The scraped silica gel was extracted mixed with methanol and was centrifuged to get the clear filtrate. The filtrate was evaporated and was made up with water. This is used as the inhibitor source for the assay.

Solvent system used for separation of Phenolics, Terpenoids and Alkaloids

.Phenolics

- Hexane-ethyl acetate-acetic acid -20:20:10 and 23:20:7
- Ethyl acetate-acetic acid -70: 30 and 90:10

Terpenoids

- Hexane-Chloroform -7:3
- Chloroform-Methanol -7:3, 85:15, 95:5 and 96:4
- Methanol-water 90:10 and 95:5

Alkaloids

- Methanol-water 7:3

Spraying reagents that were used for different groups of compounds

Phenolics

Reagents

- Folin-Ciocalteau reagent
- Sodium carbonate, 20%

Procedure

1. The TLC plate from its chamber was taken and was allowed to air dry.
2. Initially the air dried plate was sprayed with Folin-Ciocalteau reagent which was diluted to 1N.
3. After spraying, the TLC plate was allowed to dry for 5min.
4. Finally the plate was sprayed with 20% Sodium carbonate.

5. The plate was allowed to air dry and was viewed.

Terpenoids

Reagents

- Vanillin in Con H_2SO_4, 10%
- Conc. H_2SO_4 + Ethanol (2:1): Ten gram of Vanillin powder was added to 100ml of above solution. The solution was prepared fresh before use

Procedure

1. The TLC plate from its chamber was taken and was allowed to air dry.
2. Initially the air-dried plate was sprayed with 10% Vanillin in Conc. H_2SO_4.
3. After spraying, the TLC plate was placed in the oven at 100°C for 5min.
4. Finally the plate was taken out and viewed

Alkaloids

Reagent

- Dragendorff's Reagent

a) Solution A: 1.7gm Bismuth Nitrate was dissolved in 100ml water: acetic acid(8:2)
Solution B: 40 g Potassium iodide dissolved in 100 ml water

b) Solution C: 20ml acetic acid in 70ml water
5ml of solution A and 5ml of solution B were added to solution C before use.

Procedure

1. The TLC plate from its chamber was taken and was allowed to air dry.
2. Initially the air dried plate was sprayed with Dragendorffs Reagent
3. After spraying, the TLC plate was allowed to dry for 5min.
4. Finally the plate was viewed in bright light.

General screening of compounds

TLC plates placed in iodine chamber and left for 5min.Iodine will visualize most of the compounds present on the plates. Appearance of brown colored spot indicates the presence of unsaturated compounds.

Screening for Phenolics

Plates sprayed with Folin-Ciocalteau reagent and dried for 5 min, then sprayed with 20% sodium carbonate blue colored spots appeared means phenolic compound will present.

Screening for Alkaloids

Dried TLC plated sprayed with Dragendroff's reagent. Presence of red, orange, yellow or brown spots indicates the presence of alkaloids.

Screening for Terpenoids

Plates sprayed with Vanillin-H_2SO_4 reagent and then heated to 110 C for 5 min. presence of red, pink, purple, blue and spots indicate compounds is terpenes.

3.7 RP-HPLC

The purified material obtained from thin layer chromatography was subjected to RP-HPLC to purify the á-amylase inhibitor to homogeneity.

Materials

- Shimadzu HPLC model RP 4a equipped with a variable length UV detector (wave-length set between 190 to 370.
- C18, 5µ column (250 x 4.6 mm).
- Mobile phase; 70:30 acetonitrile : water containing 0.1% trifluoro acetic acid (TFA).

Method

1. Fifty microlitre of TLC purified sample was injected into the sample introduction system.
2. The compounds were eluted employing a linear gradient of 70% acetonitrile in 0.1% TFA.
3. The elution time was set for 10 min.
4. The chromatogram of the sample was obtained.

3.8 Dialysis

Materials

- Dialysis bag
- Beaker, 500mL.
- Magnetic stirrer.

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 2ml.
2) The sample was made by adding enzyme 1ml, inhibitor 500µL and buffer 500 µL.
3) The dialysis bag was then immersed in the beaker containing 500ml of phosphate buffer of pH 6.9.
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 400 µL was taken in the micro centrifuge tube and it was marked as test.
7) 200 µL of enzyme, 100 µL of inhibitor and 100 µL of buffer was taken in another micro centrifuge tubes and it was marked as control.
8) 100 µL of starch was added in both test and control tubes
9) The tubes were allowed to incubate for 5min at room temperature.
10) Freshly prepared DNS of 500 µL was added to all the tubes and the tubes were heated in the water bath for 5min.
11) After incubation the micro centrifuge tubes were allowed to cool and mixed well.
12) Finally, the absorbance was measured at 540nm and the readings were tabulated.

3.9 Dixon plot

1. A series of ten micro centrifuge tubes were taken, five tubes were labeled as control (C1-C5), and other five test tubes were labeled as test (T1-T5)
2. Apart from this three other micro centrifuge tubes were taken and labeled as $C_{substrate}$, C_{enzyme}, $C_{activity}$ respectively.
3. To all the micro centrifuge tubes labeled from T1-T5, C_{enzyme} and $C_{activity}$.200 µL of the enzyme was added using micropipette.
4. To all the micro centrifuge tubes 100uL of the buffer was added
5. Next, to all the tests the crude inhibitor was added in the varying concentrations of 25 µL ie 25, 50, 75 µL etc and was also added to all control tubes.
6. Phosphate buffer at pH-6.9 was added to all the tubes making the total volume to 500 µL. Then all the tubes were incubated in room temperature for 20mins.
7. Next 100 µL of 1% starch was added to all tubes except C_{enzyme} and the tubes were incubated at room temperature for 5min.
8. After incubation 500 µL of freshly prepared DNS was added to all micro centrifuge tubes and the tubes were boiled in the water bath for 5min.
9. After heating in water bath the micro centrifuge tubes were vortex mixed and finally the absorbance was measured
10. The above steps are repeated for different substrate concentrations. Finally a graph is plotted between inhibitor concentration and 1/V

RESULTS & DISCUSSION

Chapter-4

Results and discussion

4.1 Medicinal plants under study

Medicinal plants such as *Zizyphus mauritiana Lam, Aegle marmelos, Murraya koenigii, Gymnema sylvestre, Syzygium cumini Linn, Limonia acidissimia (new), Cassia auriculata, Tinospora cordifolia, Acalypha indica, Trigonella foenum graecum, Moringa oleifera, and Psidium guajava Var. pomiferum* are chosen for our experiments because they have been used for the treatment of diabetes since ancient days.(Panda.H,"*Handbook on herbal drugs and its plant sources"*).

The plants such as *Syzygium cumini Linn* (Villasensor *et al* ., 2006) *Cassia auriculata.*(Abesundara *et al.*, 2004), *Trigonella foenum graecum* and *Zizyphus mauritiana Lam(*www.home-remedies-for-you.com/addnl_terms.html)are already reported to have the anti-hyperglyscemic activity. Hence they are chosen to test the inhibitor activity against porcine pancreatic á-amylase.

4.2 Assay of α-Amylase Inhibitor Activity

The inhibitor activities of the organic and aqueous phases of the extracts which are prepared from 12 medicinal plants are presented in Tables: 2-43. Among the 12 medicinal plants tested in the present study *Syzygium cumini* seeds, *Psidium guajava Var. pomiferum* leaves and *Cassia auriculata* flowers strongly inhibited the porcine pancreatic á-amylase.

The organic phase of *Cassia auriculata* contains the inhibitor compound and hence inhibited almost 98% of the amylase activity even at very low inhibitor concentration (25 ì L). On the contrary the aqueous phases of *Syzygium cumini* seeds and *Psidium guajava Var. pomiferum* leaves inhibited the amylase activity. Whereas the organic phase of *Psidium guajava Var. pomiferum* leaves showed moderate inhibition (25%). The inhibitor compounds may be present in very low concentration in *Limonia acidissimia* seeds(20% inhibition), *Trigonella foenum graecum* seeds(10% inhibition), *Zizyphus mauritiana Lam* seeds (12% inhibition), *Gymnema sylvestre* leaves (3% inhibition), *Aegle marmelos* leaves (6%

inhibition), *Moringa oleifera* leaves (16% inhibition), *Acalypha indica* leaves (15% inhibition) and *Tinospora cordifolia* leaves (13% inhibition) which showed only very low inhibition of α-amylase activity.

There was no inhibition seen in aqueous phase extracts of both *Murraya koenigii and Zizyphus mauritiana Lam.* Hence the presented results indicated that some of the inhibitory secondary metabolites present in the tested medicinal plants belong to lipophilic (organic phase) and/or hydrophilic (aqueous phase) group.

4.3 Optimization of Inhibitor Concentration

The inhibitors are optimized with varying concentrations of 25ì L, 50 ì L, 75 ì L, 100 ì L and125 ì L with fixed substrate concentration. The graph was drawn between percent inhibitions of the extracts of 12 medicinal plants and varying inhibitor concentrations.(Graphs A-S)

4.4 Separation of Compounds by Thin Layer Chromatography (TLC)

Thin layer chromatography was performed for the aqueous phase extract of *Syzygium cumini* seed. The extract from the seed was directly used as the source of inhibitor sample for TLC. Different Spraying reagents were used for the identification of phenolics, terpenoids and alkaloids. TLC plate that was sprayed with Dragendorffs reagent did not showed any spots indicating the absence of alkaloids. Whereas the plates sprayed with the vanillin-sulphuric acid and Folin-Ciocalteau reagent indicated the presence of terpenoids, phenolics respectively. Preparative TLC was done to obtain the purified compound in adequate concentration, based on the Rf value silica gel in the plate was scraped into different fractions. These fractions were mixed with the HPLC grade methanol and then centrifuged. The supernatant was evaporated and dissolved in known volume of water .When this was used as the source of inhibitor for the assay the inhibitor concentration was not sufficient to inhibit the enzyme. So the process was repeated by concentrating 20mL of extract to 2mL.

The solvent system usually employed for separating phenolics was used. The phenolic compounds of the concentrated *Syzygium cumini* extract was separated by TLC and based on the Rf value the plate was divided into eight fractions. Each fraction was tested for á-amylase inhibitor activity. The fraction with Rf value between 28.5% and 43% inhibited almost 100% and the other fraction whose Rf value ranging from 43 to 57% inhibited 92.57% of the amylase activity.

4.5 RP-HPLC

Using RP-HPLC the enriched fractions with the Rf values between 28.5 to 43% and 43 to 57% are checked for the purity of TLC separated sample. The chromatogram of the two different fractions are shown in Figure f & g.

.The running time was set to 10 mins .The compound in the Rf value between 28.5 to 43% was eluted in 3.2 min and the shouldering was observed at 2.8 min. .The compound in the Rf value between 43 to 57% was eluted in 3.2 min and the shouldering was observed at 3.5 min

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 *Syzygium cumini Linn* seed extract was reversible in nature. This reversible inhibitor bind to the enzyme in a reversible manner and was removed by dialysis. The dialysed fraction restored its full activity.

4.7 Dixon plot

The inhibition type was characterized by Dixon plot with fixed substrate concentration (50, 100 &150 ì L) and varying inhibitor concentration. The graph was drawn between 1/velocity and [I], it was found that the inhibitor was non-competitive in nature. The Dixon plot is shown in Figure-e

Discussion

Oliveira et. al. (2005) investigated the effects of the *Syzygium cumini Linn* extract on glycaemia of Diabetic and non diabetic mice. Crude, ethanolic and aqueous extracts of leaves of *Syzygium cumini* reduced glycaemia of non diabetic mice. However this effect was associated with the reduction of food intake and body weight which indicates that this may

not be a genuine hypoglycaemic effect. They carried out the experiments with the extracts from the leaves of *Syzygium cumini* and no attempt was made towards the seeds of *Syzygium cumini Linn.*

Syzygium cumini seed extracts have been used for the treatment of diabetes mellitus. Stanley Mainzen prince et al. (2003) studied the effect of *Syzigium cumini* seed extract for reducing the tissue damage in diabetic rat brain. The oral administration of an aqueous *Syzygium cumini* seed extract for 6-weeks caused a significant decrease in lipids, thiobarbituric acid reactive substances and an increase in catalase and superoxide dismutase in the brain of alloxan induced diabetic rats. The oral glucose tolerance test showed that the bark of *Syzygium cumini exhibits anti-hyperglycemic activity when fed simultaneously with glucose (Villaswenor IM et. al., 2006).* At the same dosage of 5mg/20gm mouse, *Syzygium* cumini treated mice showed a significant decrease in blood glucose levels (BGLs) at 30min (alpha=0.10) and from 45min onwards at alpha=0.5. However, to our knowledge, there was no study made on the porcine pancreatic á-amylase inhibition of *Syzygium cumini* seed extracts. In the present work we have examined the effect of *Syzygium cumini* seed extracts on porcine pancreatic á-amylase inhibition.

The methanol extract of dried flowers of *Cassia auriculata* was found to have a potential alpha-glucosidase activity. It showed inhibitory activity *in vitro* preferably on maltase giving on IC(50) value of 0.023mg/mL and inhibited the maltase activity competitively. As a result of single oral administration of *Cassia auriculata* methanol extract in Sprague-Dawley rats, a significant and potent lowering of 5mg/kg, thus, concurrently suppressed insulin activity. The inhibitory effect of *Cassia auriculata* was not performed in á-amylase which is similar to alpha-glucosidase.

Gordon *et al.* (2005) tested the polyphenol rich extracts from soft fruits for their ability to inhibit α-amylase and α-glucosidase. Strawberry and Raspberry extracts were effective α-amylase inhibitors than blue berry, 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*, *Psidium guajava Var. pomiferum* and *Cassia auriculata*s showed good inhibition against α-amylase.

Eighty percent ethanolic extract of Peucedani Radix (*Peucedanum japonicum*, Umbelliferae) was subjected to successive solvent partitioning to give $CHCl_3$, *n*-BuOH and H2O soluble fractions. The *n*- BuOH fraction showed significant antihyperglycemic activity. Thus, the *n*-BuOH extract was chromatographed on a silica gel column using a gradient solvent system of EtOAc: MeOH: H_2O (9:2:0.5~0:1:0) to give four fractions (P1~P4). The P2 fraction showed significant antidiabetic activity and was chromatographed on a silica gel column eluted with EtOAc:MeOH:H_2O (9:2:0.3) to give three subfractions (P2-1~P2-3). The hypoglycemic active P2-1 and P2-2 fractions were further chromatographed. The subfraction P2-1 was purified with RP Lobar®-A column (50% MeOH) and HPLC (reverse phase; RP, 45% MeOH). In our work the anti-diabetic activity of *Syzygium cumini* was effectively enriched by TLC into fractions and it was finally checked for purity using RP-HPLC.

Table-2: Inhibitor assay of *Limonia acidissimia* seeds (Organic phase)

Inhibitor source: *Limonia acidissimia* seeds **Phase:** Organic

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.128	0.130	0.131	0.1030	0.177	0.677	0.717	0.758	0.764	0.769	0.685

Table-3: Calculation of inhibition % of *Limonia acidissimia* seeds (organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.549	0.587	0.627	0.661	0.592
Activity% = (T-C)/C_{act}	80.114	85.69	91.53	96.49	75.9
Inhibition %= 1-{(T-C)/C_{act}} %	19.86	14.31	8.67	3.51	24.1

Table-4: Inhibitor assay of *Limonia acidissimia* seeds (Aqueous phase)

Inhibitor source: *Limonia acidissimia* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.076	0.08	0.08	0.074	0.072	0.985	0.923	0.910	0.919	0.905	0.913

Table-5:Calculation of inhibition % of *Limonia acidissimia* seeds (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.909	0.843	0.83	0.845	0.833
Activity% = (T-C)/C_{act}	99.56	92.33	90.90	92.55	91.23
Inhibition % = 1-{(T-C)/C_{act}} %	0.44	7.67	9.1	7.45	8.77

Table-6: Inhibitor assay of *Trigonella foenumgraecum* seeds (Aqueous phase)

Inhibitor source: *Trigonella foenumgraecum* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.131	0.153	0.203	0.224	0.246	0.710	0.685	0.759	0.779	0.772	0.58

Table-7: Calculation of inhibition % of *Trigonella foenumgraecum* (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.579	0.532	0.556	0.555	0.526
Activity% = (T-C)/C_{act}	99.82	91.72	95.86	95.68	90.68
Inhibition % = 1-{(T-C)/C_{act}} %	0.18	8.28	4.14	4.32	9.32

Table-8: Inhibitor assay of *Trigonella foenumgraecum* seeds (Organic phase)

Inhibitor source: ***Trigonella foenumgraecum*** **Phase:** Organic

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.137	0.121	0.147	0.130	0.142	0.694	0.605	0.618	0.655	0.652	0.524

Table-9: Calculation of inhibition % of *Trigonella foenumgraecum* seeds (Organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.557	0.484	0.471	0.525	0.51
Activity% = (T-C)/C_{act}	106.29	92.36	89.88	100	97.32
Inhibition % = 1-{(T-C)/C_{act}} %	0	7.64	10.12	0	3.68

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Table-10: Inhibitor assay of *Murraya koenigii* leaves (Aqueous phase)

Inhibitor source: *Murraya koenigii* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.251	0.23	0.251	0.279	0.309	0.990	1.043	1.039	1.027	1.033	0.671

Table-11: Calculation of inhibition % of *Murraya koenigii* leaves (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.739	0.813	0.788	0.748	0.724
Activity% = $(T-C)/C_{act}$	110%	123%	117.4%	111.47%	107%
Inhibition % = $1-\{(T-C)/C_{act}\}$ %	0%	0%	0%	0%	0%

Table-12: Inhibitor assay of *Murraya koenigii* leaves (Organic phase)

Inhibitor source: ***Murraya koenigii*** **Phase:** Organic

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.216	0.202	0.190	0.2	0.197	0.659	0.669	0.772	0.773	0.762	0.659

Table-13: Calculation of inhibition % of *Murraya koenigii* leaves (Organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.443	0.467	0.582	0.573	0.565
Activity% = (T-C)/C_{act}	67.2	70.86	88.31	86.94	85.73
Inhibition % = 1-{(T-C)/C_{act}} %	32.8	29.14	11.69	13.06	14.27

Table-14: Inhibitor assay of *Zizyphus mauritiana Lam* seeds (Organic phase)

Inhibitor source: *Zizyphus mauritiana Lam* **Phase:** Organic

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.094	0.095	0.098	0.1	0.098	0.71	0.72	0.699	0.710	0.708	0.694

Table-15: Calculation of inhibition % of *Zizyphus mauritiana Lam* seeds (Organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.616	0.625	0.601	0.61	0.61
Activity% = (T-C)/C_{act}	88.76	90.05	86.59	87.89	87.89
Inhibition % = 1-{(T-C)/C_{act}} %	11.24	9.25	13.41	12.11	12.11

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Table-16 Inhibitor assay of *Zizyphus mauritiana Lam* seeds (Aqueous phase)

Inhibitor source: *Zizyphus mauritiana Lam* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.138	0.172	0.216	0.254	0.277	0.738	0.888	0.841	0.849	0.857	0.486

Table-17: Calculation of inhibition % of *Zizyphus mauritiana Lam* seeds (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.6	0.716	0.625	0.595	0.58
Activity% = (T-C)/C_{act}	123.4	147.3	128.6	122.4	119.3
Inhibition % = 1-{(T-C)/C_{act}} %	0	0	0	0	0

Table-18: Inhibitor assay of *Gymnema sylvestre leaves* (Aqueous phase)

Inhibitor source: *Gymnema sylvestre* **phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.152	0.195	0.231	0.285	0.318	0.808	0.842	0.892	0.939	0.997	0.6755

Table-19: Calculation of inhibition % of *Gymnema sylvestre* leaves (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.66	0.65	0.66	0.65	0.68
Activity% = (T-C)/C_{act}	97	96	97	96	100
Inhibition % = 1-{(T-C)/C_{act}} %	3	4	3	3	0

Table-20: Inhibitor assay of *Aegle marmelos leaves* (Aqueous phase)

Inhibitor source: *Aegle marmelos* **phase: Aqueous**

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.151	0.166	0.191	0.202	0.230	0.76	0.758	0.785	0.8	0.810	0.6375

Table-21: Calculation of inhibition % of *Aegle marmelos leaves* (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.609	0.592	0.594	0.598	0.58
Activity% = (T-C)/C_{act}	95.52	92.86	93.17	93.80	90.98
Inhibition % = 1-{(T-C)/C_{act}} %	4.48	7.14	6.83	6.2	9.02

Table-22: Inhibitor assay of *Moringa oleifera* leaves (Aqueous phase)

Inhibitor source: *Moringa oleifera* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.147	0.193	0.222	0.239	0.27	0.986	0.964	0.942	0.917	0.875	0.8145

Table-23: Calculation of inhibition % of *Moringa oleifera* leaves (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.84	0.77	0.72	0.68	0.61
Activity% = (T-C)/C_{act}	104	95	89	84	75
Inhibition % = 1-{(T-C)/C_{act}} %	0	5	11	16	25

Table-24: Inhibitor assay of *Cassia auriculata* flowers (Aqueous phase at room temp)

Inhibitor source: *Cassia auriculata* **Phase:** Aqueous at Room temperature

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	380	360	340	320	300	180	160	140	120	100	200
3	Inhibitor	20	40	60	80	100	20	40	60	80	100	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.148	0.155	0.188	0.194	0.225	0.847	0.690	0.533	0.453	0.377	0.567

Table-25: Calculation of inhibition % of *Cassia auriculata* flowers (Aqueous phase at room temp)

Calculations	T1	T2	T3	T4	T5
Test -control	0.699	0.535	0.345	0.259	0.152
Activity%=(T-C)/C_{act} %	123	94.3	60.8	45.6	26.8
Inhibition %= 1-{(T-C)/C_{act}} %	0	5.7	39.2	54.4	73.2

Table-26: Inhibitor assay of *Cassia auriculata* flowers (2nd Aqueous phase at 0-4^{0}C)

Inhibitor source: *Cassia auriculata* **Phase:** 2nd Aqueous phase at 0-4^{0}C

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	380	360	340	320	300	180	160	140	120	100	200
3	Inhibitor	20	40	60	80	100	20	40	60	80	100	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.124	0.139	0.138	0.153	0.178	0.7	0.579	0.534	0.466	0.44	0.409

Table-27: Calculation of inhibition % of *Cassia auriculata* flowers (2nd Aqueous phase at 0-4^{0}C)

Calculations	T1	T2	T3	T4	T5
Test -control	0.576	0.44	0.396	0.313	0.262
Activity%=(T-C)/C_{act} %	140	107	96.82	76.52	64.05
Inhibition %= 1-{(T-C)/C_{act}} %	0	0	3.18	23.48	35.95

Table-28: Inhibitor assay of *Cassia auriculata* flowers (2nd Organic phase at 0-4^{0}C)

Inhibitor source: *Cassia auriculata* **Phase:** Ethyl acetate stored at 0-4^{0}C (2nd organic)

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	380	360	340	320	300	180	160	140	120	100	200
3	Inhibitor	20	40	60	80	100	20	40	60	80	100	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.143	0.189	0.292	0.336	0.402	0.184	0.230	0.326	0.368	0.431	0.539

Table-29: Calculation of inhibition % of *Cassia auriculata* flowers (2nd Organic phase at 0-4^{0}C)

Calculations	T1	T2	T3	T4	T5
Test -control	0.041	0.041	0.034	0.032	0.029
Activity%=(T-C)/C_{act} %	9.6	9.6	5.31	5.93	5.38
Inhibition %= 1-{(T-C)/C_{act}} %	92.39	92.39	93.69	94.06	94.6

ethyl acetate

Table-30: Inhibitor assay of *Cassia auriculata* flowers (2nd Organic phase at room temp)

Inhibitor source: *Cassia auriculata* **Phase:** Ethyl acetate at room temperature

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	380	360	340	320	300	180	160	140	120	100	200
3	Inhibitor	20	40	60	80	100	20	40	60	80	100	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.214	0.318	0.406	0.509	0.620	0.225	0.527	0.45	0.512	0.640	0.569

Table-31: Calculation of inhibition % of *Cassia auriculata* flowers (2nd Organic phase at room temp)

Calculations	T1	T2	T3	T4	T5
Test -control	0.011	0.209	0.044	0.005	0.02
Activity%=(T-C)/C_{act} %	1.93	36.73	7.73	0.878	3.51
Inhibition %= 1-{(T-C)/C_{act}} %	98	63.26	92.26	99.12	96.48

ethyl acetate

Table-32: Inhibitor assay of *Cassia auriculata* flowers (2nd Organic phase)

Inhibitor source: *Cassia auriculata* **Phase:** Ethyl acetate (2nd organic)

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	380	360	340	320	300	180	160	140	120	100	200
3	Inhibitor	20	40	60	80	100	20	40	60	80	100	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.166	0.204	0.260	0.314	0.346	0.18	0.248	0.289	0.323	0.384	0.538

Table-33: Calculation of inhibition % of *Cassia auriculata* flowers (2nd Organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.014	0.044	0.029	0.009	0.038
Activity%=(T-C)/C_{act} %	2.60	8.17	5.39	1.67	7.06
Inhibition %= 1-{(T-C)/C_{act}} %	97.39	91.82	94.60	98.32	92.93

ethyl acetate

Table-34: Inhibitor assay of *Cassia auriculata* flowers (1st Organic phase)

Inhibitor source: *Cassia auriculata* **Phase:** Organic

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.083	0.080	0.080	0.086	0.097	0.673	0.653	0.671	0.681	0.661	0.627

Table-35:Calculation of inhibition % of *Cassia auriculata* flowers (1st Organic phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.59	0.573	0.591	0.595	0.564
Activity% = (T-C)/C_{act}	94.09%	91.38%	94.25%	94.89%	89.9%
Inhibition % = 1-{(T-C)/C_{act}} %	5.91%	8.62%	5.75%	5.11%	10.1%

Table-36: Inhibitor assay of *Syzygium cumini Linn* seeds (Aqueous phase)

Inhibitor source: Syzygium cumini Linn **Phase:** Aqueous phase

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.240	0.363	0.480	0.572	0.674	0.432	0.447	0.521	0.602	0.628	0.873

Table-37: Calculation of inhibition % of *Syzygium cumini Linn* seeds (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.192	0.084	0.041	0.03	0
Activity% = (T-C)/C_{act}	21.99%	9.62%	4.696%	3.43%	0%
Inhibition % = 1-{(T-C)/C_{act}} %	78%	90.37%	95.30%	96.56%	100%

Table-38: Inhibitor assay of *Acalypha indica* leaves (Aqueous phase)

Inhibitor source: *Acalypha indica* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.120	0.124	0.129	0.138	0.177	0.672	0.702	0.730	0.748	0.754	0.685

Table-39: Calculation of inhibition % *Acalypha indica* leaves(Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.552	0.578	0.601	0.610	0.577
Activity% = (T-C)/C_{act}	80.58	84.37	87.7	89.05	84.23
Inhibition % = 1-{(T-C)/C_{act}} %	19.42	15.63	12.3	10.95	15.77

Table-40: Inhibitor assay of *Tinospora Cordifolia* leaves (Aqueous phase)

Inhibitor source: *Tinospora Cordifolia* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	C5 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	T5 (µL)	$C_{activity}$ (µL)
1	Enzyme	-	-	-	-	-	200	200	200	200	200	200
2	Buffer (PH-7)	375	350	325	300	275	175	150	125	100	75	200
3	Inhibitor	25	50	75	100	125	25	50	75	100	125	-
	Incubation at room temp for 20 min											
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min											
5	DNS	500	500	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min											
6	Absorbance at 540nm	0.076	0.083	0.089	0.092	0.097	0.67	0.651	0.653	0.637	0.626	0.633

Table-41: Calculation of inhibition % of *Tinospora Cordifolia* leaves (Aqueous phase)

Calculations	T1	T2	T3	T4	T5
Test -control	0.5940.568	0.568	0.564	0.545	0.539
Activity% = (T-C)/C_{act}	93.83	89.73	89.09	86.09	85.15
Inhibition % = 1-{(T-C)/C_{act}} %	6.17	10.27	10.91	13.91	14.85

Table-42: Inhibitor assay of different extracts of *Psidium guajava Var. pomiferum*

Inhibitor source: *Psidium guajava Var. pomiferum*

S.No	Reagents	C1 (µL)	T1 (µL)	C2 (µL)	T2 (µL)	C3 (µL)	T3 (µL)	$C_{activity}$ (µL)
	Extract	Aqueous extract		Organic extract		Water extract		
1	Enzyme	-	200	-	200	-	200	200
2	Buffer (PH-7)	300	100	300	100	300	100	200
3	Inhibitor	100	100	100	100	100	100	-
	Incubation at room temp for 20 min							
4	Substrate (1% starch)	100	100	100	100	100	100	100
	Incubation at room temp for 5 min							
5	DNS	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min							
6	Absorbance at 540nm	0.539	0.509	0.129	1.206	0.660	0.620	1.1445

Table-43: Calculation of inhibition % of different extracts of *Psidium guajava Var. pomiferum*

Calculations	T1	T2	T3
Test -control	-	1.077	-
Activity% = (T-C)/C_{act}	0	74.53	0
Inhibition % = 1-{(T-C)/C_{act}} %	100	25.46	100

Table-44: Dialysis of *Syzygium cumini Linn* seed inhibitor

Reagents	C1 (µL)	T1 (µL)	$C_{activity}$ (µL)
Enzyme	-	200	200
Buffer (PH-7)	300	100	200
Inhibitor	100	100	-
Incubate for 20 min at room temperature			
Substrate (1% starch)	100	100	100
Incubate at room temperature for 5 min			
DNS	500	500	500
Keep at boiling water bath for 5 min			
Absorbance at 540nm	0.027	0.636	0.632

Table-45: Dixion plot of *Syzygium cumini Linn* seed inhibitor

Substrate Concentatation (Starch, 1%): 50ì L

Inhibitor Conc (ì L).	1/V
25	.848
50	1.1
75	1.54
100	2.65
125	5.24

Substrate Concentatation (Starch, 1%): 100ì L

Inhibitor Conc (ì L).	1/V
25	1.727
50	2.481
75	4.291
100	8.849
125	10.10

Substrate Concentatation (Starch, 1%): 150ì L

Inhibitor Conc (ì L)..	1/V
25	.43
50	.60
75	1.59
100	8.92
125	25.64

Table-46: Inhibitor assay of different TLC fractions of *Syzygium cumini Linn* seeds (Aqueous phase)

Inhibitor source: *Syzygium cumini Linn* **Phase:** Aqueous

S.No	Reagents	C1 (µL)	C2 (µL)	C3 (µL)	C4 (µL)	T1 (µL)	T2 (µL)	T3 (µL)	T4 (µL)	$C_{activity}$ (µL)
	Rf fraction %	0-14.2	14.2-28.5	28.5-43	43-57	0-14.2	14.2-28.5	28.5-43	43-57	
1	Enzyme	-	-	-	-	100	100	100	100	100
2	Buffer (PH-7)	300	300	300	300	200	200	200	200	300
3	Inhibitor	100	100	100	100	100	100	100	100	-
	Incubation at room temp for 20 min									
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min									
5	DNS	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min									
6	Absorbance at 540nm	0.117	0.277	0.074	0.053	0.189	0.361	0.074	0.121	0.914

Table-47: Calculation of inhibition % of different TLC fractions of *Syzygium cumini Linn* seeds

Calculations	T1	T2	T3	T4
Test -control	0.072	0.084	0	0.068
Activity% = (T-C)/C_{act}	7.87	9.19	0	7.43
Inhibition % = 1-{(T-C)/C_{act}} %	92.13	90.81	100	92.57

Table-48: Inhibitor assay of different TLC fractions of *Syzygium cumini* seeds (Aqueous phase)

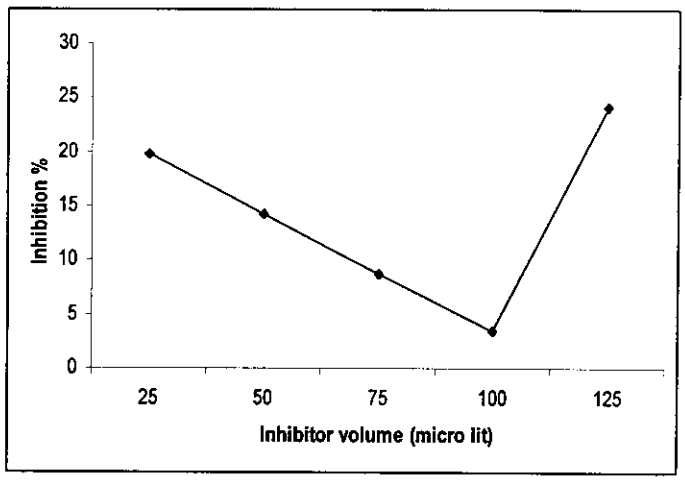
Inhibitor source: *Syzygium cumini Linn* **Phase:** Aqueous

S.No	Reagents	C5 (µL)	C6 (µL)	C7 (µL)	C8 (µL)	T5 (µL)	T6 (µL)	T7 (µL)	T8 (µL)	$C_{activity}$ (µL)
	Rf fraction %	57-71	71-86	86-100	>100	57-71	71-86	86-100	>100	
1	Enzyme	-	-	-	-	100	100	100	100	100
2	Buffer (PH-7)	300	300	300	300	200	200	200	200	300
3	Inhibitor	100	100	100	100	100	100	100	100	-
	Incubation at room temp for 20 min									
4	Substrate (1% starch)	100	100	100	100	100	100	100	100	100
	Incubation at room temp for 5 min									
5	DNS	500	500	500	500	500	500	500	500	500
	Keep in boiling water bath for 5 min									
6	Absorbance at 540nm	0.058	0.068	0.057	0.076	0.139	0.134	0.372	0.623	0.914

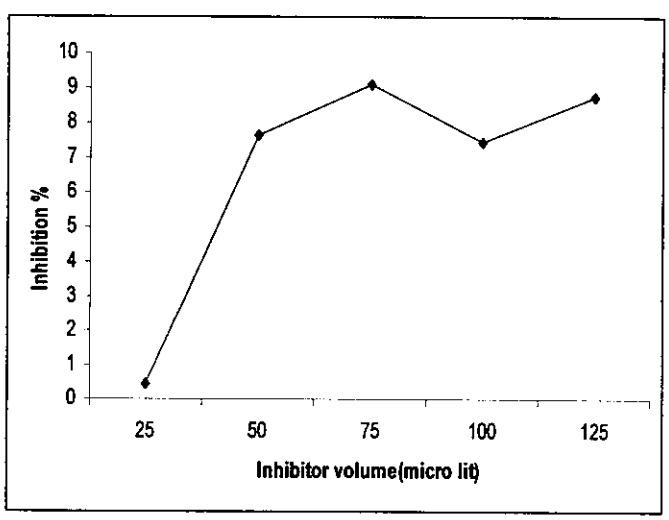
Table-49: Calculation of inhibition % of different TLC fractions of *Syzygium cumini Linn* seeds

Calculations	T5	T6	T7	T8
Test -control	0.081	0.066	0.315	0.547
Activity% = $(T-C)/C_{act}$	8.86	7.22	34.46	59.84
Inhibition % = $1-\{(T-C)/C_{act}\}$ %	91.14	92.78	65.54	40.16

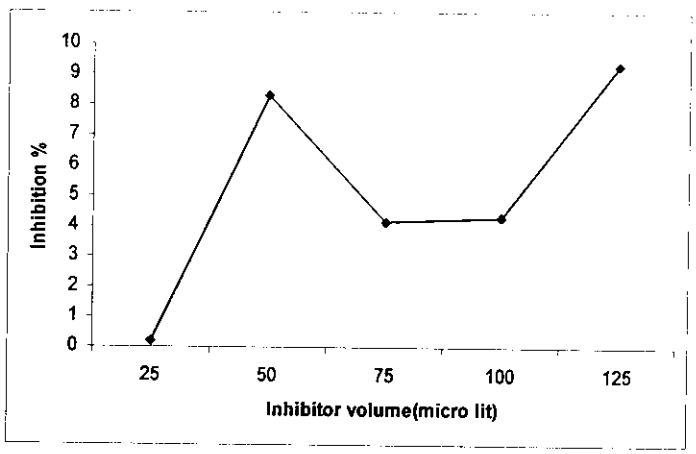
Graph-A Inhibition % Vs Inhibitor volume of *Limonia acidissimia* seeds (Organic phase)



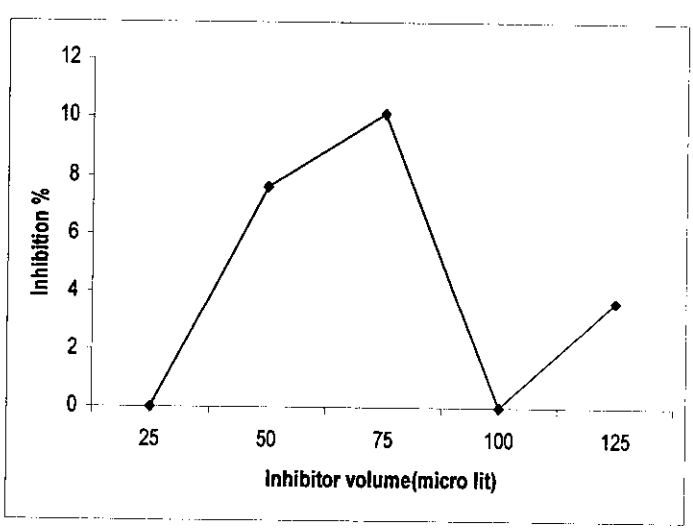
Graph-B Inhibition % Vs Inhibitor volume of *Limonia acidissimia* seeds (Aqueous phase)



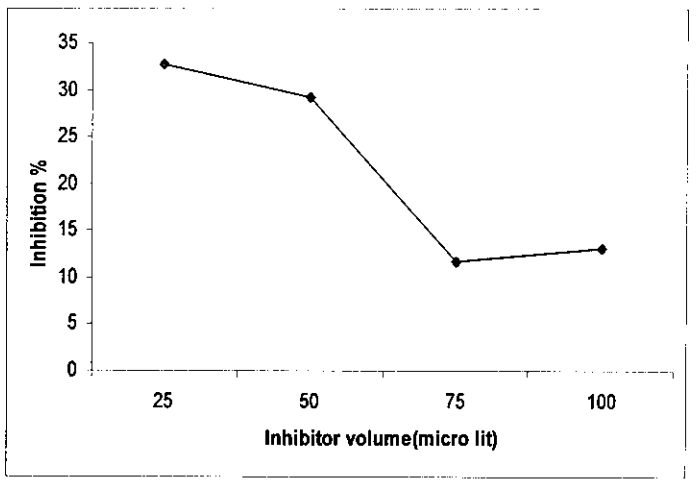
Graph-C Inhibition % Vs Inhibitor volume of *Trigonella foenumgraecum* (Aqueous phase)



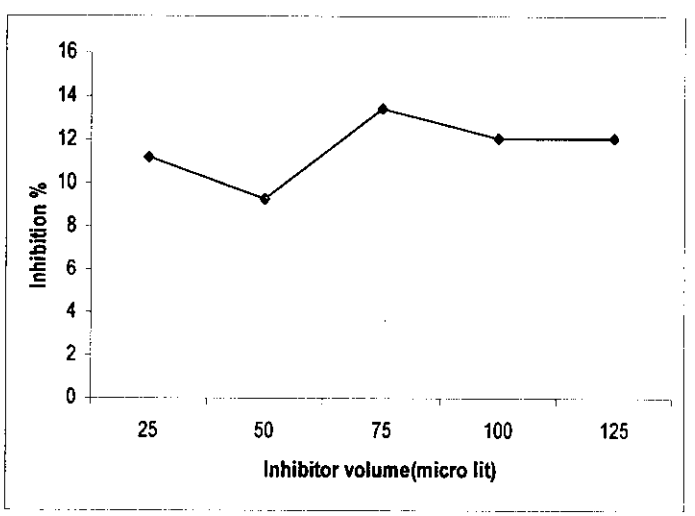
Graph-D Inhibition % Vs Inhibitor volume of *Trigonella foenum graecum* seeds (Organic phase)



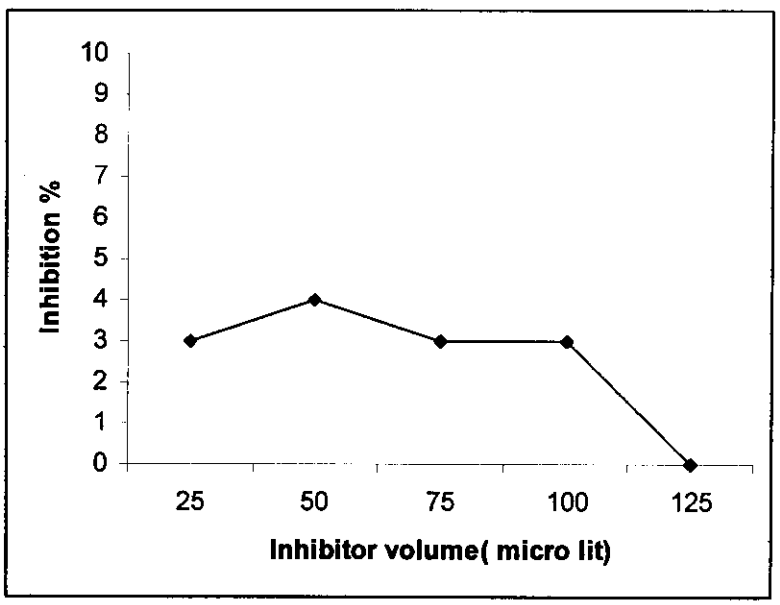
Graph-E: Inhibition % Vs Inhibitor volume of *Murraya koenigii* leaves (Organic phase)



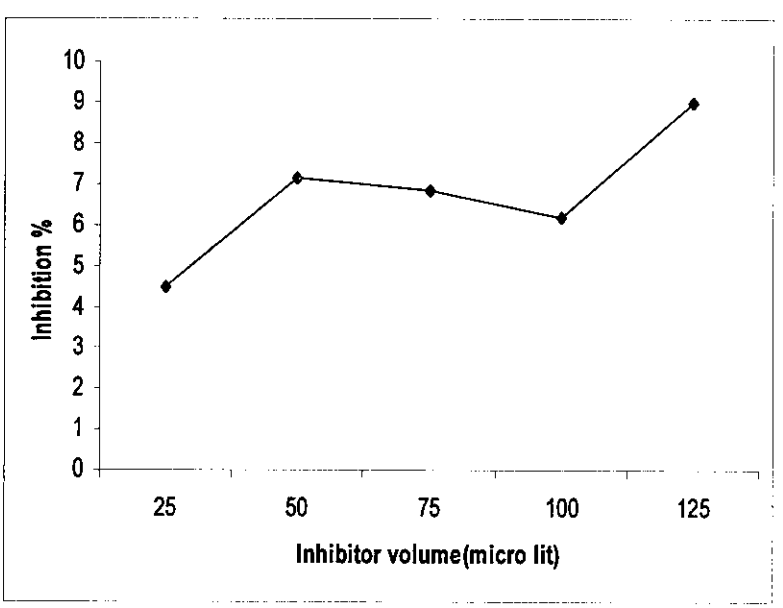
Graph-F: Inhibition % Vs Inhibitor volume of *Zizyphus mauritiana Lam* seeds (Organic phase)



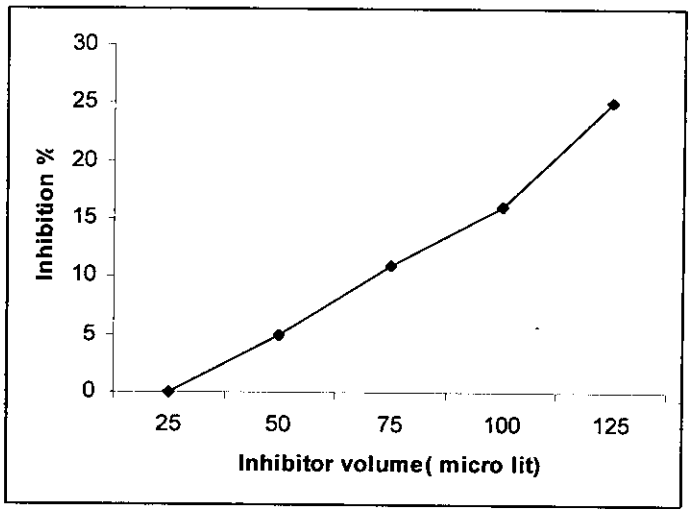
Graph-G Inhibition % Vs Inhibitor volume *Gymnema sylvestre* leaves (Aqueous phase)



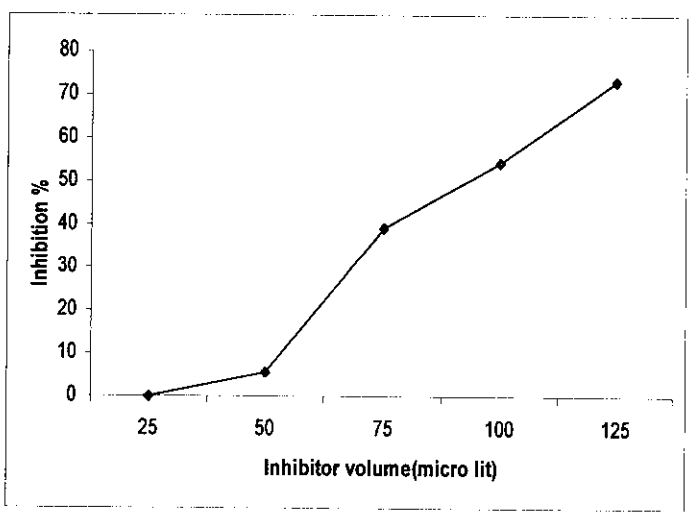
Graph-H: Inhibition % Vs Inhibitor volume of *Aegle marmelos leaves* (Aqueous phase)



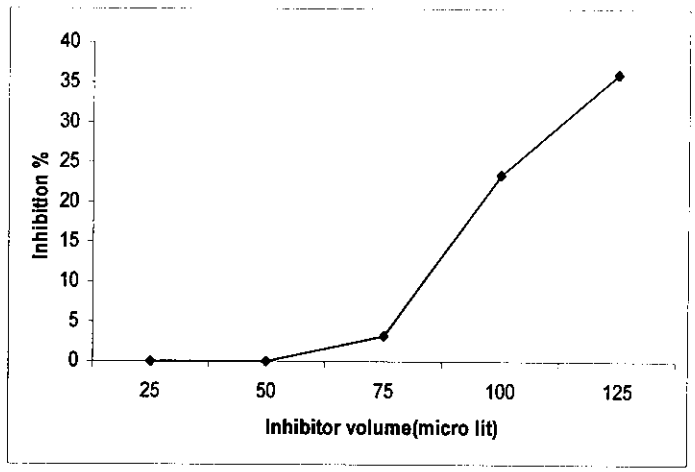
Graph-I: Inhibition % Vs Inhibitor volume of *Moringa oleifera* leaves (Aqueous phase)



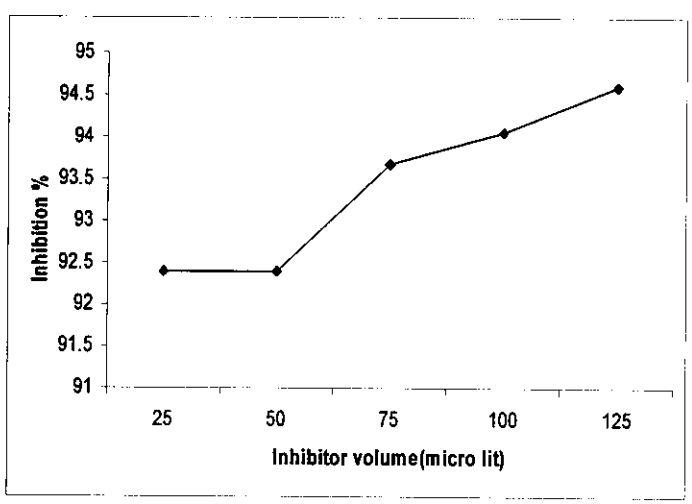
Graph-J: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (Aqueous phase at room temp)



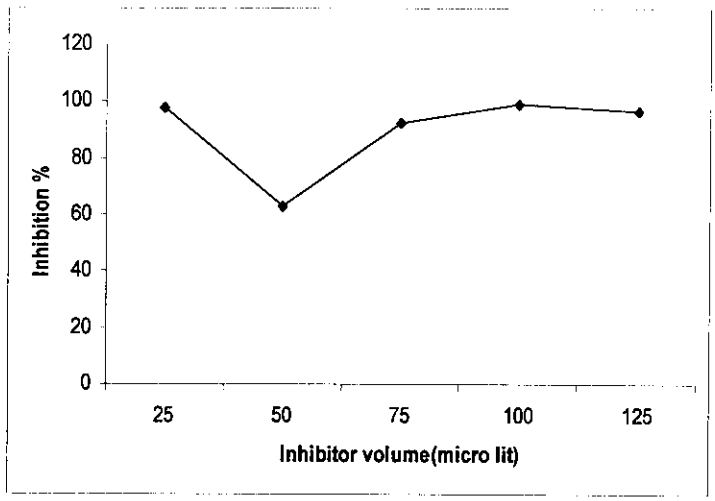
Graph-K: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (2nd Aqueous phase at 0-4^{0}C)



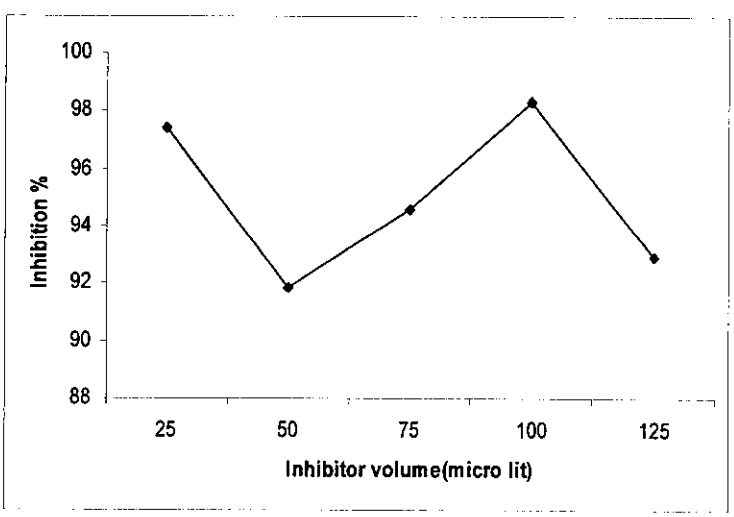
Graph-L: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (2nd Organic phase at 0-4^{0}C)



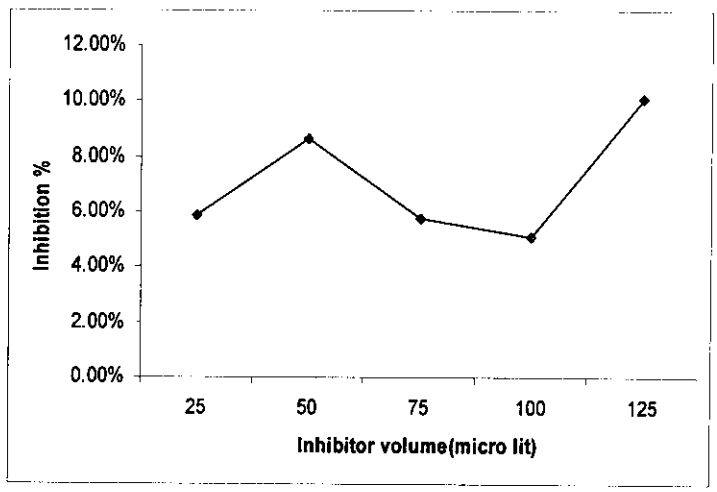
Graph-M: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (2^{nd} Organic phase at room temp)



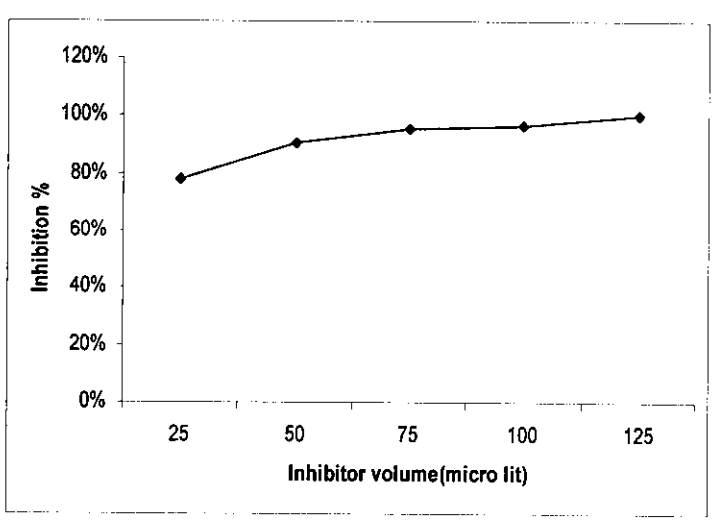
Graph-N: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (2^{nd} Organic phase)



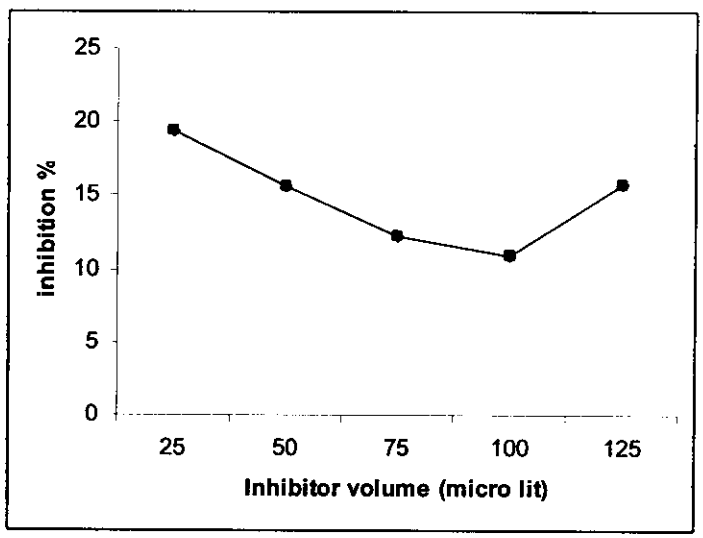
Graph-N: Inhibition % Vs Inhibitor volume of *Cassia auriculata* flowers (1st Organic phase)



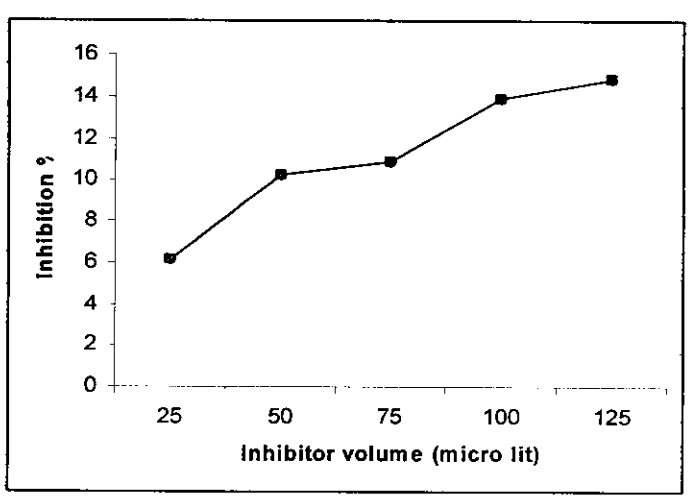
Graph-O: Inhibition % Vs Inhibitor volume of *Syzygium cumini Linn* seeds (Aqueous phase)



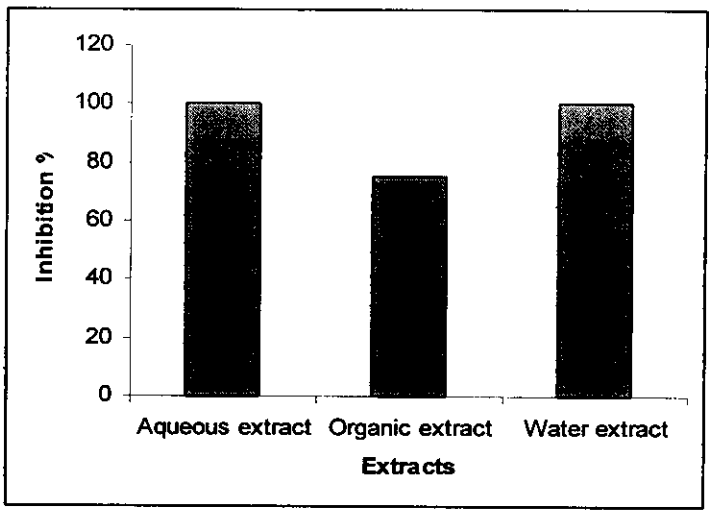
Graph-P: Inhibition % Vs Inhibitor volume of *Acalypha indica* seeds (Aqueous phase)



Graph-Q: Inhibition % Vs Inhibitor volume of *Tinospora Cordifolia* leaves (Aqueous phase)



Graph-R: Inhibition % Vs Inhibitor volume of *Psidium guajava Var. pomiferum* leaves (Different extracts)



Graph-S: Inhibition % Vs Inhibitor volume of *Syzygium cumini* leaves from different TLC fractions (Aqueous phase)

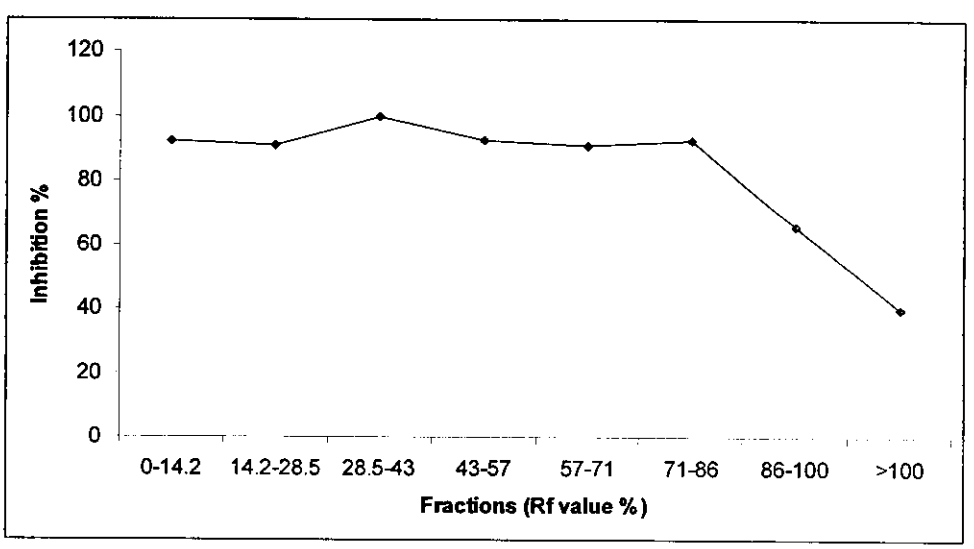
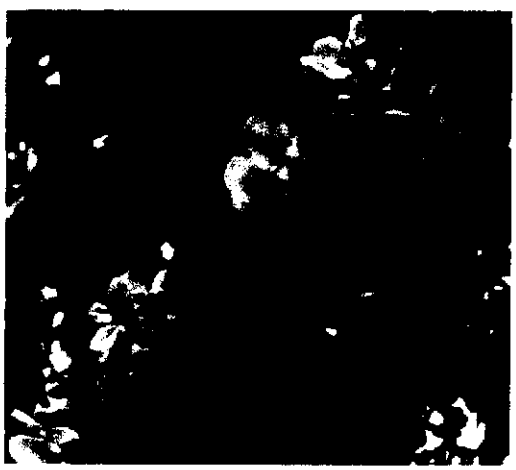


Figure-b: Medicinal Plants

Syzygium cumini Linn

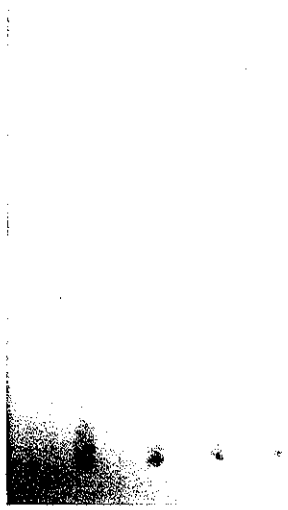
Cassia auriculata



Psidium guajava Var. pomiferum

Figure-c: Separation of compounds by TLC

Phenolics



Terpenoids **Alkaloids**

Figure-c: Dialysis of *Syzygium cumini Linn* seed inhibitor

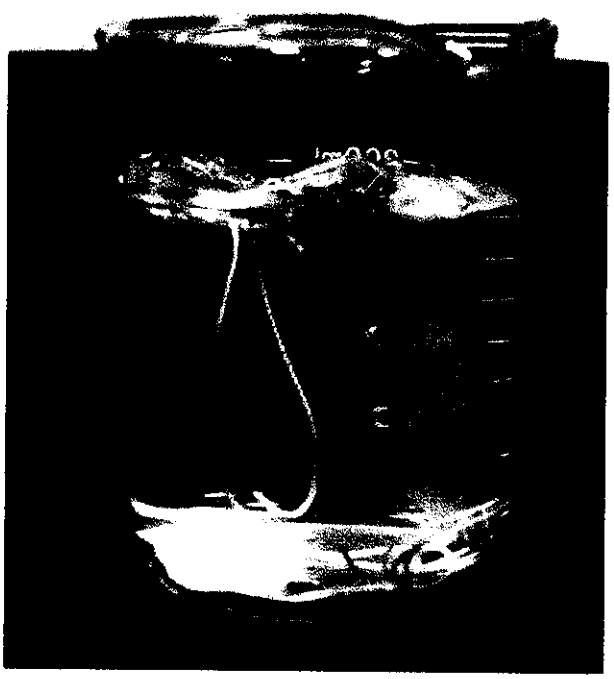


Figure-d: Dixon plot of *Syzygium cumini Linn* seed inhibitor

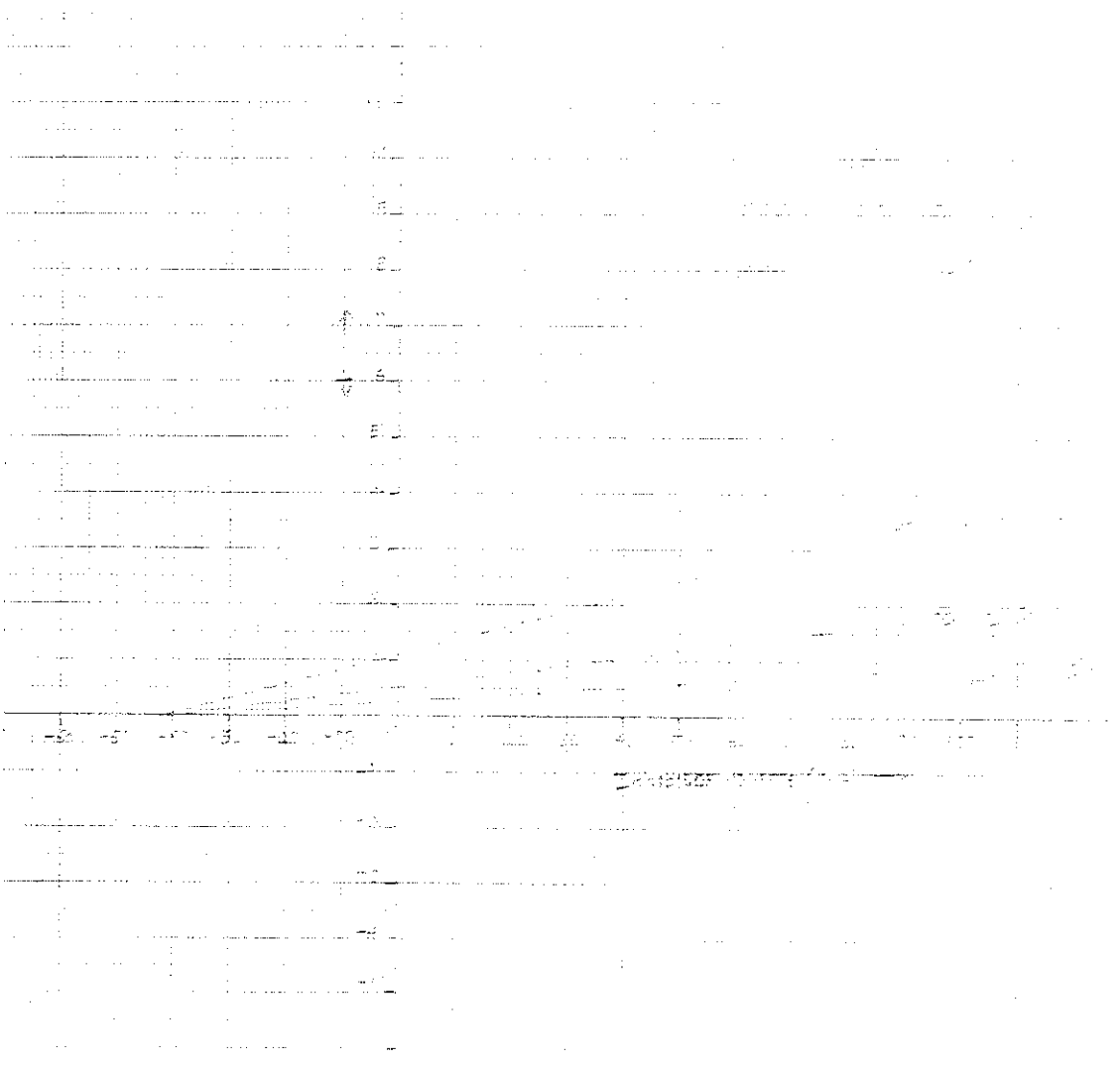


Figure-f: RF-HPLC chromatogram & contour view of *Syzygium cumini Linn* seed inhibitor fraction (Rf- 28.5 to 43%)

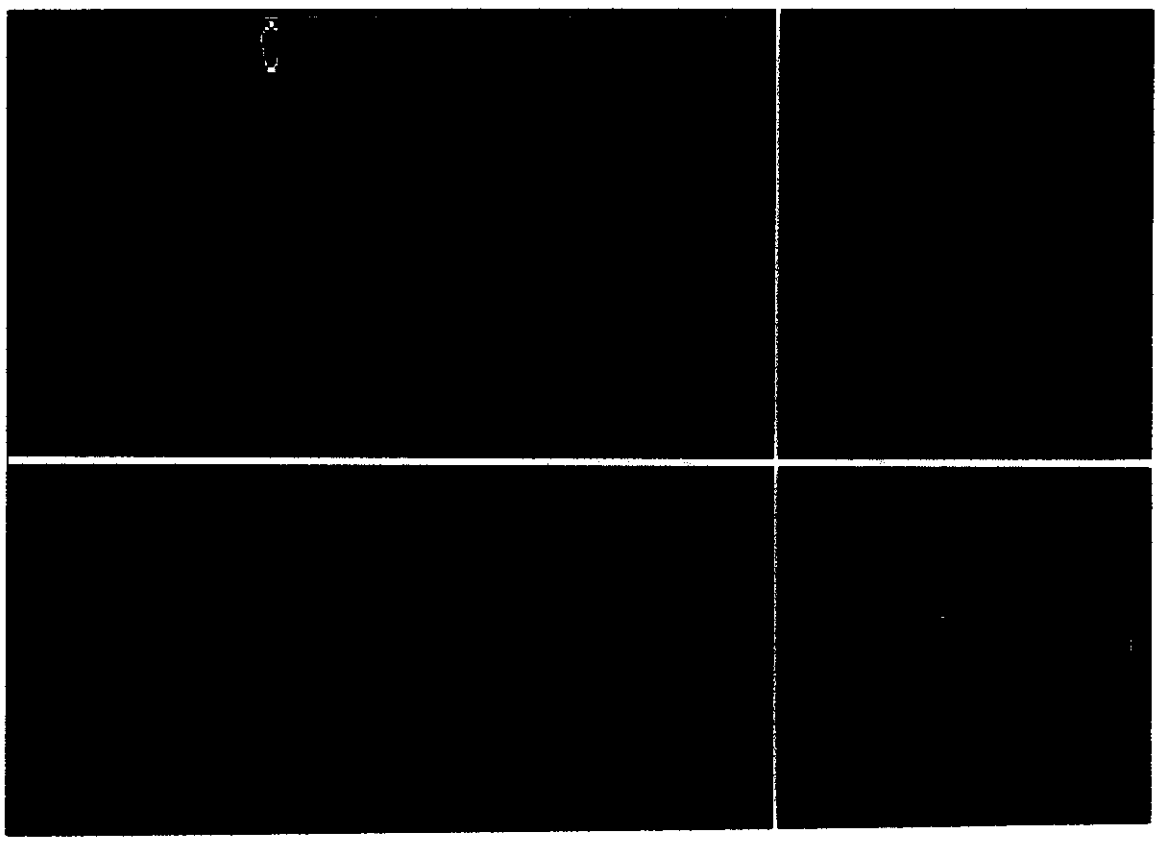


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Figure-g: RF-HPLC chromatogram & contour view of *Syzygium cumini Linn* seed inhibitor fraction (Rf- 43 to 57%)

CONCLUSION

Chapter-5

Conclusion

Natural á-amylase inhibitors from medicinal plants offer an attractive therapeutic approach to the treatment of diabetes by decreasing glucose release from starch. Powerful synthetic á-amylase inhibitors are available, but cause various side effects. So, phenolic alpha-amylase inhibitors from medicinal plant extracts are potentially safer. We have identified a potent reversible, non-competitive á-amylase inhibitor from the aqueous extract of *Syzygium cumini linn* seed. The anti-diabetic activity of the extract was then effectively enriched by preparative thin layer chromatography into different fractions and it was finally checked for purity using RP-HPLC.

Chapter-6

Future perspectives

The TLC purified sample was sent to the central drug research institute (CDRI), lucknow to record LC-MS. The mass spectra of the separated compounds obtained through RP-HPLC will be used to identify the compounds. Also attempts will be made to collect pure fractions from the preparative HPLC column directly and test verify the á-amylase inhibitor activity.

REFERENCES

Chapter-7

References

Ahmed, B., A. J. Al-rehailly., T. A. Al-Howiriny., K. A. El-Sayed and M. S. Ahmad. 2003. Scropolioside-D2 and Harpagoside-B: Two New Iridoid Glycosides from "*Scrophularia deserti* and Their Antidiabetic and Antiinflammatory Activity". *Biol. Pharm. Bull.* **26**(4): 462-467.

Alexander-Lindo, R. L., E.Y. St. A. Morrison and M. G. Nair. 2004. "Hypoglycaemic Effect of Stigmast-4-en-3-one and its Corresponding Alcohol from the Bark of *Anacardium occidentale* (Cashew)". *Phytother. Res.* **18**: 403–407.

Antia, B.S., J.E. Okokon and P.A. Okon .2005. "Hypoglycemic activity of aqueous leaf extract of *Persea americana* Mill". *Indian J. Pharmacol.* **37**(5): 325-326.

Beckman Model 330 HPLC Manuel, Beckman Instruments, Fullerton, CA.

Bidlingmeyer, B.A. and Warren, F.V. Jr. *Analytical Chemistry*, 1984, Vol. **56**, pp. 1583-96.

Brieteneder, H. and C.A. Radauer, 2004. "Classification of plant food allergens". J. *Allergy and Clin.Immunol.*, May, **113**: 821-830.

Bright, F.V. *Analytical Chemistry*, 1988, Vol. **60**, pp. 1031-1039.

Brown, P.R. *Analytical Chemistry*, 1990, Vol. **62**, pp. 995-1008.

Can, A., N. Akev., N. Ozsoy., S. Bolkent., B. P. Arda., R. Yanardag and A. Okyar. 2004. "Effect of *Aloe vera* Leaf Gel and Pulp Extracts on the Liver in Type-II Diabetic Rat Models". *Biol. Pharm. Bull.* **27**(5): 694—698.

Choi, S.B and Sunmin Park. 2002. "A steroidal Glycoside from *Polygonatum odaratum* (Mill) Druce. Improves insulin resistance but not alter insulin secretion in 90% Pancreatectomate Rats". *Biosci. Biotechnol. Biochem.* **66**(10): 2036-43.

Choi, S.Z., S. O. Lee., K. U. Jang., S. H Chung., S. H.Park., H. C. Kang., E. Y. Yang., H. J. Cho and K. R. Lee. 2005. "Antidiabetic Stilbene and Anthraquinone Derivatives from *Rheum undulatum". Arch. Pharm Res.* **28**(9): 1027-1030.

Conforti, F., .M. R. Loizzo., G. A. Statti and F. MenichiniI. 2005. "Comparative Radical Scavenging and Antidiabetic Activities of Methanolic Extract and Fractions from *Achillea ligustica"* ALL. *Biol. Pharm. Bull. 28*(9):1791-1794.

Conforti, F., G. Statti., M. R. Loizzo., G. Sacchetti., F. Poli and F. Menichini. 2005." *In Vitro* Antioxidant Effect and Inhibition of a-Amylase of Two Varieties of *Amaranthus caudatus* Seeds". *Biol. Pharm. Bull.* **28**(6): 1098—1102.

Fuentes, N. L., H. Sagua., G. Morales., J. Borquez., A. S. Martin., J. Soto and L. A. Loyola. 2005. "Experimental Antihyperglycemic Effect of Diterpenoids of Llareta *Azorella compacta* (Umbelliferae) Phil in Rats". *Phytother. Res.* **19**: 713–716.

Ghosh, R., Kh. Sharatchandra., S. Rita and I. S. Thokchom. 2004. "Hypoglycemic activity of Ficus hispida (bark) in normal and diabetic albino rats". *Indian J. Pharmacol.* **36**(4): 222-225.

Gibbs, B. F and I. Alli. 1998. "Characterization of a purified Ü-amylase inhibitor from white kidney beans (*Phaseolus vulgaris*)". *Food Research International.***31**(3): 217-225.

Glajch, J.L. and Kirkland, J.J.; *Analytical Chemistry*, 1983, Vol. **55**, pp. 319-32.

Gray, A. M and P. R. Flatt. 1999. "Insulin-secreting activity of the traditional antidiabetic plant *Viscum album* (mistletoe)". *Journal of Endocrinology* **160:** 409–414.

Hiroshi Oneda, Seungjae Lee and Kuniyo Inouye "Inhibitory Effect of 0.19 α-Amylase Inhibitor from Wheat Kernel on the Activity of Porcine Pancreas α-Amylase and Its Thermal Stability" *J. Biochem*, 2004, Vol. 135, No. 3 421-427

Hou, C. C., S. J. Lin., J.T. Cheng and F. L. Hsu. 2003. "Antidiabetic Dimeric Guianolides and a Lignan Glycoside from *Lactuca indica". J. Nat. Prod.* **66**: 625-629.

Jayapragasam, B., S. K. Vareed., L. K. Olson and M.G.Nair. 2005. "Insulin Secretion by Bioactive Anthocyanins and Anthocyanidins Present in Fruits". *J. Agric. Food Chem.* **53**: 28-31.

Jones, D.G. *Analytical Chemistry*, 1985, Vol. **57**, pp. 1057-1073.

Kamalakkannan, N and P. S. M. Prince. 2004. "Antidiabetic and Anti-oxidant Activity of *Aegle marmelos* Extract in Streptozotocin-Induced Diabetic Rats". *Pharmaceutical Biology.* **42**(2): 125–130.

Kandra, L., G. Gyemant., A. Zajacz and G. Battab. 2004. "Inhibitory effects of tannin on human salivary *a*-amylase". *Biochemical and Biophysical Research Communications.* **319**: 1265–1271.

Knox, J.H. and Kauer, B.; *High Performance Liquid Chromatography*; Brown. P.R. and Hartwick, R.A. Eds.; Wiley Interscience: New York, 1989, Chapter **4**.

Lee, S.O., S. Z. Choi., J. H. Lee., S. H Chung., S. H. Park., H. C.Kang., E. Y. Yang., H. J. Cho and K. R. Lee. 2004. "Antidiabetic Coumarin and Cyclitol Compounds from *Peucedanum japonicum". Arch. Pharm. Res.* **27**(12): 1207-1210.

Mallavadhani, U.V and G. Sahu. 2003. Pterostilbene: "A Highly Reliable Quality- Control Marker for the Ayurvedic Antidiabetic Plant 'Bijasar'". *Chromatographia.* **58**: 307–312 .

Mantena, S. K., S. Mutalik., H. Srinivasa., G. S. Subramanian., K. R. Prabhakar., K. R. Reddy., K. K. Srinivasan and M. K. Unnikrishanan. 2005. "Antiallergic, Antipyretic, Hypoglycemic and Hepatoprotective Effects of Aqueous Extract of *Coronopus didymus* LINN". *Biol. Pharm. Bull.* **28**(3): 468—472.

Mant, C.T. and Hodges R.S. eds. "High-Performance Liquid Chromatography of Peptides and Proteins: Separations, Analysis, and Conformation", *CRC Press*: Boston, 1991.

Marshall J. J and C. M. Lauda "Purification and properties of phaseolamin, an inhibitor of alpha-amylase, from the kidney bean, Phaseolus vulgaris" *JBC,* Vol. **250**, Issue 20, 8030-8037, Oct, 1975

McClure, W.F. *Analytical Chemistry*, 1994, Vol. **66**, pp. 43-54.

Nogowski, L., K. W. Nowak., P. Kaczmarek and P. Mackowiak. 2002. "The Influence of Coumestrol, Zearalenone, and Genestein administration on insulin receptors and insulin secretion in ovaryectomized Rats". *Journal of Signal Receptor and Transduction.* **22**(1–4) : 449–457.

Norberg, Å., N. K. Hoa., E. Liepinsh., D. V.Phan., N. D. Thuan., H. Jornvall., R. Sillard.and C. G. Ostenson. 2004."A Novel Insulin-releasing Substance, Phanoside, from the Plant *Gynostemma pentaphyllum". The Journal of biochemical chemistry.* **279**(40): 41361–41367.

Novotny, M. *Analytical Chemistry*, 1989, Vol. **60**, pp. 500-10.

Octavio, L. and D. Rigden, 2002. Plant "-amylase inhibitors and their interaction with "-amylases. *Eur. J. Biochem.*, **269**: 397-412.

Octivio, L. and D. Rigden, 2000. Activity of wheat "- amylase inhibitors towards bruchid "-amylase and structural explanation of observed specificities. *Eur. J. Biochem.*, **267**: 2166-2173.

Ogundipe, O.O., J.O. Moody., T.O. Akinyemi and A. Raman. 2003. "Hypoglycemic potentials of methanolic extracts of selected plant foods in alloxanized mice". *Plant Foods for Human Nutrition.* **58**: 1–7.

Pari, L and G. Saravanan. 2002. "Antidiabetic effect of Cogent db, a herbal drug in alloxan-induced diabetes mellitus". *Comparative Biochemistry and Physiology Part C.* **131** :19-25.

Pari, L and M. Latha. 2004. "Antihyperglycaemic Effect of *Scopavia dulcis:* Effect on Key Metabolic Enzymes of Carbohydrate Metabolism in Streptozotocin-Induced Diabetes". *Pharmaceutical Biology.* **42** (8): 570-576.

Preet, A., B. L. Gupta., M. R. Siddiqui., P. K. Yadava and N.Z. Baquer. 2005. "Restoration of ultrastructural and biochemical changes in alloxan-induced diabetic rat sciatic nerve on treatment with Na_3VO_4 and *Trigonella* – a promising antidiabetic agent". *Molecular and Cellular Biochemistry.* **278**: 21–31.

Reddy, G. B.S and N. Dhananjaya. 2000. "Chemical Investigation of Mycale mytilorum and a Study on Toxicity and Antidiabetic Activity of 5-Octadecylpyrrole-2-carboxaldehyde". *Bioorganic & Medicinal Chemistry.* **8**(27). 36.

Reddy, S. V., A. K. Tiwari., U. S. Kumar., R. J. Rao and J. M. Rao. 2005. "Free Radical Scavenging, Enzyme Inhibitory Constituents from Antidiabetic Ayurvedic Medicinal Plant *Hydnocarpus wightiana* Blume". *Phytother. Res.* **19** : 277–281.

Regnier, F.E. *Analytical Chemistry*, 1983, Vol. **55**, pp. 1298-1306.

Richardson, M., 1991. *Methods in Biochemistry*, volume. **5**, Academic press, pp: 259-305.

Sathissekar, D and S. Subramanian. 2005. "Beneficial Effects of *Momordica charantia* Seeds in the Treatment of STZ-Induced Diabetes in Experimental Rats". *Biol. Pharm. Bull.* **28**(6): 978—983.

Schoeff, M.S. and Williams, R.H. *Principles of Laboratory Instruments*, Mosby: St. Louis, 1993.

Singh, J. and M. Bblundel, 2001. "Albumin and globulin proteins of Wheat Flour: Immunogical and Nterminal sequence characterization". J. *Cereal Sci.*, 2001. Vol. **34,I1**, July, P: 85-103

Trivedi, N. A., B. Mazumdar., J. D. Bhatt and K. G. Hemavathi. 2004."Effect of shilajit on blood glucose and lipid profile in alloxan- alloxaninduced induced diabetic rats". *Indian J. Pharmacol.* **36**(6): 373-376.

Tsutsumi, T., S. Kobayasi., Y. Y. Liu and H Kondani. 2003. "Anti-hyperglycemic Effect of Fangchinoline Isolated from *Stephania Tetrandra* Radix in Streptozotocin-Diabetic Mice". *Biol. Pharm. Bull.* **26**(3): 313-317.

Tzu-Liua, C., H. Hsea., C.K. Liia., P.S. Chena and L.Y. Sheenb. 2005. "Effects of garlic oil and diallyl trisulfide on glycemic control in diabetic rats". *European Journal of Pharmacology.* **516**: 165 – 173.

Utsonamia, H., T. Yamakava., J. Kamai., K. Kadonosono and S.I. Tonoko. 2005."Anti hyperglycemic effects of plum in a Rat model of obecity and Type-2 Diabetis,Wister fatty acid".*Biomedical Research.***25**(5): 193-200.

Valencia, A., A. E. Bustillo., G. E. Ossa and M. J. Chrispeels. 2000. "Amylases of the coffee berry borer (*Hypothenemus hampei*) and their inhibition by two plant amylase inhibitors". *Insect Biochemistry and Molecular Biology.* **30**: 207–213.

Wang, J., Z. Lou., Z. Zhu., Y. Chai and Y. Wu. 2005. A Rapid High-Performance Liquid Chromatographic Method for Qua Wellinder, B.S.; Kornfelt, T.; Sorensen, H.H.; *Analytical Chemistry*, 1995, Vol. **67**, pp. 39-43.

Wiegand, G., Epp, O. & Huber, R. (1995) "The crystal structure of porcine pancreatic alpha-amylase in complex with the microbial inhibitor Tendamistat". *J. Mol. Biol.* **247**, 99-110.

Yajima, H., E. keshima., M. Shiraki., T. Kanaya., D. Fujiwara., H. Odai., N. T.Kasaoka., O. Ezaki., S. Oikawa and K. Kondo. 2004. Isohumulones, "Bitter Acids Derived from Hops, Activate Both Peroxisome Proliferator-activated Receptor and Reduce Insulin Resistance". *The Journal of Biochemical Research.* **279**(32): 33456–33462.

Yanardag, R., S. Bolkent., A. T. Luoguz and Ö. Ö. Sacan. 2003."Effects of *Petroselinum crispum* Extract on Pancreatic B Cells and Blood Glucose of Streptozotocin-Induced Diabetic Rats". *Biol. Pharm. Bull.* **26**(8): 1206-1210.

Yoon, S. H and J. F. Robyt. 2003. "Study of the inhibition of four alpha amylases by acarbose and its 4IV-a-maltohexaosyl and 4IV-a-maltododecaosyl analogues". *Carbohydrate Research.* **338**: 1969- 1980.