ISOLATION AND IDENTIFICATION OF α-AMYLASE INHIBITOR(S) FROM GUAVA LEAVES

(*Psidium guajava Var. pomiferum*)

A PROJECT REPORT

Submitted by

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in partial fulfillment for the award of the degree

of

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in

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APRIL 2007

ANNA UNIVERSITY: CHENNAI 600 025
BONAFIDE CERTIFICATE

Certified that this project report entitled **"ISOLATION AND IDENTIFICATION OF α-AMYLASE INHIBITOR(S) FROM GUAVA LEAVES (*Psidium guajava Var. pomiferum*)"** is the bonafide work of **"Ms. Padhmavathy Y, Ms. Poornima Murthi K** and **Mr. Ratish M"** who carried out the project work under my supervision.

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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. Certain α-amylase inhibitors, better known as 'Starch Blockers', are increasingly being considered for the control of diabetes. These are substances that inhibit the digestive pancreatic α-amylase enzyme required to break down dietary starches thereby reducing hyperglycemia. We have identified α-amylase inhibitory activity in different parts of the Guava plant. The aqueous phase of *Psidium guajava Var. pomiferum* leaf powder extract showed potent inhibitor activity against the porcine pancreatic α-amylase enzyme. The non-proteinaceous inhibitor was found to be non-competitive and reversible. The anti-diabetic activity of the extract was then enriched by preparative TLC into fractions with different Rf values and it was finally checked for purity using RP-HPLC and the inhibitor sample has been sent to CDRI (Central Drug Research Institute), Lucknow for further LC-ESI-MS analysis. The antioxidant capacities of Guava leaf powder extract was confirmed by FRAP, CUPRAC and DPPH assays. An organoleptic test was performed for four varieties of 'Herbal tea' prepared from guava leaf powder. A preliminary clinical study was performed on human volunteers using Guava leaf powder filled capsules.

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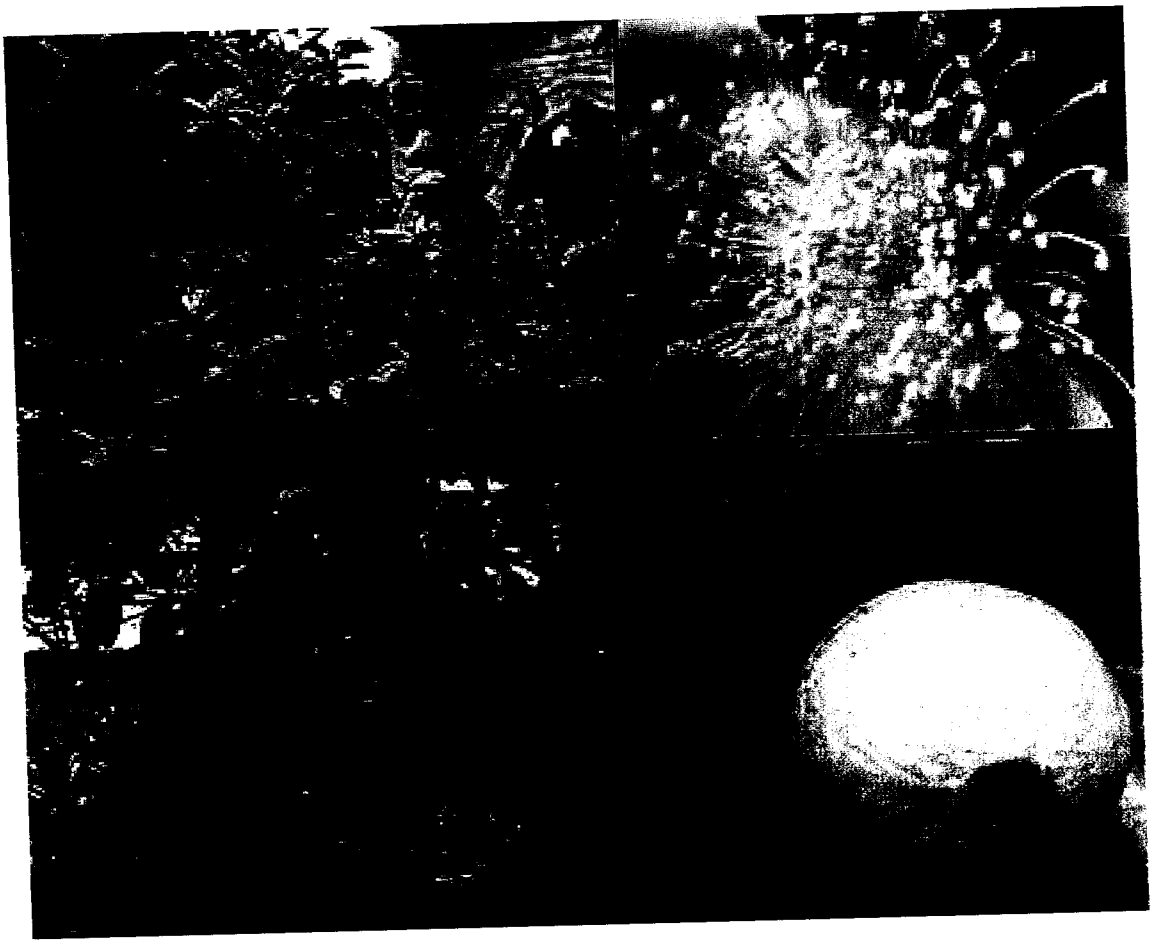
1. INTRODUCTION



1. INTRODUCTION

Diabetes mellitus is one of the oldest diseases known to mankind and yet with the tremendous scientific advances witnessed, medical science cannot claim that it knows all that needs to be known about this disease, including its management. This is the main reason for the persistent interest all over the world to explore alternative remedies from the so-called "alternative systems" of medicine. 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. Diabetes can cause many complications. Acute complications (hypoglycemia, ketoacidosis or nonketotic hyperosmolar coma) may occur if the disease is not adequately controlled. Serious long-term complications include cardiovascular disease, chronic renal failure, retinal damage, nerve and microvascular damage, which may cause erectile dysfunction (impotence) and poor healing. Poor healing of wounds, particularly of the feet, can lead to gangrene which can require amputation. Adequate treatment of diabetes, as well as increased emphasis on blood pressure control and lifestyle factors (such as smoking and keeping a healthy body weight), may improve the risk profile of most aforementioned complications.

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. 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 of 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, research on hypoglycemic compounds from medicinal plants has become an important aspect.

Recent advances in understanding 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. α-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 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.

Scientific classification	
Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Myrtales
Family:	Myrtaceae
Genus:	***Psidium*** L.
Species	
More than 100 known	

Guava (from Spanish **Guayaba**; **Goiaba** in Portuguese) is a genus of about 100 species of tropical shrubs and small trees in the myrtle family Myrtaceae, native to the Caribbean, Central America and northern South America.Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of Vitamin C in 100 g of fruit) and contains appreciable amounts of Vitamin A as well. It is both an anti-diabetic and an antioxidant. Many medicinal plants have been reported to show the effects of anti-diabetic activity. The present

investigation was initiated in *Psidium guajava Var. pomiferum* with the following objectives:

1. To estimate the α-amylase enzyme inhibition in various parts of the guava plant [leaf, stem, fruit].
2. To isolate and identify the structure of the inhibitor compound(s) in the guava leaf.
3. To perform an organoleptic test for herbal tea prepared from guava leaf powder.
4. To test the effect of guava leaf powder filled capsules on selected human volunteers, as a preliminary study.
5. To analyze the antioxidant properties of leaf powder by FRAP, CUPRAC and DPPH assays.

2. LITERATURE REVIEW

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2.1 Diabetes

The term *diabetes* was coined by Aretaeus of Cappadocia. It is derived from the Greek word *diabaínein* that literally means "passing through," or "siphon", a reference to one of diabetes' major symptoms — excessive urine production. The disease was well known to the ancient Indian medical experts. All the renowned classic texts of Ayurveda like Charaka Samhita (1000 B.C.), Sushruta Samhita (600 B.C.) and subsequent works refer to this disease under the term *Madhumeha* or *Ikshumeha,* literally meaning 'sugar in the urine' (Sharma P.V *et al.,* 1983). Apart from detailed description of its etiopathogenesis (according to Ayurvedic concepts), the two types of diabetic patients (obese and lean) and a definite familial prediction to the disease are referred to in Ayurveda, besides the importance given to dietary regulations, physical exercises and baths, in addition to the use of a number of plant drugs in the management of the disease (Mehta.K.C, 1982). In 1675 Thomas Willis added *mellitus* from the Latin word meaning a sweet taste. This had been noticed in urine by the ancient Greeks, Chinese, Egyptians, and Indians. In 1776 Matthew Dobson confirmed that the sweet taste was because of an excess of a kind of sugar in the urine and blood of people with 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.

Diabetes, without qualification, usually refers to *Diabetes mellitus*, but there are several rarer conditions also named diabetes. The most common of these is *Diabetes insipidus* (*insipidus* meaning "without taste" in Latin) in which the urine is not sweet; it can be caused by either kidney or pituitary gland damage.

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 mellitus—formerly known as insulin-dependent diabetes (IDDM), childhood diabetes or also known as juvenile diabetes, is characterized by loss of the insulin-producing beta cells of the islets of Langerhans of the pancreas leading to a deficiency of insulin. Type 1 diabetes is an autoimmune disease. 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. It should be noted that there is no known preventive measure that can be taken against type 1 diabetes. Most people

affected by type 1 diabetes are otherwise healthy and of a healthy weight when onset occurs. Diet and exercise cannot reverse or prevent type 1 diabetes. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages. This type of diabetes can affect children or adults but was traditionally termed "juvenile diabetes" because it represents a majority of cases of diabetes affecting children.

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. Without insulin, ketosis and diabetic ketoacidosis can develop and coma or death will result.

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. Type 2 diabetes mellitus — previously known as adult-onset diabetes, maturity-onset diabetes, or non-insulin-dependent diabetes mellitus (NIDDM)—is due to a combination of defective insulin secretion and *insulin resistance* or *reduced insulin sensitivity* (defective responsiveness of tissues to insulin), which almost certainly involves the insulin receptor in cell membranes. At this stage hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver, but as the disease progresses the impairment of insulin secretion worsens, and therapeutic replacement of insulin often becomes necessary. There are numerous theories as to the exact cause and mechanism for this resistance, but central obesity (fat concentrated around the waist in relation

to abdominal organs) is known to predispose individuals for insulin resistance, possibly due to its secretion of adipokines that impair glucose tolerance. Obesity is found in approximately 55% of patients diagnosed with type 2 diabetes.

Type 2 diabetes is increasingly being diagnosed in children and adolescents. Type 2 diabetes may go unnoticed for years in a patient before diagnosis, as visible symptoms are typically mild or non-existent, without ketoacidotic episodes, and can be sporadic as well. However, severe long-term complications can result from unnoticed type 2 diabetes, including renal failure, vascular disease (including coronary artery disease), vision damage, etc

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.

Gestational Diabetes

Some women develop gestational diabetes late in pregnancy. Although this form of diabetes usually disappears after the birth of the baby, women who have had gestational diabetes have a 20 to 50 % chance of developing type 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. The hormones of pregnancy or a shortage of insulin causes gestational diabetes. Women with gestational diabetes may not experience any symptoms.

2.3 Treatments of diabetes

Type 1 diabetes

The most common cause of beta cell loss leading to type 1 diabetes is autoimmune destruction, accompanied by antibodies directed against insulin and islet cell proteins. The principal treatment of type 1 diabetes, even from the earliest stages, is replacement of insulin. Ketosis and diabetic ketoacidosis can develop without insulin and as a result, coma or death may occur.

Currently, type 1 diabetes can be treated only with insulin, with careful monitoring of blood glucose levels using blood testing monitors. Emphasis is also placed on lifestyle adjustments (diet and exercise). Apart from the common subcutaneous injections, it is also possible to deliver insulin by a pump, which allows continuous infusion of insulin 24 hours a day as needed at meal times.

Type 1 treatment must be continued indefinitely. The average glucose level for the type 1 patient should be as close to normal (80–120 mg/dl, 4–6 mmol/l) as possible. Values above 200 mg/dl (10 mmol/l) are often accompanied by discomfort and frequent urination leading to dehydration. Values above 300 mg/dl (15 mmol/l) usually require immediate

treatment and may lead to ketoacidosis. Low levels of blood glucose, called hypoglycemia, may lead to seizures or episodes of unconsciousness.

Type 2 Diabetes

Type 2 diabetes is usually first treated by attempts to change physical activity, the diet (generally to decrease carbohydrate intake) and weight loss. These can restore insulin sensitivity even when the weight loss is modest. However, the underlying tendency to insulin resistance is not lost, and so attention to diet, exercise, and weight must continue. The usual next step is treatment with oral antidiabetic drugs. As insulin production is initially unimpaired in Type 2, oral medication (often used in various combinations) can still be used to improve insulin production (e.g., sulfonylureas), to regulate inappropriate release of glucose by the liver (and attenuate insulin resistance to some extent (e.g., metformin), and to substantially attenuate insulin resistance (e.g., thiazolidinediones). If these fail (cessation of beta cell insulin secretion is not uncommon amongst Type 2), insulin therapy will be necessary to maintain normal or near normal glucose levels. A disciplined regimen of blood glucose checks is recommended in most cases.

Gestational diabetes

Gestational diabetes mellitus (GDM) occurs in about 2%–5% of all pregnancies. It is temporary and fully treatable but, if untreated, may cause problems with the pregnancy, including macrosomia (high birth weight), foetal malformation and congenital heart disease. It requires careful medical supervision during the pregnancy.

Foetal/neonatal risks associated with GDM include congenital anomalies such as cardiac, central nervous system, and skeletal muscle malformations. Increased foetal insulin may inhibit foetal surfactant production and cause respiratory distress syndrome. Hyperbilirubinemia may result from red blood cell destruction. In severe cases, perinatal death may occur. Induction may be indicated with decreased placental function.

2.4 Natural treatments for diabetes

Scientific interest in 'alternative' or 'traditional system' in this area as also literature surveys and updates on *Diabetes mellitus* have concentrated, mainly on the screening of plant drugs (from all possible sources) for their blood-sugar lowering effect. Very few plants have been studied further, in depth, for investigating their site & mechanism of action and for possible development as anti-diabetic drugs. In most animal studies, water extracts or alcoholic extracts of the plants have been screened. Among these plants, some like *Allium cepa* (Onion), *Allium sativum* (garlic), *Syzygium cumini* (Syn. *Eugenia jambolana;* (black plum), *Momordica charantia* (bitter gourd) *Gymema sylvestre*, *Pterocarpus marsupium*, *Psidium guajava spp* etc., have attracted more attention of the scientists as well as laymen, in recent years (Chaudry R.R *et al.*, 1970).

Natural 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

(Satyavati G.V *et al.,* 1976)

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. Pregnant or nursing women should not use fenugreek.

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.

2.5 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 means pills taken by mouth (oral) that lower (hypo) the blood glucose (glycemia) level (Atta-Ur-Rahman and Khurshid Zaman, 1989).

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 intestine.

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.

2.6 Anti-diabetic compounds from plants

Many plants have been identified so far that display anti-diabetic potential. Among these plants, some like *Phyllanthus amarus* (Ali H *et al.*, 2006), *Allium cepa* (Onion), *Allium sativum* (garlic), *Syzygium cumini*

(Syn. *Eugenia jambolana* (black plum), *Momordica charantia* (bitter gourd) *Gymema sylvestre, Pterocarpus marsupium, Psidium guajava spp* etc., have been studied extensively (Chaudry R.R *et al.,* 1970 and Jain R.C *et al.,* 1973). α-amylase inhibitors or 'Starch Blockers' have been extensively studied in plants. The α-amylase inhibitor may be proteinaceous or non-proteinaceous in nature.

2.6.1 *Lactuca indica*

Three novel sesquiterpene lactones, Lactucain A1, B2, and C3, and a new furofuran lignan, lactucaside 4, were isolated from *Lactuca indica* along with nine known compounds, 11*â*, 13-dihydrolactucin, cichoriosides B, quercetin, quercetin 3-*O*-glucoside, rutin, apigenin, luteolin, luteolin7-*O*-glucuronide, and chlorogenic acid. Among these compounds, latucain C3 and lactucaside 4 showed significant antidiabetic activity. Compound 1 was obtained as an amorphous powder (Hou *et al.,* 2003).

2.6.2 *Syzygium cumini*

The seeds of *Syzygium cumini* are used by the Ayurvedic physicians (and also in Indian folklore) in the treatment of diabetes mellitus. The hypoglycaemic activity of the seeds has been studied by several workers in animal models (Shrotri D S *et al.,* 1963, Bhaskaran R and Santhakumari G, 1986). In alloxan diabetic rats, S. *cumini* seed extract led to a decrease in the levels of blood glucose, urea and serum triglyceride levels (Giri J *et al.,* 1985). The aqueous extract of *Syzygium cumini* or *Eugenia jambolana* seeds showed inhibition against the porcine pancreatic α-amylase (Karthic *et al.,*

2007 – unpublished). The compounds identified from the seed extract of *Syzygium cumini* were stilbene C-glucoside, betulinic acid, 3,5,7,4`-tetrahydroxy flavanone and tetragalloyl glucose, a hydrolysable tannin. The crude aqueous extract prepared from 27g of *Syzygium cumini* seed powder was found to be equivalent to 25mg of acarbose.

2.6.3 *Azorella compacta*

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 (Fuentes *et al.*, 2005). 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 anti-hyperglycemic 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 β cells of pancreatic islets, while mulinolic acid may act upon glucose utilization or production in the liver.

2.6.4 *Gymnema sylvestre*

Gymnema sylvestre is yet another plant reputed specially in South India for its use as an antidiabetic drug. Adrenaline-induced hyperglycemia in rats was countered by injection of the leaf extract (Gupta S S *et al.,* 1961). Orally, the leaves significantly reduced the hyperglycemia induced in rats by anterior pituitary extract. In alloxan diabetic rabbits, the leaf extract of the plant reduced the blood sugar level and also reversed the hepatic changes produced by hyperglycemia (Shanmugosundaram K R *et al.,* 1981). The alcohol-water extract of *G. sylvestre* restored the elevated protein-bound polysaccharide components & glycosaminoglycans in serum & tissues of alloxan diabetic rats, and it was suggested that the plant apparently restores the synthesis of sulfated glyosaminolycans and thereby could be of possible assistance in preventing vascular complications of diabetes (Rathi A N *et al.,* 1981).

2.6.5 *Peucedanum japonicum*

The anti-diabetic 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) (Lee *et al.*, 2004). The results showed that coumarin and a cyclitol, that is, peucedanol 7-*O*-β-D-glucopyranoside and *myo*-inositol. Their structures were identified by spectroscopic methods. Compound 1 showed 39% inhibition of postprandial hyperglycemia at 5.8mg/kg dose, and compound 2 also significantly inhibited postprandial hyperglycemia by 34% ($P<0.05$).

Extracts of herbs with promising anti-diabetic 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 mobile phase 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 those of other interfering components of the mobile phase. 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.

2.6.6 *Vigna sublobata*

Alpha-amylase inhibitor protein, which inhibits the activity of insect (*Callosobruchus analis*) alpha-amylase, was characterized from *V. sublobata* (Kokiladevi E *et al.,* 2005). The molecular weight of purified inhibitor protein was 14 kDa by SDS-PAGE. The inhibitor is non-glycosylated protein and its N-terminal sequence is similar (A P S P V...) to *Phaseolus vulgaris* alpha-AI-1. Its pI value is 6.0 and largely localised in cotyledons. The inhibitory activity decreased during germination from days one to five.

2.7 Guava and diabetes

Scientific investigations on the medicinal properties of guava leaf products date back to the 1940s. The Tikuna Indians decoct the leaves or bark of guava as a cure for diarrhea. In fact, an infusion or decoction made from the leaves and/or bark has been used by many tribes for diarrhea and dysentery throughout the Amazon, and Indians also employ it for sore throats, vomiting, stomach upsets, for vertigo, and to regulate menstrual periods. Tender leaves are chewed for bleeding gums and bad breath, and it is said to prevent hangovers (if chewed before drinking). A decoction of the bark and/or leaves or a flower infusion is used topically for wounds, ulcers and skin sores (Rastogi R.P *et al.,* 1982). Flowers are also mashed and applied to painful eye conditions such as sun strain, conjunctivitis or eye injuries. Most scientific evidence examines the clinical efficacy of guava in treating GI disorders.

The pharmacological actions and the medicinal uses of aqueous extracts of guava leaves in folk medicine include the treatment of various types gastrointestinal disturbances such as vomiting, diarrhea, inhibition of the peristaltic reflex, gastroenteritis, spasmolytic activity, dysentery, diabetes, abdominal distention, flatulence and gastric pain (Karnick C.R, 1972). These extracts have also been indicated to cause disturbances of the central nervous system: insomnia, convulsions and epilepsy. Bronchitis, asthma attacks, cough, pulmonary diseases could be also treated with guava teas and could also be useful as anti-inflammatory and hemostatic agent (Satyavati, G.V, *et al.,* 1987).

Guava tea has displayed many medical effects among users such as blood sugar lowering, weight control and slimming, anti-oxidant, cholesterol lowering, intestinal health promoter (Pastore R *et al.*, 2006). Anti-stomach-ulcer and anti-cancer functions are not confirmed yet, but are apparently predictable. Guava tea is so effective because the total polyphenol released from the leaves is 113mg/g. Polyphenols inhibits glucose absorption from intestine villus to blood circulation, therefore prevents overshoot of post-prandial glucose. It also prevents down-shoot of fasting glucose caused by delayed insulin after meal. So it is very good for blood glucose control of people with mild diabetes (Aiman R, 1970). Polyphenols inhibit the activity of amylase, a starch-digesting enzyme found in saliva and in the intestines (Horigome T *et al.*, 1988).

Because of the inhibition function of glucose absorption, it surely cuts down on calories intake to our body. Many Japanese companies promote guava tea as a slim tea. So it is suitable for calorie control of sweet teeth and people with overweight or obesity problems. Because guava tea contains no caffeine, it's also good for blood glucose control of elder people, overweight children, and people with osteoporosis. In a word, it's a multifunctional tea, especially good for daily health care for women (Luo, M, 1999).

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

activity is attributed to these flavonoids (Zhongjun M.A, *et al.*, 2004). The flavonoids have demonstrated antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava; it is able to relax intestinal smooth muscle and inhibit bowel contractions (Koo Hui Miean and Suhaila Mohamed, 2001). In addition, other flavonoids and triterpenes in guava leaves show anti-spasmodic activity.

Guava's main plant chemicals include: alanine, alpha-humulene, alpha-hydroxyursolic acid, alpha-linolenic acid, alpha-selinene, amritoside, araban, arabinose, arabopyranosides, arjunolic acid, aromadendrene, ascorbic acid, ascorbigen, asiatic acid, aspartic acid, avicularin, benzaldehyde, butanal, carotenoids, caryophyllene, catechol-tannins, crataegolic acid, D-galactose, D-galacturonic acid, ellagic acid, ethyl octanoate, essential oils, flavonoids, gallic acid, glutamic acid, goreishic acid, guafine, guavacoumaric acid, guaijavarin, guajiverine, guajivolic acid, guajavolide, guavenoic acid, guajavanoic acid, histidine, hyperin, ilelatifol D, isoneriucoumaric acid, isoquercetin, jacoumaric acid, lectins, leucocyanidins, limonene, linoleic acid, linolenic acid, lysine, mecocyanin, myricetin, myristic acid, nerolidiol, obtusinin, octanol, oleanolic acid, oleic acid, oxalic acid, palmitic acid, palmitoleic acid, pectin, polyphenols, psidiolic acid, quercetin, quercitrin, serine, sesquiguavene, tannins, terpenes, and ursolic acid (Misra and Seshadri, 1968). Diatrol II, which contains guava leaf powder, can help diabetes symptoms as well as stomach, dysentery, and other digestion maladies.

Guava leaf provides antioxidant effects beneficial to the heart, heart protective properties, and improved myocardial function. In two randomized human studies, the consumption of guava fruit for 12 weeks was

shown to reduce blood pressure by an average 8 points, decrease total cholesterol levels by 9%, decrease triglycerides by almost 8%, and increase "good" HDL cholesterol by 8%. The effects were attributed to the high potassium and soluble fiber content of the fruit (Atta-ur-Rahman *et al.*, 1989). In other animal studies guava leaf extracts have evidenced analgesic, sedative, and central nervous system (CNS) depressant activity, as well as a cough suppressant actions. The fruit or fruit juice has been documented to lower blood sugar levels in normal and diabetic animals and humans. Most of these studies confirm the plant's many uses in tropical herbal medicine systems. However, the reaction mechanisms involved in the inhibition of alpha-amylase by plant protein inhibitors are not clearly understood. Alpha-amylase and its inhibitors are drug-design targets for the development of compounds for treatment of diabetes (Octivio and Rigden, 2000).

2.8 Antioxidant capacity of Guava

Natural antioxidants, particularly in fruits and vegetables have gained increasing interest among consumers and the scientific community because epidemiological studies have indicated that frequent consumption of natural antioxidants is associated with a lower risk of cardiovascular disease and cancer. The defensive effects of natural antioxidants in fruits and vegetables are related to three major groups: vitamins, phenolics, and carotenoids. There is a considerable epidemiological evidence indicating association between diets rich in fresh fruits and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer (Jimenez-Escrig, A, *et al.*, 2001).

Free-radicals are generated continuously in the body due to metabolism and disease (Lee *et al.*, 1995). In order to protect themselves against free radicals, organisms are endowed with endogenous (catalase, superoxide dismutase, glutathione peroxidase/ reductase) and exogenous (C and E vitamins, β - carotene, uric acid) defences; yet these defence systems are not sufficient in critical situations (oxidative stress, contamination, UV exposure, etc.) where the production of free radicals significantly increases. It is generally assumed that the active dietary constituents contributing to these protective effects are the antioxidants (vitamins, carotenoids, polyphenols, sterols). *Psidium guajava Linn*, belonging to the family of *Myrtaceae*, has been used as health tea. Its leaf contains copious amounts of phenolic phytochemicals which inhibit peroxidation reaction in the living body, and therefore can be expected to prevent various chronic diseases such as diabetes, cancer, heart-disease (Kimura.Y *et al.*, 1983). Furthermore, decreasing of free-radicals has antioxidizing effect in the body, meaning these guava leaf polyphenols can prevent arterial sclerosis, thrombosis, cataract and inhibit senescence of the body and skin.

Many people habitually take medicinal decoction of guava leaf for long for treatment of diarrhoea, and therefore, the safety of guava leaves have empirically been confirmed. People in China use guava leaf as anti-inflammatory and haemostatic agent (Liu, 1988). It was reported that the leaves of *P. guajava Linn* contain an essential oil rich in cineol, tannins and triterpenes. In addition, three flavonoids (quercetin, avicularin, and guaijaverin) have been isolated from the leaves (Hidetoshi Arima and Genichi Danno, 2002 and Bilyk A *et al.*, 1985). The antioxidant activity of phenolic compounds is determined by their molecular structure and, more

specifically, by the position and degree of hydroxylation of the ring structure. The antioxidative activity is conventionally used to indicate the ability of antioxidant to scavenge some radicals (Cao.G *et al.*, 1993). Phenolic compounds are typical active oxygen scavengers in foods and have been evaluated by several methods. One among tests proposed for assessment of antioxidative activity (AOA) is DPPH free-radical colorimetry (You Chen *et al.*, 1999), whose color changes from purple to yellow in the presence of antioxidants. The kinetics of decolorization reactions directly relate to the types of antioxidants and to their different concentrations. The more rapidly the absorbance decreases, the more potent is the antioxidant activity of the antioxidants in terms of hydrogen donating ability (Yen and Du, 1998). The rapid reduction of DPPH radical by antioxidants allows the evaluation of antioxidant power of different antioxidants.

Guava has antioxidant properties attributed to the polyphenols found in its leaves. Ascorbic acid and phenolics are known as *hydrophilic antioxidants*, while carotenoids are known as *lipophilic antioxidants*. Guava (*Psidium guajava L.*) fruit is considered a highly nutritious fruit because it contains a high level of ascorbic acid (50–300 mg/100 g fresh weight), which is three to six times higher than oranges. Red-eshed Brazilian guava has several carotenoids such as phytofuene, b-carotene, b-cryptoxanthin, g-carotene, lycopene, rubixanthin, lutein, and neochrome reported that Indonesian guava is an excellent source of provitamin A carotenoids. Phenolic compounds such as myricetin and apigenin (Miean and Mohamed, 2001), ellagic acid, and anthocyanins are also at high levels in guava fruits (Misra and Seshadri, 1968 and Leong L P *et al.*, 2002). Therefore, producing guava specially bred for higher levels of antioxidant compounds is a realistic

approach to increase dietary antioxidant intake. Therefore, the assay for screening germplasm and hybrids should be simple, inexpensive, rapidly performed and provide a high degree of precision.

Several assays (Hinneburg I, *et al.,* 2006) have been frequently used to estimate antioxidant capacities in fresh fruits and vegetables and their products and foods for clinical studies including 2,2 - azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Miller N.J. and Rice-Evans, C.A., 1997), 2,2 - diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams et al., 1995; Gil *et al.,* 2002), ferric reducing antioxidant power (FRAP) (Benzie and Strain, 1999; Guo *et al.,* 2003), and the oxygen radical absorption capacity (ORAC) (Cao *et al.*, 1993 and Prior *et al.*, 2003). The ORAC assay is said to be more relevant because it utilizes a biologically relevant radical source (Prior *et al.,* 2003). These techniques have shown different results among crop species and across laboratories. No correlation of antioxidant activity was reported between the FRAP and ORAC techniques among most of the 927 freeze-dried vegetable samples, whereas these methods revealed high correlation in blueberry fruit (Connor *et al.,* 2002). Awika *et al.* (2003) observed high correlation between ABTS, DPPH, and ORAC among sorghum and its products. One of our objectives was to perform the DPPH, FRAP and DDPH assays to estimate antioxidant activities in guava leaf powder extracts.

3. MATERIALS & METHODS

3. MATERIALS AND METHODS

3.1 Extraction of compounds from different parts of the guava plant

(Arnon D I, 1949)

3.1.1 Chemicals and materials required

- Petroleum ether
- Acetone
- NaCl, 10%
- Fresh leaves – tender, young and mature, stem, fruit-young and mature

3.1.2 Apparatus and glass wares required

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

3.1.3 Procedure

The method of Arnon D I (1949) was adopted with some modifications:

1. Using a pestle and mortar 5g of the desired part of the guava plant (fruit, leaf and stem) 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 NaCl solution.
4. The separating funnel was shaken carefully. The layers were allowed to

separate and the lower one was allowed to drain and collected in a beaker. The upper layer was evaporated at room temperature and the same volume was made up with water. This upper layer was named as 'organic phase 1'. The lower layer is again treated with the equal volume of ethyl acetate. Again the phases are allowed to separate using separating funnel. The lower aqueous layer is named as 'Aqueous phase'. The upper organic layer is evaporated and is made up with the equal volume of water. It is named as 'organic phase 2'.

5. The experiment was repeated with stem parts and fruits and the extracts were collected and stored at 4^0C till further use.

3.2 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.

3.3 α Amylase inhibition assay - DNS method

(Bernfield P, 1955)

3.3.1 Principle

The method of Bernfield (1955) was adopted with modifications. This method is 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 eg., 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:

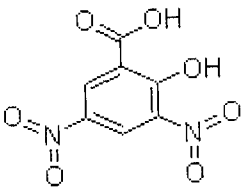
Oxidation

. Aldehyde group [-CHO] → Carboxyl group [-COOH]

←

Reduction

. 3, 5-dinitrosalicylic acid → 3-amino, 5-nitrosalicylic acid



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. The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid. However, it 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. 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.

3.3.2 Materials required

- Micro centrifuge tubes
- Pipettes
- Spectrophotometer
- Water bath

3.3.3 Reagents required

- **Starch solution, 1%** (Freshly prepared)
- **Dinitrosalicylic acid reagent (DNS reagent):** 1 g of dinitrosalicylic acid was dissolved in 20 ml of 2N NaOH and 50 ml water. Then 30 g Na-K tartarate was added and made up to 100 ml with water.

3.3.4 Procedure

1. Two micro centrifuge tubes were taken, one was labelled as control (C) and the other as Test (T).
2. To the test 200 µl of the working standard enzyme was added.
3. Next to both the micro centrifuge tubes phosphate buffer (pH 6.9) was added – 300 µl to control and 100 µl to test.
4. 100 µl inhibitor extract (from various parts of the guava plant) was

added to all the tubes.

5. To a test tube labelled 'Blank' (B) add 400 µl buffer and to another tube labelled 'C_{act}' add 200 µl enzyme and 200 µl buffer.
6. The tubes were then incubated at room temperature (RT) for 20 min.
7. Freshly prepared substrate (starch 1%) of 100 µl was added to all the tubes.
8. The tubes were again incubated at room temperature (RT) for 5 min.
9. 500 µl DNS reagent was added to all the tubes to arrest the reaction.
10. The tubes were then kept in boiling water bath for 5 min.
11. Cool the tubes and measure the Optical Density (OD) at 540 nm.

3.4 Ammonium Sulphate precipitation

(Sadasivam and Manickam, 2004)

3.4.1 Principle

The solubility of proteins is markedly affected by the ionic strength of the medium. As the ionic strength is increased, protein solubility at first increases. This is referred to as 'Salting in'. However, beyond a certain point, the solubility begins to decrease and this is known as 'Salting out'. At a low salt concentration, the solubility of a protein is very high. However, as we keep increasing the salt concentration, the proteins begin to precipitate out. It is observed that the hydrophobic proteins precipitate out first, with the hydrophilic proteins precipitating at a much higher salt concentration. In lab scale purification strategies, ammonium sulphate precipitation is often used as a first purification and concentrating procedure. When the protein is in the solution, water molecules surround the protein forming hydrogen bond with the protein. When ammonium sulphate is added, it takes up the water molecules around the protein, exposing the

hydrophobic sides on the protein. Because hydrophobic groups tend to be together, the protein will aggregate and thus come out of the solution.

3.4.2 Procedure

1. Two 50ml beakers were taken, washed and labeled as "Water extract" and "Hot Water extract" respectively.
2. To each of the beaker, 10grams of guava leaf powder was added and mixed with 50ml of water.
3. The beaker labeled as "Hot Water extract" was taken, stirred well and kept at the hot plate maintained at 100^0C to boil.
4. The beaker labeled as "Water extract" was stirred well the contents of the beaker were filtered using Whatmann filter paper.
5. When the contents in the beaker labeled "Hot Water extract" began to boil, it was removed from the hot plate and the contents were filtered using Whatmann filter paper.
6. Both the filtrates were collected and labelled as above respectively.
7. To the cold water extract, 14.7 grams of ammonium sulphate was added and to the heated extract, 21 grams of ammonium sulphate was added little by little at 0^0C and under constant stirring conditions.

[**Note:** The ammonium sulphate quantity was measured with respect to 100% saturation. From the standard value table, 70.7g Ammonium Sulphate must be added to 1l of the initial solution to achieve 100% saturation (S.Sadasivam and A.Manickam, 2004)]

8. Both the beakers were allowed to stand at 0^0C for half an hour to facilitate precipitation.
9. After half an hour, the contents of both the beakers were transferred into centrifuge tubes and were centrifuged at 6000rpm for 20 min.

10. After centrifugation, the supernatant of the tubes were collected and checked for inhibitor activity. The pellet in the centrifuge tube was collected separately, dissolved in buffer and subject to dialysis.

3.5 Dialysis

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

3.5.1 Materials

- Dialysis bag
- Beaker, 500ml
- Magnetic stirrer

3.5.2 Procedure

Dialysis was carried out by the method of Harris *et al.*, (1989):

1) The dialysis bag was taken and the one end of the bag was tightly tied and the other end was tied after adding the sample of 2 ml.
2) The sample was made by adding enzyme 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 5 min 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.

1) After incubation the micro centrifuge tubes were allowed to cool and mixed well. Finally, the absorbance was measured at 540nm and the readings were tabulated.

3.6 Separation of Compounds by Thin Layer Chromatography (TLC)

(Roger *et al.*, 1987)

3.6.1 Materials

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

3.6.2 Procedure for preparation of TLC plates

1. Clean 20 x 20 cm glass plates were 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 in benzene.
2. For one plate about 20 g of silica gel G was weighed and transferred to a wide-mouthed conical flask.
3. To the conical flask about 30-35 ml water was added (Quantity of water may 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 or 0.5 mm.
5. The plates that were coated with the silica gel were dried in air at room temperature. After that plates were activated by keeping it in the oven at 110°C for 2 hr.

6. Without disturbing the silica gel layer, the standard compound and the test samples were spotted with the help of a capillary tube or a microlitre syringe.
7. About 120ml of the solvent mixture of the corresponding ratio was added to the TLC tank. In order to saturate the chamber, a filter paper was wetted and was placed over the inner sides of the tank
8. The TLC plate was placed inside the chamber and tightly covered by means of the lid.
9. When the solvent system reaches the top of the plate, it was removed and air-dried.
10. Finally the plate was sprayed uniformly with the spraying reagent and the spots were noted.
11. The Rf values of the spots were calculated and the corresponding silica gel fraction was scraped.
12. For preparative TLC, the sample was applied as a streak, developed and then a portion of the TLC plate was sprayed to detect the sample. Then the corresponding region of silica gel was scraped.
13. The scraped silica gel was extracted by mixing with methanol and then centrifuged to get the clear filtrate. The filtrate evaporated and was made up with water. This was used as the inhibitor source for the assay.

3.6.3 Solvent system used for separation of the following compounds

Phenolics

- Chloroform-Acetic acid, 9:1, 8:2, 7:3
- Methanol-Water-Acetic Acid, 7:2:1, 8:1:1
- Chloroform-Methanol-Acetic Acid, 8:1:1, 5:3:2

- Chloroform-Ethyl Acetate-Methanol, 3:4:3, 6:2:2
- Ethyl Acetate-Methanol, 7:3, 6:4

Terpenoids

- Chloroform-Methanol 5:5, 6:4

Alkaloids

- Chloroform-Methanol, 9.6:4
- Aceton-Methanol-Ammonium hydroxide, 7:2:2
- Methanol-Water, 7:3

Flavonoids

- Hexane-Ethyl acetate-Acetic acid, 4:4:2, 2:6:2, 2.5:5:2.5
- Hexane-Ethyl acetate, 7.5:2.5
- Chloroform–Methanol-Acetic acid, 7:1.5:1.5

3.6.4 Spraying reagents for different groups of compounds

Phenolics/Flavonoids

Reagents

- Folin-Ciocalteau reagent (Freshly prepared)
- Sodium carbonate, 20%

Procedure

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

Terpenoids

Reagents

- Vanillin in Con H_2SO_4. 10%(w/v)
- 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.

(or)

- Mix 1g Vanillin along with 75ml Methanol, 15ml Acetic acid and 5ml Con H_2SO_4.

Procedure

1. The TLC plate from its chamber was taken and 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 110°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

Solution C: 20ml glacial 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 allowed to air dry.

2. Initially the air dried plate was sprayed with Dragendorff's Reagent
3. After spraying, the TLC plate was allowed to dry for 5min.
4. Finally the plate was viewed in bright light.

3.6.5 General screening of compounds

TLC plate was 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/Flavonoids**

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

3.7 RP-HPLC

The purified material obtained from preparative thin layer chromatography was subjected to RP-HPLC to purify the α-amylase inhibitor to homogenity.

3.7.1 Materials

- HPLC (Shimadzu model LC 8A) equipped with a variable length UV detector (wave-length set at a 190 to 370nm) and a microprocessor.
- Column – phenomenex luna ODS C18, Analytical 5μ partials (250 x 4.6 mm).
- Sonicator-ultrasonic LC60H (LABLINE ELMA).
- Filter –cellulose acetate membrane, porosity 0.22μm.
- Mobile phase; 70:30 acetonitrile (HPLC grade):Double distilled water containing 0.1% trifluoro acetic acid(TFA).

3.7.2 Method: (Knox J H *et al.*, 1989)

1. Solvents were degassed before use.
2. The column was equilibrated with mobile phase.
3. Fifty microlitre of TLC purified sample was filtered through membrane filter (0.22 μm) and then injected into the sample introduction system.
4. The compounds were eluted employing a linear gradient of 70% acetonitrile in 0.1% TFA.
5. The elution time was set for 25min.
6. Flow rate was adjusted to 1ml/min.
7. The chromatogram of the sample was recorded.

3.8 Lineweaver Burk (LB) plot

(Lineweaver H and Burk D, 1934)

The determination of values for K_m and V_m with high precision

can be difficult. Many experiments are used to generate pairs of V and [S] data. Consequently, other methods of generating inhibition kinetics parameters are suggested such as LB Plot, Eadie-Hofstee plot, Hanes-Woolf plot and Dixon plot. A graph of 1/[S] Vs 1/V gives a correct estimate of the nature of inhibition.

1. A series of five micro centrifuge tubes were taken and labeled as T1 – T5.
2. Apart from this two other micro centrifuge tubes were taken and labeled as Blank and $C_{activity}$ respectively.
3. To all the micro centrifuge tubes labeled from T1-T5 and $C_{activity}$, 200 µl of the enzyme was added using micropipette.
4. To all the micro centrifuge tubes 100 µl of the Phosphate buffer was added.
5. Next, to all the tests (T1 – T5) the crude inhibitor was added in the varying concentrations of 10, 25, 50, 75, 100 and 150 µl.
6. Pure, double distilled water was added to all the tubes making the total volume to 500 µl. Then all the tubes were incubated in room temperature for 20 min.
7. Next 20 µl of 1% starch was added to all tubes and the tubes were incubated at room temperature for 5 min.
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 at 540 nm.
10. The above steps are repeated for different substrate concentrations (40, 60, 80 and 100 µl). A graph is plotted between 1/[S] and 1/V.

3.9 Ferric ion reducing antioxidant power (FRAP) assay

(Benzie I F F and Strain J J, 1999)

3.9.1 Principle

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this method the Fe^{3+} is reduced to Fe^{2+} in the presence of antioxidants (Reductants) in the extracts. The blue colour formed is colorimetrically measured at 700 nm. The increase in the absorbance is directly proportional to the concentration of total antioxidants present in the sample.

3.9.2 Reagents

a) Sample preparation

In a clean dry conical flask, 5g of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) was weighed and 50ml of Ethyl Acetate was added. This was kept in an orbital shaker for overnight. The contents were filtered with Whatmann filter paper and the filtrate was collected. The solvent in the filtrate was evaporated and 50mg of the dried content was dissolved in 50ml of distilled water. From this, a series of dilution (1:20 [100µg], 2:20 [200 µg] to 10:20 [1000 µg]) is prepared for experimental analysis.

b) 0.2M Phosphate buffer (pH = 6.6)

26.5ml of Na_2HPO_4 was mixed with 73.5ml of NaH_2PO_4

(0.2M Na_2HPO_4 = 35.6g/L, 0.2M NaH_2PO_4 = 31.2g/L)

c) 1% potassium ferricyanide

In a clean dry 100ml standard flask, 1g of potassium ferricyanide was weighed and made upto the mark with distilled water.

d) 10% TCA

In a clean dry 100ml standard flask, 10g of TCA was weighed and made upto the mark with distilled water.

e) 0.1% $FeCl_3$

In a clean dry 100ml standard flask, 0.1g of $FeCl_3$ was weighed and made upto the mark with distilled water.

3.9.3 Procedure

1.Two ml (concentration varying from 100 to 1000µg) of the extract was pipetted out into a series of tubes.

2.Two ml of phosphate buffer (pH = 6.6) and 2ml of 1% potassium ferricyanide was added to all the tubes.

3.To a "Blank" tube, 4ml of phosphate buffer (pH = 6.6) and 2ml of 1% potassium ferricyanide were added.

4.All the tubes were boiled at 50^0C for 20 minutes.

5.The reaction was arrested by adding 2ml of 10% TCA in all the tubes.

6.The tubes were centrifuged at 650g for 10 minutes and 4ml of supernatant was pipetted out.

7.To this 0.8ml of 0.1% $FeCl_3$ was added.

8. The blue colour formed was colorimetrically read at 700nm and an increase in the OD reading showed an increased antioxidant activity in the leaf extract.

).The same procedure was followed for Vit C and E.

3.10 Cupric ion reducing antioxidant capacity (CUPRAC) assay

(Tutem E *et al.*, 1991)

3.10.1 Principle

Antioxidant activity assay methods existing in the literature based on the measurement of radical scavenging activity of antioxidant compounds suffer from the difficulties encountered in the formation and stability of colored radicals. Copper (II)-neocuproine [Cu(II) - Nc] reagent is used here as the chromogenic oxidizing agent. Because the copper (II) (or cupric) ion reducing ability of polyphenols is measured, the method is "cupric reducing antioxidant capacity" abbreviated as the CUPRAC method. This method is advantageous over the ferric reducing antioxidant power (FRAP) method because the redox chemistry of copper(II) as opposed to that of ferric ions involves faster
kinetics. The chromogenic oxidizing reagent of the developed CUPRAC method, that is, bis (neocuproine) copper (II) chloride [Cu (II) - Nc], reacts with polyphenols [Ar(OH)*n*] in the manner

2*n* Cu(Nc)2^2+ + Ar(OH)n = 2*n* Cu(Nc)2^+ + Ar(dO)*n* + 2*n* H^+ (1)

where the liberated protons may be buffered with the relatively concentrated ammonium acetate buffer solution. In this reaction, the reactive Ar-OH groups of polyphenols are oxidized to the corresponding quinones and Cu (II) - Nc is reduced to the highly colored Cu (I) - Nc chelate showing maximum absorption at 450 nm. In this reaction, each flavonoid (in the

aglycon form) having n phenolic -OH groups theoretically acts as a $2n$-e donor.

3.10.2 Reagents

a) $CuCl_2$ reagent

Dissolved 0.034g of $CuCl_2$ in 20ml of distilled water.

b) Neocuproine reagent

Dissolved 0.031g of neocuproine in 2ml of distilled water.

c) Ammonium acetate

Dissolved 1.54g of ammonium acetate in 20ml of distilled water.

3.10.3 Procedure

1.One ml (concentration varying from 100 to 1000µg) of the extract was pipetted out into a series of test tubes.

2.One ml of $CuCl_2$, 1ml neocuproine, 1ml Ammonium Acetate and 1ml Ethanol was added to all the tubes.

3.All the tubes were incubated at 30^0C for 30 min.

4.The bluish green colour formed was colorimetrically read at 450nm and an increase in the OD reading showed an increased antioxidant activity in the leaf extract.

5.The same procedure was followed for Vitamins C and E.

3.11 DPPH (2, 2 - diphenyl-1-picrylhydrazyl) assay

(Brand-Williams W *et al.*, 1995)

3.11.1 Principle

Free Radicals are a group of active molecules with unpaired electrons that react with biological membrane, resulting in cell damage. Antioxidants are capable of taking up these free radicals. In doing so, they

protect healthy cells from damage and abnormal growth. In this DPPH assay system, due to its odd electron, the stable DPPH free radical produces a color with strong absorbance at 517 nm. When DPPH is placed in an assay system containing free radical scavengers such as flavonoids, the color vanishes. The change in absorbance produced in this reaction is a measurement of antioxidant scavenging capacity of test samples. When $DPPH^{\bullet}$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep violet to light yellow) were measured at 517 nm with a UV/visible light spectrophotometer.

% ANTIOXIDANT CAPACITY = [(Control – Test) / Control] * 100

3.11.2 Reagents

a) DPPH Reagent

3.15 mg DPPH (2, 2 - diphenyl-1-picrylhydrazyl) was mixed in 10 ml methanol to get the active reagent. This must be freshly prepared before the assay.

3.11.3 Procedure

1.Two ml (concentration varying from 100 to 1000µg) of the extract was pipetted out into a series of test tubes.

2.Half (0.5) ml of DPPH reagent was added to all the tubes. 2 ml methanol and 0.5 ml DPPH served as 'Control' and 2 ml methanol served as 'Blank'.

3.All the tubes were incubated in dark at room temperature for 30 min.

4.The purple colour formed was colorimetrically read at 517 nm and a decrease in the OD reading showed an increased antioxidant activity in the leaf extract.

5.The same procedure was followed for Vitamin C.

3.12 Herbal tea preparation & organoleptic test

3.12.1 Preparation of herbal tea

Tea is a beverage made by steeping processed leaves, buds, or twigs of the tea bush (*Camellia sinensis*) in hot water for a few minutes. The processing can include oxidation, heating, drying and the addition of other herbs, flowers, spices, and fruits. There are four basic types of true tea: black tea, oolong tea, green tea and white tea. The term 'Herbal tea' usually refers to infusions of fruit or of herbs that contain no *C. sinensis*. (Alternative terms for herbal tea that avoid the word "tea" are *tisane* and *herbal infusion*).

- **Sample 1** → Pure Guava leaf powder decoction + sugar to taste
- **Sample 2** → Sample 1 + mint powder
- **Sample 3** → Sample 1 + cardamom powder
- **Sample 4** → Sample 1 + lime

3.12.2 Organoleptic test

'Organoleptic' means relating to perception by a 'sensory organ'. It involves the use of sense organs for testing of any food item. Eg., Red chillies, wine, pepper, etc., A herbal tea was prepared using guava leaf powder and an organoleptic evaluation of the same was conducted by thirteen volunteers on 06/02/07 in the Bio-chemistry laboratory. The tea was evaluated with senses of sight, smell and taste, rather than by a scientific or chemical evaluation. The score card for evaluation was prepared as follows:

SCORE CARD FOR ORGANOLEPTIC EVALUATION OF HERBAL TEA

Criteria	Standard Scores	Score after evaluation			
		Sample 1	2	3	4
1. COLOUR AND APPEARANCE					
Good/Acceptable	3				
Fair/Moderately acceptable	2				
Poor/Not acceptable	1				
2.CONSISTENCY					
Good/Acceptable	3				
Fair/Moderately acceptable	2				
Poor/Not acceptable	1				
3.FLAVOUR					
Good/Acceptable	3				
Fair/Moderately acceptable	2				
Poor/Not acceptable	1				
4.TASTE					
Good/Acceptable	3				
Fair/Moderately acceptable	2				
Poor/Not acceptable	1				
5.MOUTH FEEL					
Good/Acceptable	3				
Fair/Moderately acceptable	2				
Poor/Not acceptable	1				

3.13 Preliminary clinical trial using guava leaf powder filled capsules

- Empty soft gelatin capsules were purchased and filled under sterile conditions with guava leaf powder.
- Three volunteers of different age groups were chosen based on their existing blood glucose levels and given these filled capsules with a fixed dosage regimen of 5 →

  Pre Breakfast - 1 no

  Pre Lunch - 2 nos

  Pre Dinner - 2 nos

Their 'fasting' blood glucose was monitored for a period of 20-30 days. Promising results were obtained from this preliminary study.

4. RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Alpha- amylase inhibitory assay

The different parts of the Guava plant were checked for α-amylase inhibitory activity by DNS (3, 5 di Nitro Salicylic acid) method using 1% starch as substrate. The Stem, Fruit – young and mature, Whole leaf – tender, young and mature, Mature leaf powder – Water Extract and Hot Water Extract were checked for α-amylase inhibition. The enzyme used was porcine pancreatic α-amylase (PPA). The table for Maltose calibration curve and % α-amylase inhibition in different parts of Guava plant are shown in Tables 4.1.1, 4.1.2, 4.1.3 and Fig. 4.1.1, 4.1.2

TABLE 4.1.1 - Maltose Calibration Curve

S.No	Maltose (ml)	Conc. of Maltose (ug)	Distilled water (ml)	DNS (ml)		40% Rochelle's salt (ml)	O.D 540nm
1	0	0	2.0	1.0		1.0	0.000
2	0.2	20	1.8	1.0	Boiling water bath for 5 mins	1.0	0.020
3	0.4	40	1.6	1.0		1.0	0.032
4	0.6	60	1.4	1.0		1.0	0.046
5	0.8	80	1.2	1.0		1.0	0.055
6	1.0	100	1.0	1.0		1.0	0.074

Fig. 4.1.1 - Maltose Calibration Curve

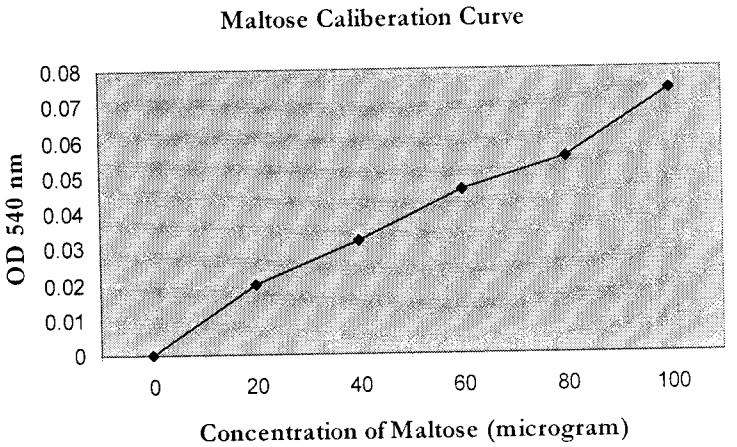


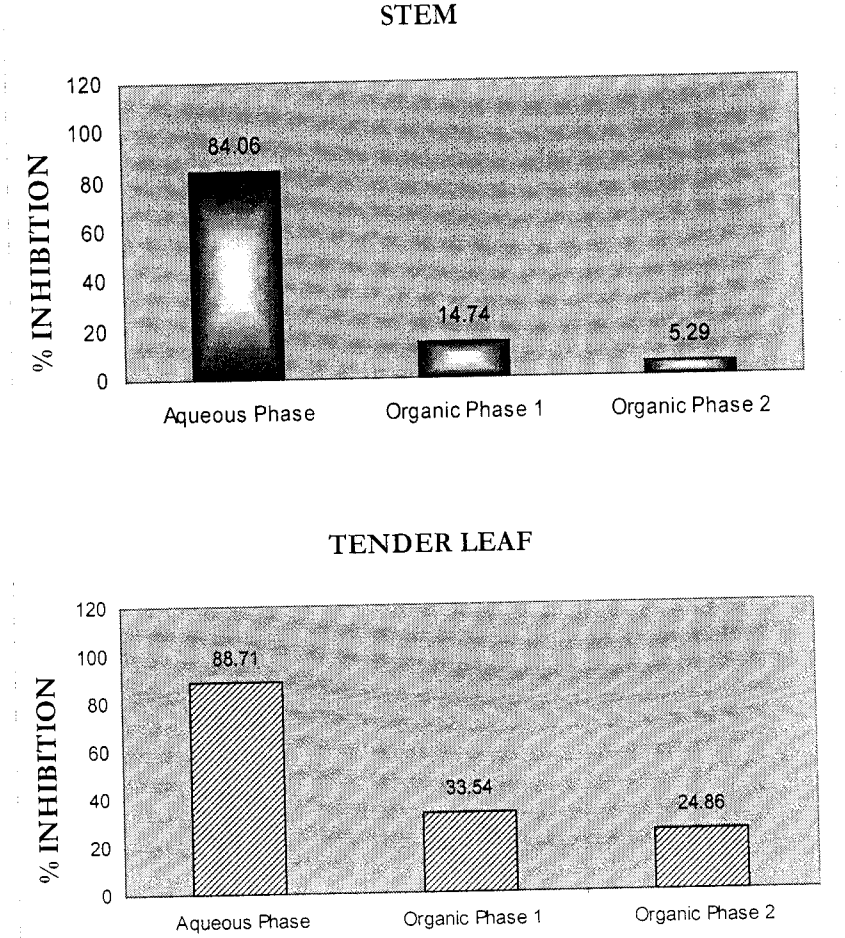
TABLE 4.1.2 -% α-Amylase Inhibition in different parts of Guava Plant

Part of the plant	System phase	% α-amylase Inhibition			
		Set 1	Set 2	Set 3	Mean
Tender leaf	Aqueous	88.69	87.67	89.78	88.71
	Organic 1	32.29	34.06	34.26	33.54
	Organic 2	23.98	24.25	26.36	24.86
Young leaf	Aqueous	85.83	84.33	86.24	85.47
	Organic 1	18.53	21.39	18.94	19.62
	Organic 2	15.60	17.58	15.27	16.15
Mature leaf	Aqueous	86.24	83.79	85.56	85.20
	Organic 1	16.42	19.21	15.68	17.10
	Organic 2	12.40	13.97	11.65	12.67
Stem	Aqueous	82.08	85.89	84.20	84.06
	Organic 1	13.01	16.08	15.12	14.74
	Organic 2	4.97	5.79	5.11	5.29
Young fruit	Aqueous	87.47	86.51	87.88	87.29
	Organic 1	20.78	24.59	21.05	22.14
	Organic 2	4.16	5.95	5.59	5.23
Mature fruit	Aqueous	89.23	87.81	87.26	88.10
	Organic 1	29.84	31.06	33.58	31.49
	Organic 2	25.41	25.89	26.57	25.96

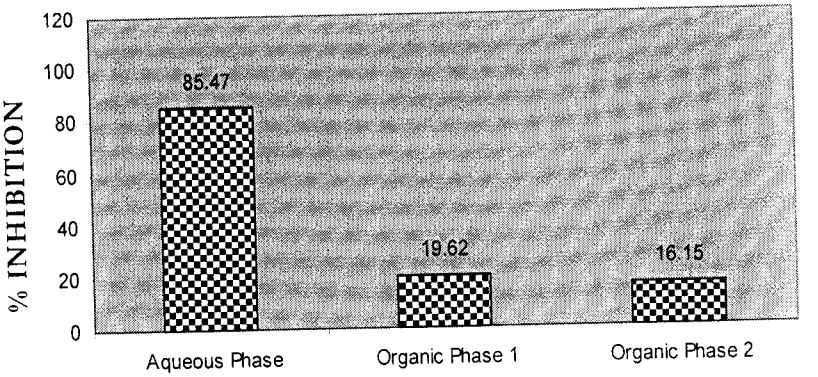
TABLE 4.1.3 - % α-Amylase Inhibition in different powder extracts of Guava leaves

EXTRACT	% α-amylase Inhibition			
	Set 1	Set 2	Set 3	Mean
Water Extract	92.78	91.28	93.39	92.48
Hot Water Extract	88.69	88.97	88.82	88.83

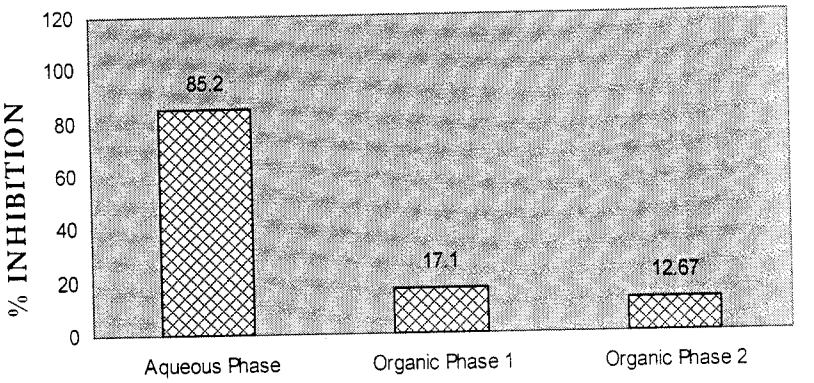
Fig. 4.1.2 - % α-Amylase Inhibition in various parts of the guava plant



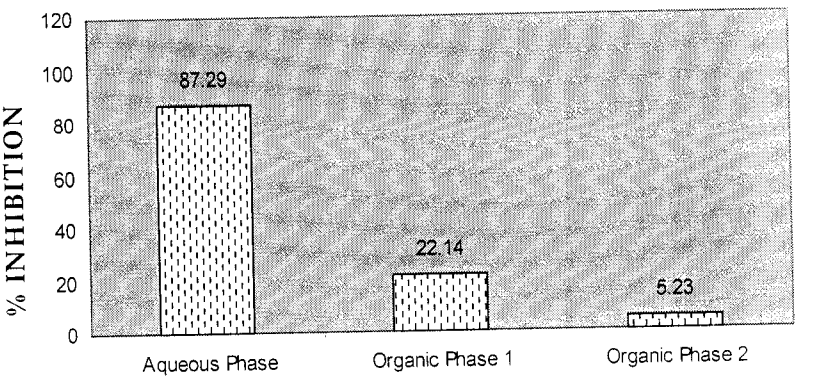
YOUNG LEAF



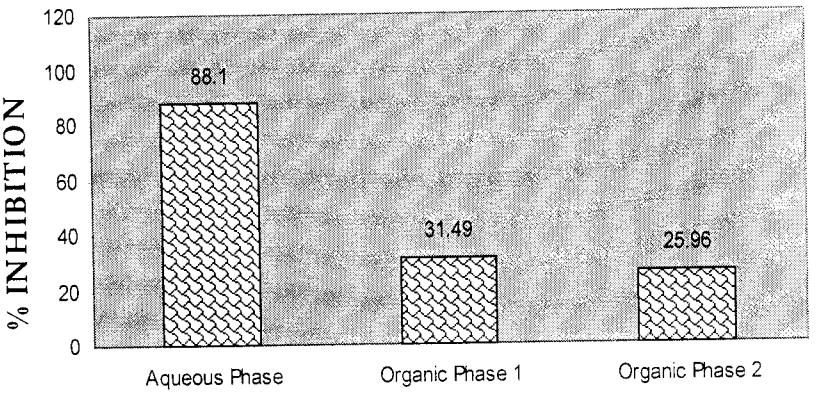
MATURE LEAF



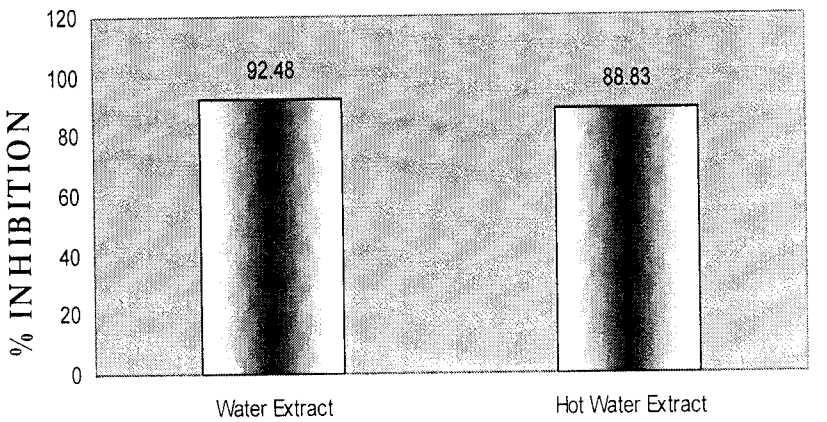
YOUNG FRUIT



MATURE FRUIT



% INHIBITION IN WATER EXTRACTS



The result of the assays implied that the water extract of the mature leaves from the plant showed the maximum α-amylase inhibition of about 92%. This was followed by the hot water extract which showed approx. 88% inhibition. The result indicated that the inhibitor was considerably thermostable as the % inhibition reduced only to a negligible amount on heating. Inhibitory activity was found maximum (greater than

85% inhibition) in all the aqueous phases of the extracted fractions. Ojewole J.A, (2005) and Oh *et al.,* (2005) reported maximum hypoglycemic activity in aqueous extracts of guava plants. Almost the entire Guava plant (inclusive of Stem) showed significant α-amylase inhibition. Contrary to the aqueous extracts, the organic extracts showed negligible amount of inhibition (around 5-25%). So, our focus was on the water extracts for the remainder of the project. The ranking of the % inhibition in various extracts are as shown in Table 4.1.4

TABLE 4.1.4 - Ranking of α-Amylase inhibition % in different extracts analyzed

Part and phase of the extract	% α-amylase Inhibition
Water Extract	92.48
Hot Water Extract	88.83
Tender leaf – Aqueous phase	88.71
Mature fruit – Aqueous phase	88.10
Young fruit – Aqueous phase	87.29
Young leaf – Aqueous phase	85.47
Mature leaf – Aqueous phase	85.20
Stem – Aqueous phase	84.06

The % inhibition with varying inhibitor volume of both water and hot water extracts in both Aqueous and Organic phases were also determined. The results are shown in Tables 4.1.5, 4.1.6 and Fig. 4.1.3,

4.1.4, 4.1.5, 4.1.6. In both the extracts, the % inhibition increased with increasing volume of the inhibitor.

TABLE 4.1.5 - % α-Amylase Inhibition with varying inhibitor volume (Water Extract)

S.No	Water Extract-Aqueous Phase Inhibitor Concentration (in µl)	% α-Amylase Inhibition	
		Aqueous phase	Organic phase
1.	10	52.16	2.65
2.	25	76.48	3.15
3.	40	78.15	4.75
4.	50	93.52	8.52

TABLE 4.1.6 - % α-Amylase Inhibition with varying inhibitor volume (Hot Water Extract)

S.No	Hot Water Extract-Aqueous Phase Inhibitor Concentration (in µl)	% α-Amylase Inhibition	
		Aqueous phase	Organic phase
1.	10	15.56	3.83
2.	25	67.47	2.90
3.	40	79.32	3.52
4.	50	97.59	11.05

Fig. 4.1.3 - % α-Amylase inhibition Vs Aqueous phase (Water Extract) inhibitor volume

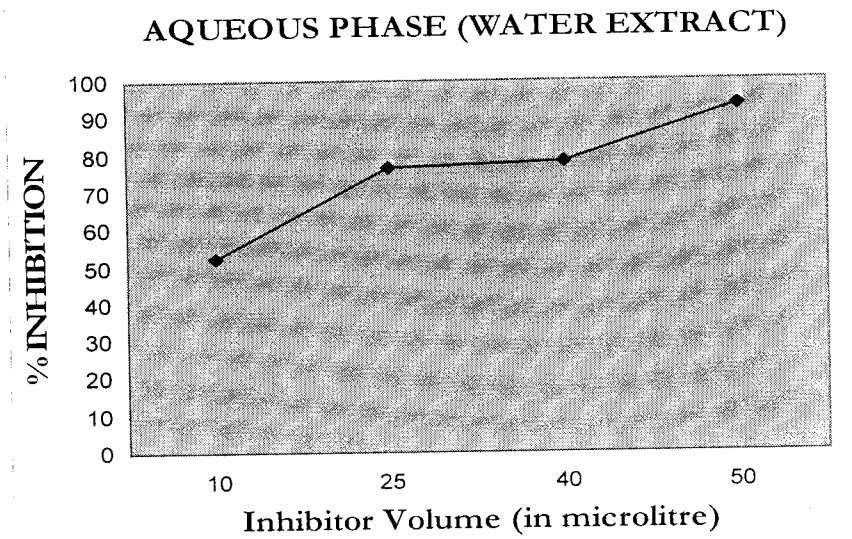


Fig. 4.1.4 - % α-Amylase inhibition Vs Organic phase (Water Extract) inhibitor volume

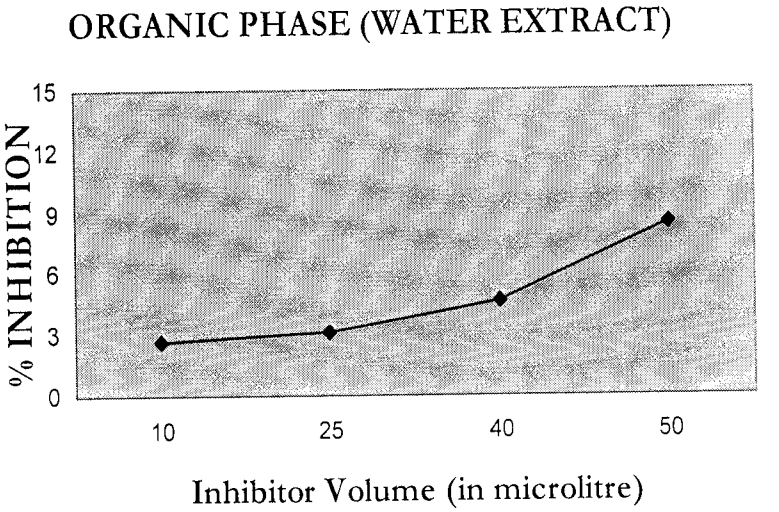


Fig. 4.1.5 - % α-Amylase inhibition Vs Aqueous phase (Hot Water Extract) inhibitor volume

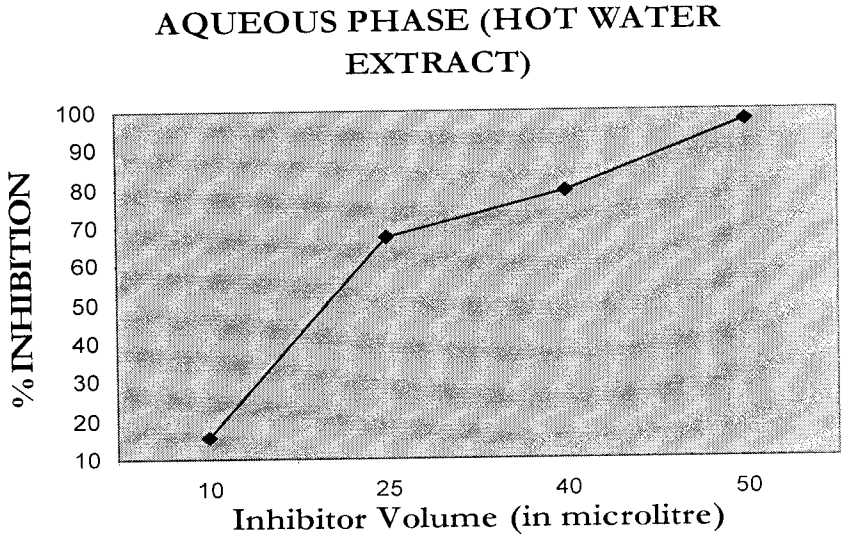
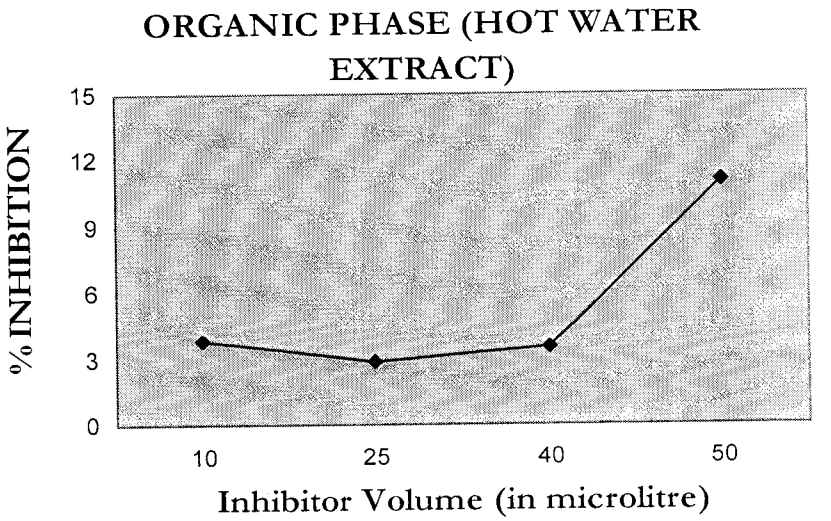


Fig. 4.1.6 - % α-Amylase inhibition Vs Organic phase (Hot Water Extract) inhibitor volume



4.2 Ammonium sulphate precipitation and dialysis

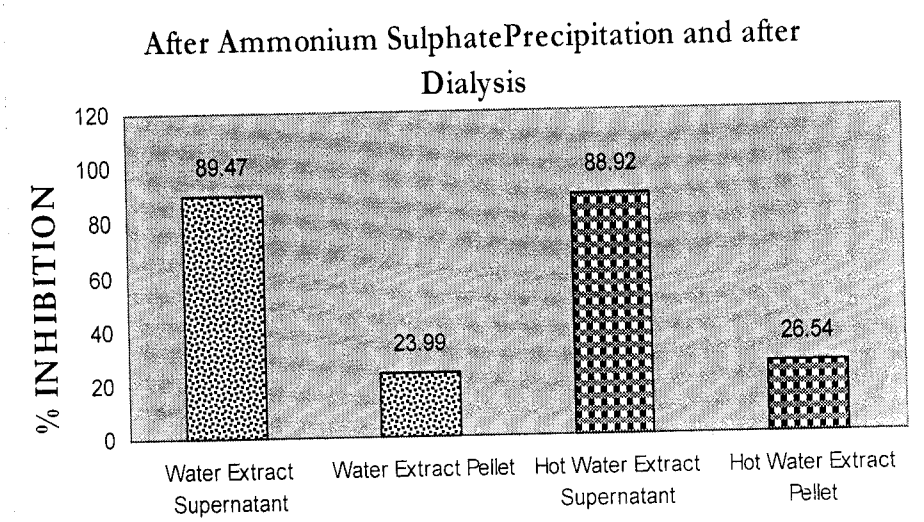
The Hot water extract and the water extract of Guava leaf powder were subject to Ammonium Sulphate precipitation to check the nature of inhibitor ie., whether it is proteinaceous or non-proteinaceous. The extracts along with ammonium sulphate at 100% saturation (Sadasivam and Manickam, 2004) were centrifuged for 30 min at 6000 rpm and the supernatant was checked for any α-amylase inhibition. It was found that the supernatant had % inhibition similar to that of the crude extract. This indicated that the inhibitor was non- proteinaceous in nature.

Dialysis experiment was performed to find out the nature of the inhibitor and the results showed that the inhibitor from *Psidium guajava Var. pomiferum* extract was non-proteinaceous in nature. The reversible inhibitor binds to the enzyme in a reversible manner without any formation of covalent bonds. Dialysis was performed to the pellet obtained from ammonium sulphate precipitation and minimal amount (approx 25%) of α-amylase inhibitory activity was observed by assay. This confirmed that the inhibitor is a non-proteinaceous one. The table for % inhibition after Ammonium sulphate precipitation and dialysis is shown in Table 4.2 and Fig. 4.2.

TABLE 4.2 - % α-Amylase Inhibition after Ammonium Sulphate Precipitation and after Dialysis

S.No	Nature of Extract	% α-Amylase Inhibition			
		Set I	Set II	Set 111	Mean
1.	Hot Water Extract - Supernatant	88.97	90.80	86.99	88.97
2.	Water Extract - Supernatant	89.58	88.90	89.92	89.47
	AFTER DIALYSIS				
3.	Hot Water Extract - Pellet	27.45	26.54	25.63	26.54
4.	Water Extract - Pellet	22.86	23.98	25.13	23.99

Fig. 4.2 - % α-Amylase Inhibition after Ammonium Sulphate Precipitation and after Dialysis



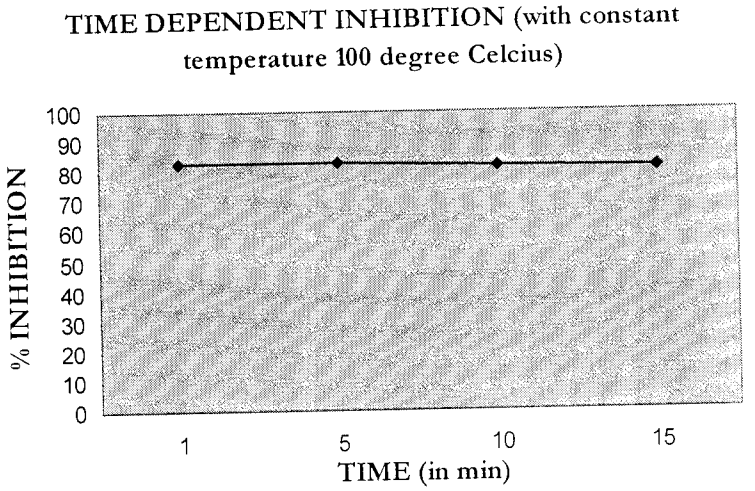
4.3 Stability analysis of the inhibitor

The time dependent stability of the inhibitor was also tested following microwave exposure at varying exposure times of the crude extract at a constant temperature of about 100°C. The inhibitory activity showed no significant decrease with varying exposure times of 1, 5, 10 and 15 min. The results are as shown in Table 4.3.1 and Fig. 4.3.1

TABLE 4.3.1 - Time Dependent α-Amylase inhibition (at constant Temperature of 100^0C)

S.No	Time (in min) at constant temperature 100^0C	% α-Amylase Inhibition
1.	1	83.17
2.	5	83.11
3.	10	82.14
4.	15	81.23

Fig. 4.3.1 - % α-Amylase inhibition Vs Time (in min) at constant Temperature of 100^0C

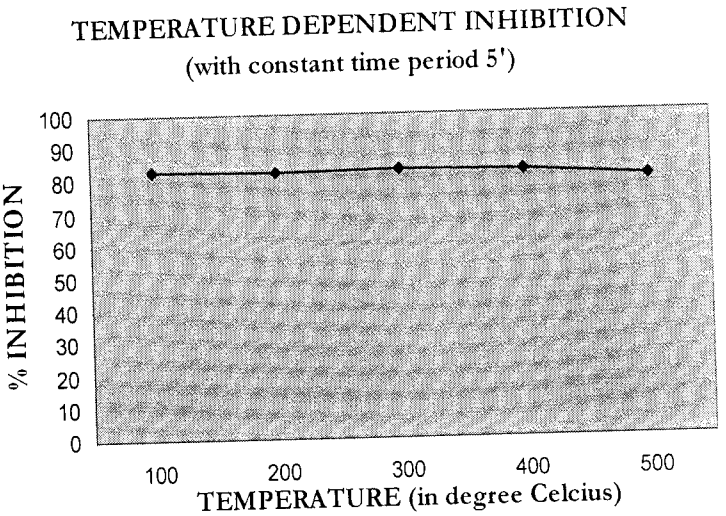


The thermostability of inhibitor was tested at varying temperatures from 100-500°C and the inhibition was prominent even at such high temperatures (around 85%). A linear pattern of inhibition was observed. The results are as shown in Table 4.3.2 and Fig. 4.3.2

TABLE 4.3.2 - Temperature Dependent α-Amylase inhibition (at constant Time period of 5 min)

S.No	Temperature (in ^{0}C) with constant time interval of 5 min	% α-Amylase Inhibition
1.	100	83.11
2.	200	82.39
3.	300	82.91
4.	400	82.46
5.	500	80.32

Fig. 4.3.2 - % α-Amylase inhibition Vs Temperature (in^{0}C) at constant Time period of 5 min

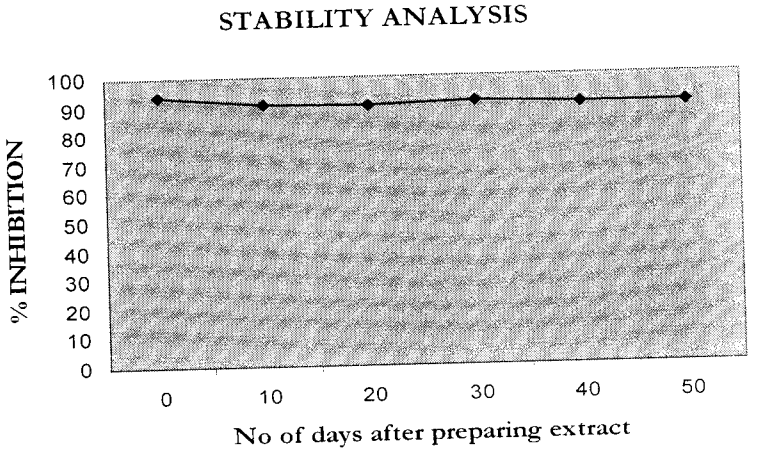


The stability of the inhibitor was also tested with sample after ten, twenty, thirty, forty and fifty days after preparing the extract. The % inhibition was in accord with the fresh extract values (around 90%). The inhibition pattern was once again observed to be linear. The table for % inhibition after storage of the Water Extract inhibitor sample (constant temperature 8-10°C) for a number of days after extract preparation is shown in Table 4.3.3 and Fig. 4.3.3

TABLE4.3.3 - Stability analysis of the α-Amylase inhibitor compound (Stored at 8-10^{0}C)

S.No	No. of Days after Extract preparation	% α-Amylase Inhibition
1.	0 – Fresh Extract	93.52
2.	10	91.07
3.	20	90.61
4.	30	91.32
5.	40	90.48
6.	50	90.09

Fig. 4.3.3 - % α-Amylase inhibition Vs No of days after preparation of Water Extract



4.4 LB Plot of the inhibitor sample

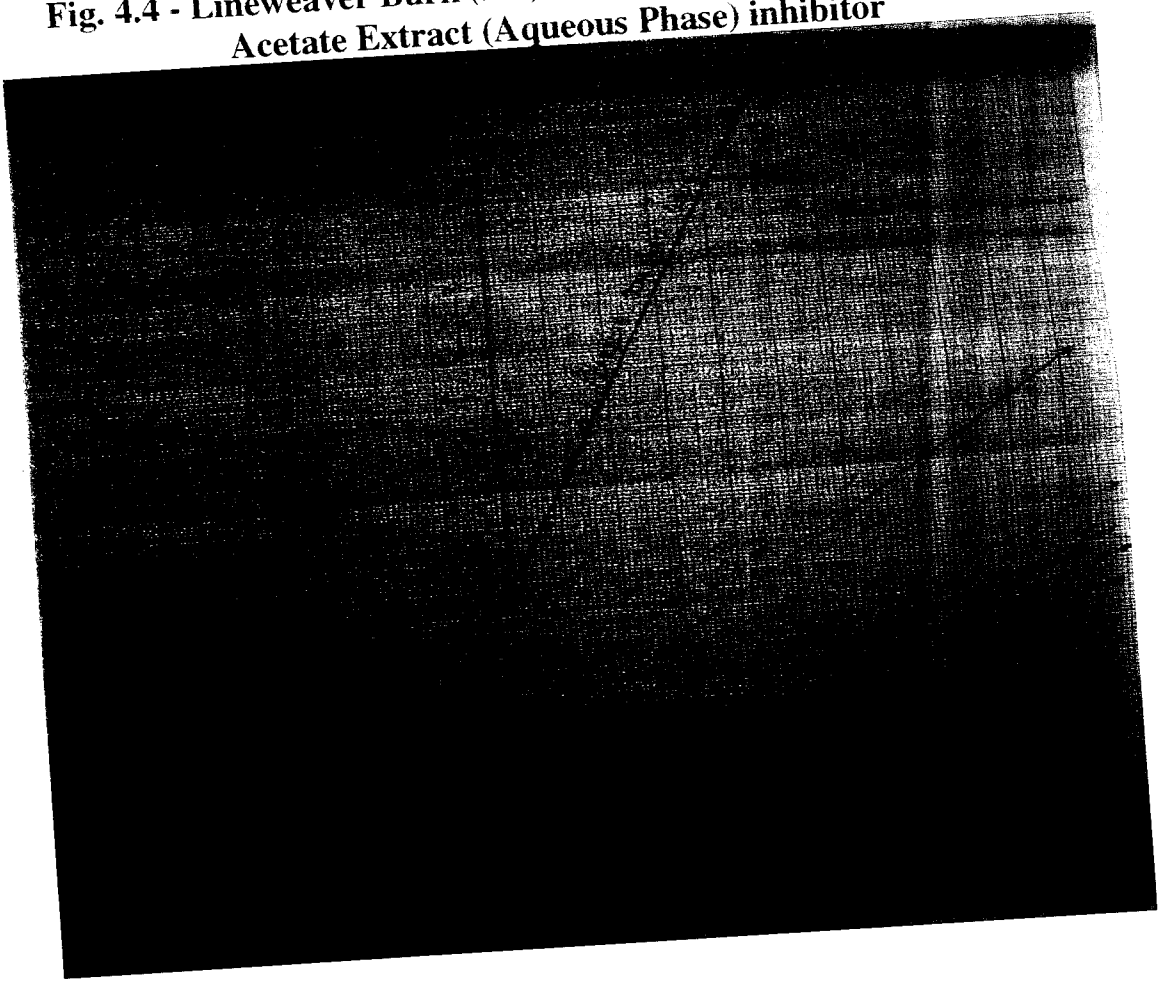
Lineweaver Burk (LB) plot was plotted using the varying concentrations of the substrate with the constant amount of the inhibitor and comparing it with commercially available acarbose as standard. The graph was drawn between 1/Velocity and 1/[S]. The inhibitior was found to be reversible and non-competitive as K_m values of the inhibitor sample at varying volumes remained constant (K_m= 40.00). Starch blockers from *Syzygium cumini Linn* seed fraction inhibited porcine pancreatic α-amylase in non-competitive and reversible fashion (Karthic *et al.*, 2007 – unpublished). The LB Plot is shown in Table 4.4 and Fig. 4.4.

TABLE 4.4 - Lineweaver-Burk (LB) Plot of *Psidium guajava Var. pomiferum* - Leaf powder Water Extract Inhibitor

xed ibitor lume n µl)	10µl					25µl					50µl				
in µl	20	40	60	80	100	20	40	60	80	100	20	40	60	80	100
D 540 nm	.409	.432	.387	.372	.178	.670	.629	.597	.581	.838	1.230	1.256	1.199	1.003	1.13
ug/ml-nin)	640	560	500	480	240	870	820	780	760	1100	1500	1600	1500	1300	1400
1/V* 0^-3	1.56	1.78	2.00	2.08	4.17	1.15	1.22	1.28	1.32	.909	.67	.63	.67	.77	.71

ixed hibitor olume in µl)	75µl					100µl					150µl				
S] in µl	20	40	60	80	100	20	40	60	80	100	20	40	60	80	100
O.D 540 nm	.70	1.02	.82	.87	.696	.818	.804	.471	.660	.921	.867	.889	.618	.727	.760
V (µg/ml-min)	960	1300	1060	1140	900	1060	1040	600	860	1200	1140	1260	800	900	980
1/V* 10^-3	1.04	.77	.94	.88	1.11	.943	.96	1.67	1.16	.83	.88	.79	1.25	1.1	1.01

Fig. 4.4 - Lineweaver Burk (LB) Plot of Guava Leaf powder-Ethyl Acetate Extract (Aqueous Phase) inhibitor



4.5 Standardisation of the inhibitor

With varying concentrations of the inhibitor, the enzyme activity was found out and then a graph was plotted to determine IC_{50} value (IC_{50} is the concentration of the inhibitor required to inhibit 50% of the enzyme activity). This was then compared with the IC_{50} value from the graph plotted with varying concentration of acarbose. The results are shown in Table 4.5.1, 4.5.2 and Fig. 4.5.1, 4.5.2. It was standardized that approximately **2.34g** of the crude guava leaf powder extract was equivalent to **25mg** of acarbose (dosage per day).

TABLE 4.5.1 - % α-Amylase Inhibition with varying Acarbose Inhibitor volume

ACARBOSE CONCENTRATION (µg)	α-AMYLASE INHIBITION (%)	OD 540nm	
		CONTROL	TEST
1	16	0.80	1.375
2	30	0.123	1.198
5	75	0.443	0.833
10	81	0.532	0.826

TABLE 4.5.2 -% α-Amylase Inhibition with varying Water Extract Inhibitor volume

CRUDE EXTRACT CONCENTRATION (µg)	α-AMYLASE INHIBITION (%)	OD 540nm	
		CONTROL	TEST
100	17	0.210	1.487
200	34	0.321	1.335
300	45	0.144	0.993
400	70	0.476	0.945
500	80	0.670	0.975

Fig. 4.5.1 - % α-Amylase inhibition Vs Acarbose concentration

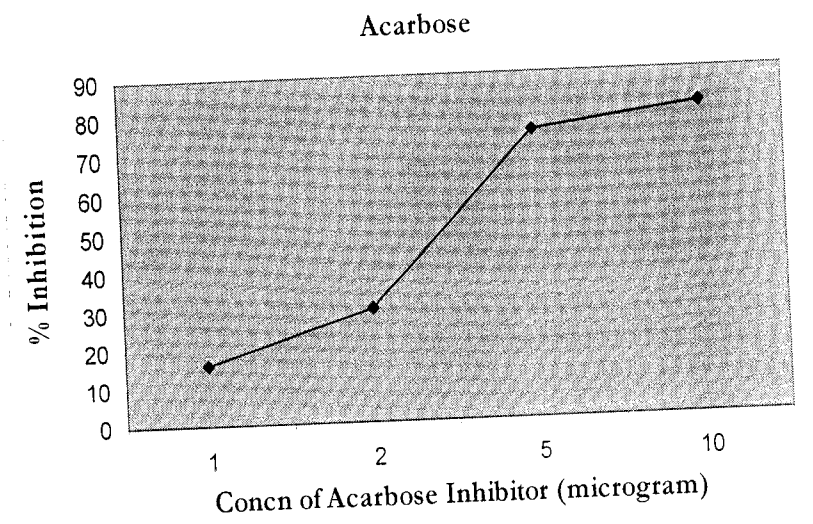
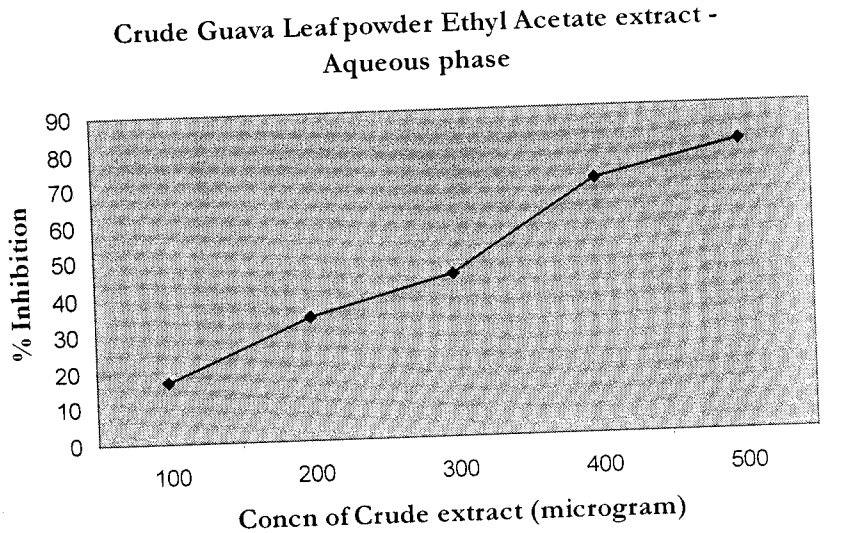


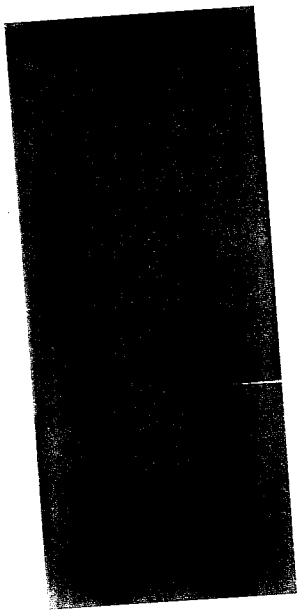
Fig. 4.5.2 - % α-Amylase inhibition Vs Crude Guava leaf powder Extract concentration



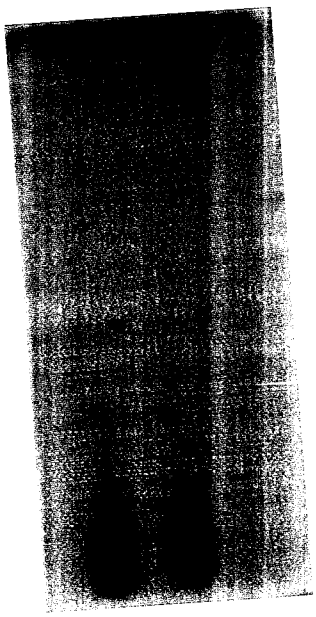
4.6 Separation of Compounds by Thin Layer Chromatography (TLC)

The Aqueous phase of Water extract was taken and TLC was performed with various solvents. The extract was directly used as the source of inhibitor sample for TLC. Different Spraying reagents were used for the identification of phenolics, flavonoids, 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/flavonoids respectively. The results are shown in Fig. 4.6.1.

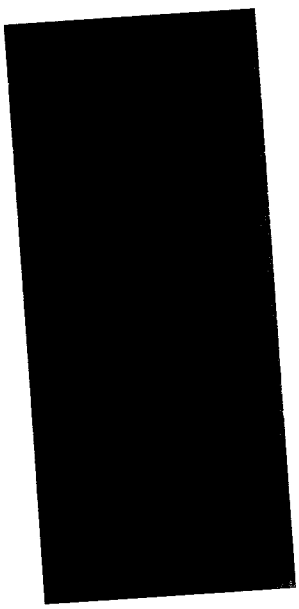
Fig. 4.6.1 - Separation of compounds by TLC



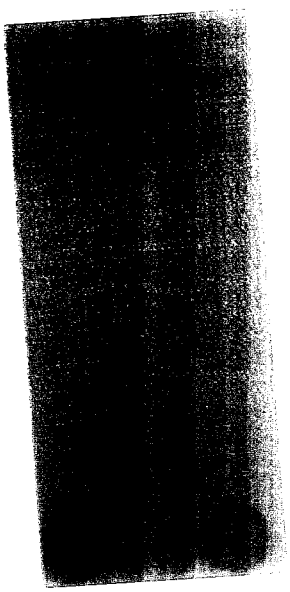
PHENOLICS



FLAVONOIDS

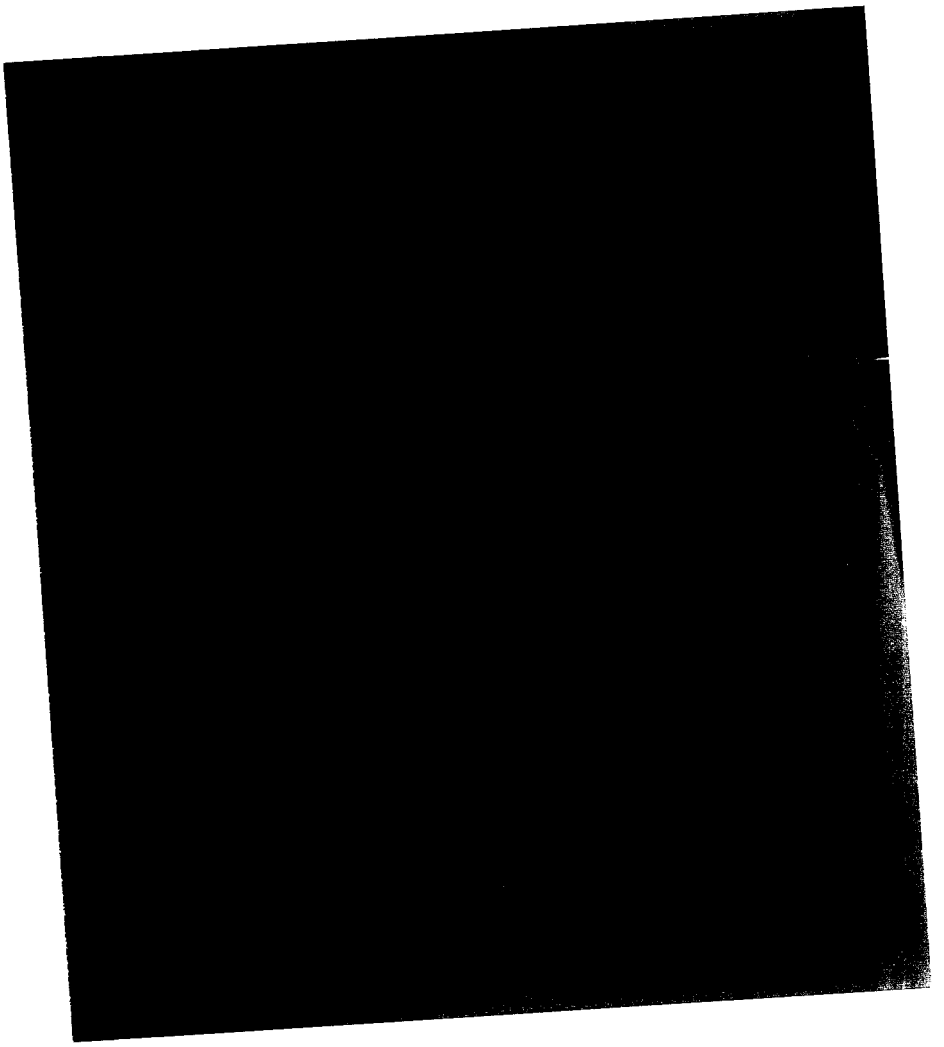


TERPENOIDS



ALKALOIDS

Fig. 4.6.2 - Preparative TLC for enrichment of inhibitor concentration



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 ethyl acetate and then centrifuged. The supernatant was evaporated and dissolved in equal volume of water. This was used as the source of inhibitor for the assay. A preparative TLC plate is shown in Fig. 4.6.2. Phytochemical studies have identified more than 20 compounds in guava

extracts (Osman *et al.,* 1974; Begum *et al.,* 2002). The major constituents of its leaves were identified to be tannins, β-sitosterol, maslinic acid, essential oils, triterpenoids and flavonoids (Liang Q, *et al.,* 2005).

The solvent system usually employed for separating phenolics was used. The phenolic and terpenoid compounds of the *Psidium guajava Var. pomiferum* extract was separated by TLC and based on the Rf value the plate was divided into five fractions. Each fraction was tested for α-amylase inhibitor activity. The Phenolic fraction with Rf value between 26% to 50% and the terpenoid fraction whose Rf value ranging from 51% to 75% inhibited 63.62% and 70.97% of the amylase activity respectively. The results are shown in Tables 4.6.1, 4.6.2 and Fig. 4.6.3, 4.6.4.

The polyphenol rich extracts from soft fruits have the ability to inhibit α-amylase and α-glucosidase. The best solvent system for phenolics [Chloroform:EthylAcetate:Methanol – 3:4:3] and terpenoids [Chloroform: Methanol – 5:5] were found and the compounds were isolated and are sent for RP-HPLC. The RP-HPLC chromatogram in different views are shown in Fig. 4.6.5, 4.6.6, 4.6.7, 4.6.8.

TABLE 4.6.1- % α-Amylase Inhibition in different Rf Value fractions of Phenolics

S.No	Rf Values (in %)	% Inhibition
1.	0 – 25	61.60
2.	26 – 50	63.62
3.	51 – 75	60.33
4.	76 – 100	25.05
5.	>100	10.48

TABLE 4.6.2 - % α-Amylase Inhibition in different Rf Value fractions of Terpenoids

S.No	Rf Values (in %)	% Inhibition
1.	0 – 25	48.52
2.	26 – 50	67.53
3.	51 – 75	70.97
4.	76 – 100	65.89
5.	>100	9.02

Fig. 4.6.3 - % α-Amylase inhibition Vs TLC Rf Value fractions for Phenolics

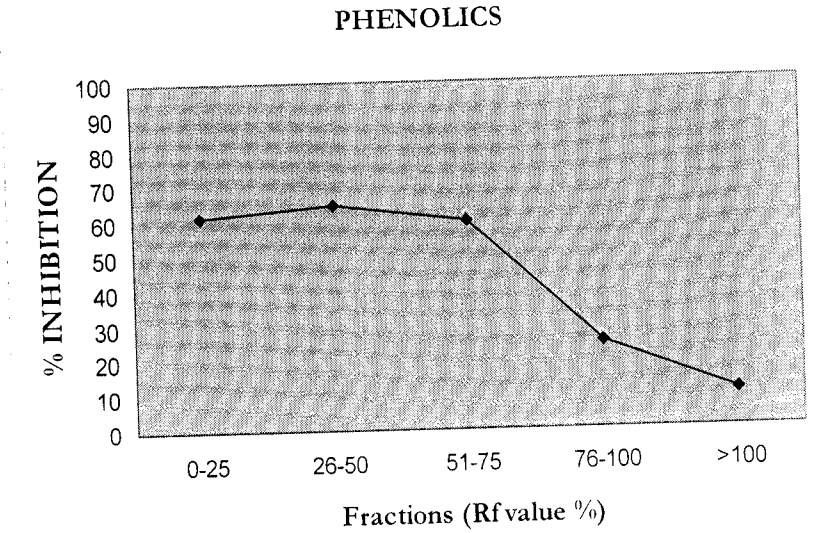
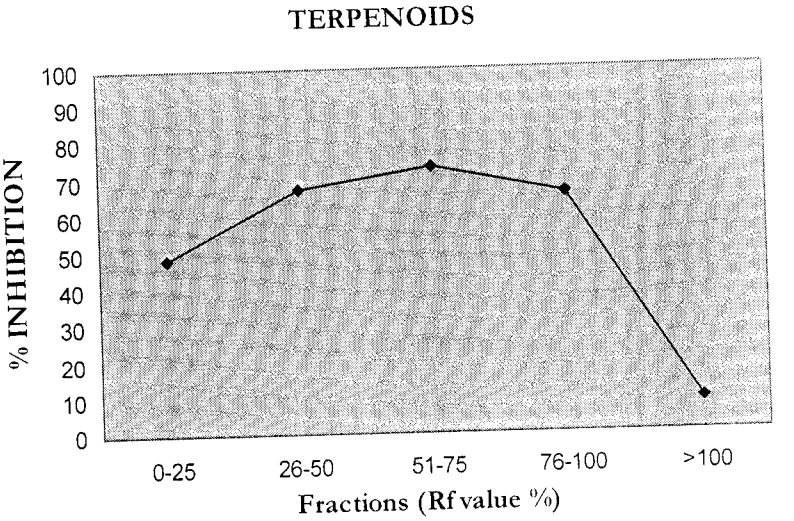


Fig. 4.6.4 - % α-Amylase inhibition Vs TLC Rf Value fractions for Terpenoids



4.7 Antioxidant Assays

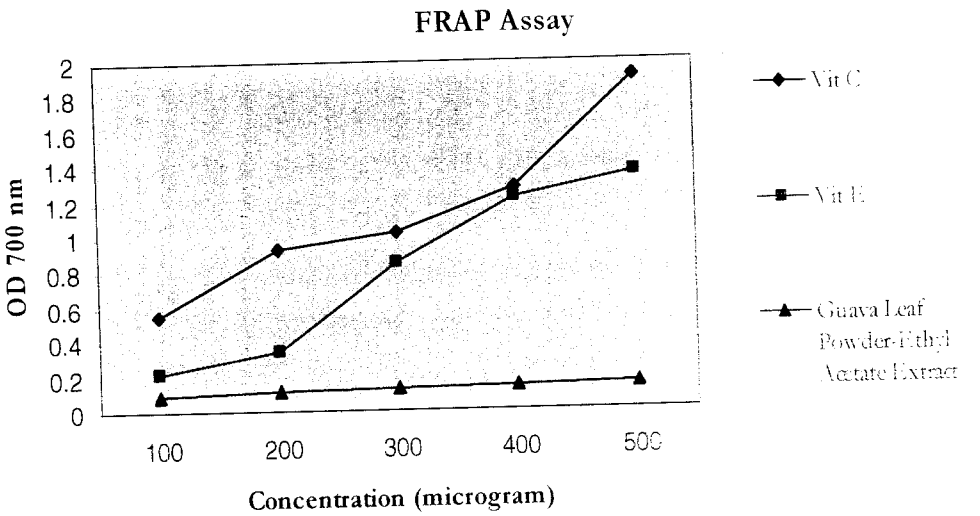
4.7.1 FRAP Assay

The FRAP assay was done according to Benzie and Strain (1996) with some modifications. Guava Leaf powder extracts were allowed to react with Potassium Ferricyanide. The reaction was arrested after 20 min incubation at 50^0C with 10% TCA. The tubes were centrifuged at 650g for 10 minutes and 4ml of supernatant was treated with 0.8ml of 0.1% $FeCl_3$ was added. The blue colour developed [ferrous tripyridyltriazine complex] was colorimetrically read at 700 nm (Kriengsak *et al.*, 2006) and an increase in the OD reading showed an increased antioxidant activity in the leaf extract. However, the antioxidant activity of the leaf powder extract was less when compared with standard antioxidants such as Vitamin C and E. The results are as shown in Table 4.7.1 and Fig. 4.7.1

TABLE 4.7.1 - FRAP Assay

S No.	Concentration (µg)	OD 700 nm		
		Vitamin C	Vitamin E	Guava leaf powder extract in Ethyl acetate
1.	100	0.554	0.213	0.088
2.	200	0.934	0.349	0.111
3.	300	1.027	0.856	0.123
4.	400	1.279	1.216	0.138
5.	500	1.912	1.353	0.145

Fig. 4.7.1 - FRAP Antioxidant Assay results comparison between Vitamin C, E and Guava Leaf Powder-Ethyl Acetate Extract



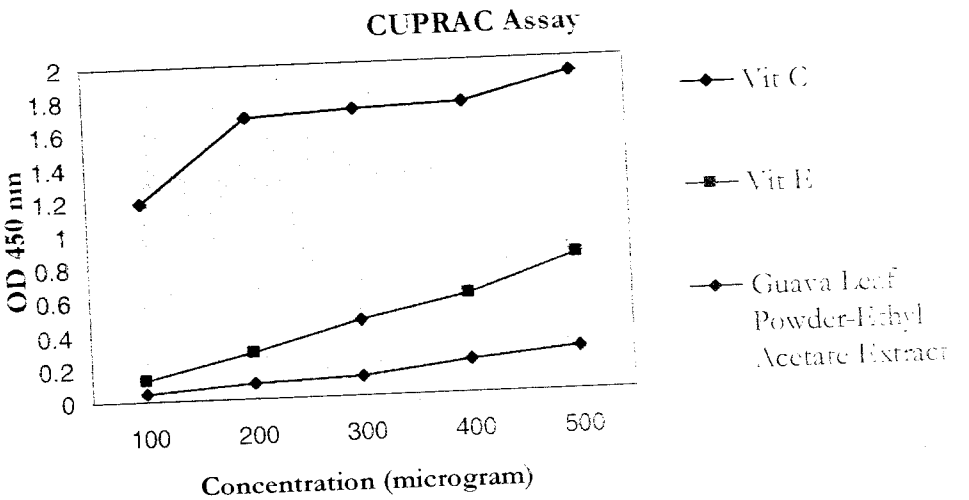
4.7.2 CUPRAC Assay

CUPRAC Assay was performed with Guava Leaf powder extract in Ethyl acetate and compared with standard Vitamin C and E. The results are as shown in Table 4.7.2 and Fig. 4.7.2. Copper (II)-neocuproine [Cu(II) - Nc] reagent is used here as the chromogenic oxidizing agent which is reduced to the highly colored Cu (I) - Nc chelate showing maximum absorption at 450 nm (Tutem E *et al.*, 1991). An increase in the OD reading showed an increased antioxidant activity in the leaf extract. However, the antioxidant activity of the leaf powder extract was less when compared with standard antioxidants such as Vitamin C and E.

TABLE 4.7.2 - CUPRAC Assay

S No.	Concentration (µg)	OD 450 nm		
		Vitamin C	Vitamin E	Guava leaf powder extract in Ethyl acetate
1.	100	1.184	0.213	0.052
2.	200	1.682	0.349	0.092
3.	300	1.722	0.856	0.115
4.	400	1.742	1.216	0.194
5.	500	1.912	1.353	0.257

Fig. 4.7.2 - CUPRAC Antioxidant Assay results comparison between Vitamin C, E and Guava Leaf Powder-Ethyl Acetate Extract



4.7.3 DPPH Assay

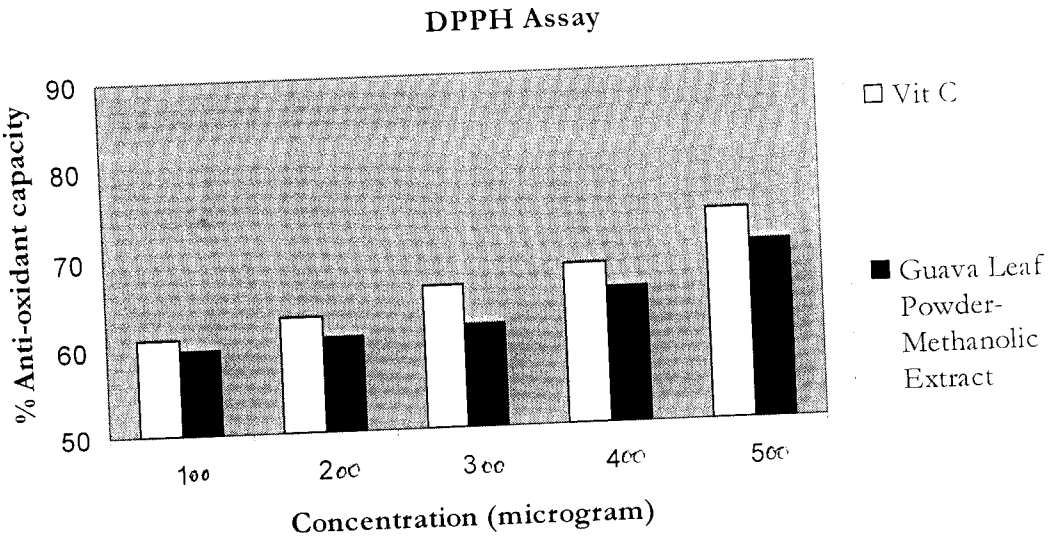
The DPPH assay was done according to the method of Brand-Williams *et al.*, (1995) with some modifications. $DPPH^{\bullet}$ reacts with an antioxidant compound, which can donate hydrogen and gets reduced. Guava Leaf Powder extracts were allowed to react with 0.5 ml of DPPH reagent. The 'Control' tube showed maximum OD value at 517nm due to absence of free radical scavenging (Kriengsak *et al.*, 2006). On the contrary, the OD values decreased with increasing concentration of test samples providing a strong evidence of free radical scavenging. The changes in colour (from deep violet to light yellow) were measured at 517 nm on a UV/visible light spectrophotometer. The % antioxidant capacity was compared with standard Vitamin C. The values were similar to those observed in Vitamin C. The results are as shown in Table 4.7.3 and Fig. 4.7.3

TABLE 4.7.3 - DPPH Assay

OD 517 nm of 'CONTROL' = 1.541

S No.	Concentration (µg)	OD 517 nm		% Anti-oxidant Activity [(C-T)/C]	
		Vit C	Guava Leaf Powder Extract in Methanol	Vit C	Guava Leaf Powder Extract in Methanol
1.	100	0.602	0.619	60.93	59.83
2.	200	0.566	0.605	63.27	60.74
3.	300	0.522	0.591	66.13	61.65
4.	400	0.491	0.535	68.14	65.28
5.	500	0.404	0.461	73.78	70.08

Fig. 4.7.3 - DPPH Antioxidant Assay results - % Antioxidant Capacity Vs Concentration for Vitamin C and Methanolic Extract of Guava Leaf powder



4.8 Herbal tea preparation and Organoleptic test

Herbal tea, using Guava leaf powder, was prepared in four different flavours and an organoleptic test was performed on 6th February, 2007 in the Bio-Chemistry laboratory. The four different flavours were:

- **Sample 1** → Pure Guava leaf powder decoction + sugar to taste
- **Sample 2** → Sample 1 + mint powder
- **Sample 3** → Sample 1 + cardamom powder
- **Sample 4** → Sample 1 + lime

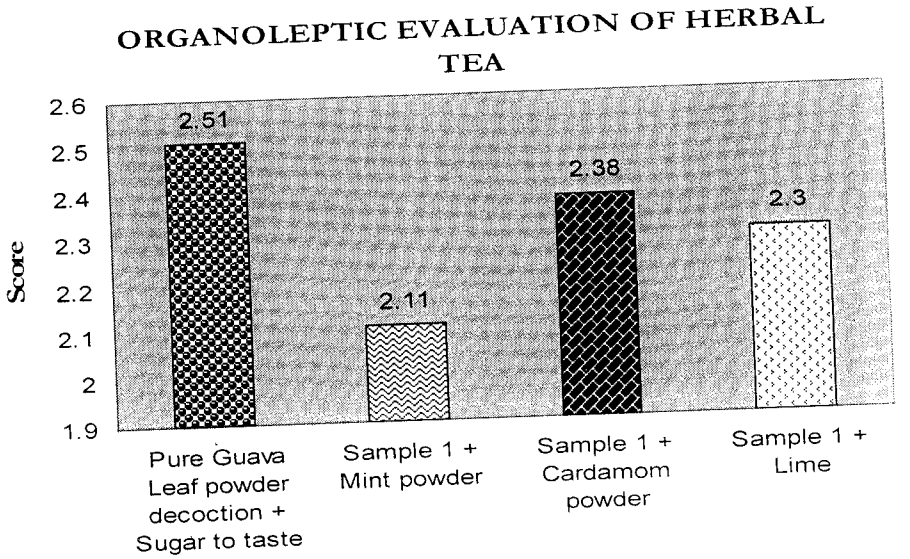
The results of this test, shown in Table 4.8 and Fig. 4.8, indicated that plain tea prepared using Guava leaf powder decoction along with little sugar to taste [Sample 1] was the most palatable when compared with other flavours. Studies confirm that tea catechins—potent antioxidants—are effective in suppressing increases of glucose and insulin

concentrations in the blood. Since blood sugar tends to increase with age, this effect is an extremely important anti-aging benefit. Tea polyphenols inhibit the activity of α-amylase, found in saliva and in the intestines. Starch is broken down more slowly, and the rise in serum glucose is minimized. Green Tea and its extract in the form of a nutritional supplement helps in calorie and insulin control.

TABLE 4.8 - Results of Organoleptic Evaluation of Herbal Tea

Sample	Details	Average Score (max = 3)
1	Pure Guava leaf powder decoction + sugar (to taste)	2.51
2	Sample 1 + Mint powder	2.11
3	Sample 1 + Cardamom powder	2.38
4	Sample 1 + Lime	2.30

Fig. 4.8 - Organoleptic Evaluation of Herbal Tea samples



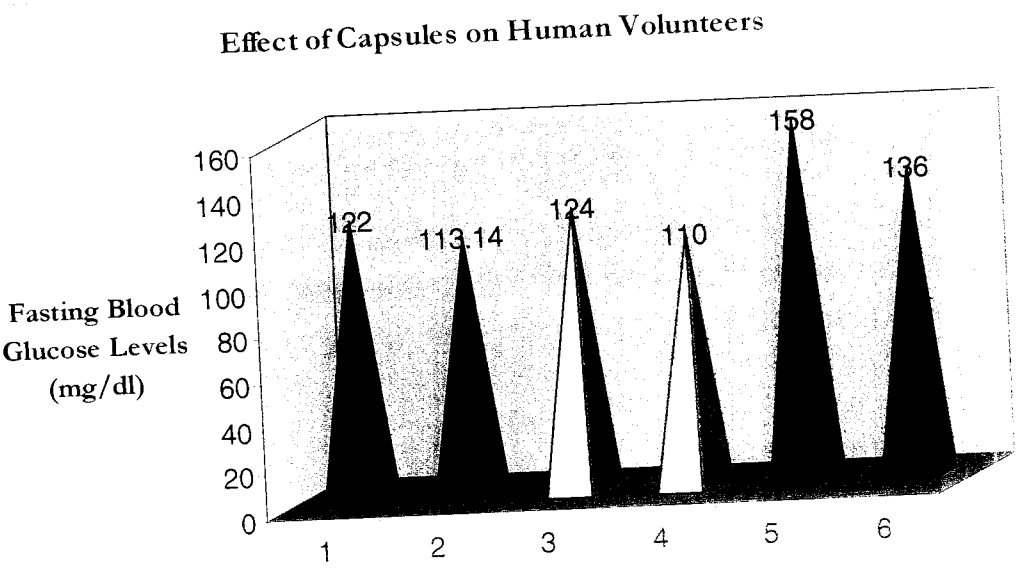
4.9 Preliminary clinical trial using Guava Leaf powder filled capsules

Soft gelatin capsules were filled with 150mg of Guava leaf powder each and distributed to three volunteers whose blood sugar level were marginal (approx 110 mg/dl "fasting") or above normal (more than 120 mg/dl "fasting"). The dosage regimen was set as 5. Volunteers of different age groups were selected. The blood glucose levels ('fasting') were measured before and after the capsule consumption. The results were obtained after a period of one month. The blood sugar level consistently decreased in all the volunteers and maintained around normal. The results of this preliminary study are shown in Table 4.9 and Fig. 4.9.

TABLE 4.9 - Effect of Capsules on Volunteers

Volunteer No	Age (in years)	"Fasting" Blood Glucose Levels (in mg/dl)		No of days of Capsule consumption
		Before Capsule consumption	After Capsule consumption	
1	39	122	113.14	30
2	78	124	110	30
3	47	158	136	15

Fig. 4.9 - Effect of Capsules on Volunteers – Blood Glucose levels before and after the testing duration



Key

1.Volunteer No.1 [Age 39] Blood Glucose Levels BEFORE Capsule Consumption.
2.AFTER Capsule Consumption.

3.Volunteer No.2 [Age 78] Blood Glucose Levels BEFORE Capsule Consumption.
4.AFTER Capsule Consumption.

5.Volunteer No.3 [Age 47] Blood Glucose Levels BEFORE Capsule Consumption.
6.AFTER Capsule Consumption.

5. CONCLUSION & FUTURE PERSPECTIVES

5. CONCLUSION

Alpha-amylase inhibitors from natural sources like medicinal plants offer an attractive therapeutic approach towards effective treatment of diabetes by decreasing glucose release from starch. Powerful, synthetic α-amylase inhibitors are available but cause various side effects. So, phenolic and terpenoidal α-amylase inhibitors from medicinal plant extracts are potentially safe. We have identified a potent, reversible, non-competitive α-amylase inhibitor from the aqueous extract of *Psidium guajava Var. pomiferum* leaves. 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. The preparative RP-HPLC purified sample has been sent for identification of chemical structure. The antioxidant potential of Guava leaf powder was also confirmed by FRAP, CUPRAC and DPPH assays.

FUTURE PERSPECTIVES

The RP-HPLC purified sample has been sent to the Central Drug Research Institute (CDRI), Lucknow to record LC-ESI-MS. The mass spectra of the separated compounds will be used to identify the chemical structure of the compounds. The preparative HPLC purified sample has to be tested for its anti-diabetic activity in experimental animals on comparison with the dry leaf powder with human volunteers, clinical trial has to be done systematically.

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