IDENTIFICATION OF α- AMYLASE INHIBITORS FROM *Psidium guajava* AND INVESTIGATE ITS ANTIDIABETIC EFFECTS ON ALLOXAN-INDUCED DIABETIC RATS

PROJECT REPORT

Submitted by

SANTHOSH. M

Register No: 0820203012

in partial fulfillment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE-06.

(An Autonomous Institution affiliated to Anna University, Coimbatore)

MAY 2010

ANNA UNIVERSITY: COIMBATORE

BONAFIDE CERTIFICATE

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE-641 006

Department of Biotechnology

PROJECT WORK -PHASE II

MAY 2010

This is to certify that the project entitled **IDENTIFICATION OF α-AMYLASE INHIBITORS FROM *Psidium guajava* AND INVESTIGATE ITS ANTIDIABETIC EFFECTS ON ALLOXAN-INDUCED DIABETIC RATS** is the bonafide record of project work done by **SANTHOSH. M Register No: 0820203012** of M.Tech during the year 2009-2010.

Dr. S.SADASIVAM

Dean – Biotechnology

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore - 641 006

Dr. S.SADASIVAM

Dean - Biotechnology

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore - 641 006

Submitted for the Project Viva-Voce examination held on 17.5.10

Internal Examiner

17.5.10

External Examiner

DECLARATION

I affirm that the project work titled **IDENTIFICATION OF α- AMYLASE INHIBITORS FROM *Psidium guajava* AND INVESTIGATE ITS ANTIDIABETIC EFFECTS ON ALLOXAN-INDUCED DIABETIC RATS** being submitted in partial fulfillment for the award of **M.Tech (Biotechnology)** is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

SANTHOSH. M

0820203012

I certify that the declaration made by the above candidate is true.

Signature of the Guide,

Dr. S.SADASIVAM

Dean - Biotechnology

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

I am very much grateful to my **Parents** and the **Almighty** for showering their blessings on me without which none of these would have been possible.

I wish to extend my sincere thanks to our principal **Dr.S. Ramachandran** for his incredible support for all my toil regarding the project.

With immense pleasure, I wish to avail this opportunity and evoke on record the ineffable personal indebtness and deep sense of gratitude to my internal guide **Dr.S. Sadasivam, Dean**-Department of Biotechnology, Kumaraguru College of Technology, Coimbatore for giving me an opportunity to carry out my project work outside the campus.

I would like to thank and express my gratitude to **Mr. T. Sathish Kumar, Senior Lecturer,** Department of Biotechnology and all the staff members of Department of Biotechnology, Kumaraguru College of Technology, Coimbatore for their valuable suggestions and guidance.

I am elated to accord my deep sense of gratitude to **Mr. K. G. Prasanth, Assistant Professor,** Department of Pharmacology, PSG College of Pharmacy and **Mr. B. Balaji, Research Scholar,** Department of Pharmacology, PSG College of Pharmacy for their meticulous guidance, continuous encouragement and suggestions rendered throughout my project work.

It gives me immense pleasure to express my sincere thanks to **Dr. N. Saraswathy, Assistant professor,** Department of Biotechnology, Kumaraguru College of Technology and my special thanks to my beloved brothers **Mr. Vijaya Bhaskar M and Mr. Ramesh M, Mrs. K. Boomalatha, Ms. N. Anitha, Mr. Elango, Mr. K. Ramesh, Mr. V. Palanisamy** and my **friends** and my **juniors** who were the pillars of support during the hardships of my project.

I wish to thank all the teaching and non-teaching members of the Department of Biotechnology, Kumaraguru College of Technology, for their help throughout my project work.

SANTHOSH M

CONTENTS

TABLE OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	ACKNOWLEDGEMENT	IV
	ABSTRACT	IX
	LIST OF TABLES	X
	LIST OF FIGURES	XI
1.	**Introduction**	1
1.1	Objectives	6
2.	**Review of Literature**	7
2.1	Diabetes	7
2.2	Pathophysiology	8
2.3	Types of Diabetes	9
2.3.1	Type 1 Diabetes	9
2.3.2	Type 2 Diabetes	9
2.3.3	Gestational Diabetes	10
2.3.4	Other specific forms of Diabetes	11
2.4	Treatments of Diabetes	11

	2.4.1	Mechanism and present drugs for therapy of diabetes mellitus	13
2.5		Natural Treatments for Diabetes	15
2.6		Guava and Diabetes	17
	2.6.1	Phytochemistry	17
	2.6.2	Biological Activity and Clinical Research	18
2.7		Antioxidant capacity of guava	19
2.8		Laboratory Animal	22
	2.8.1	Strains	23
2.9		Induction of diabetes in rats	24
	2.9.1	The mechanism of alloxan action	24
3.		**Materials and Methods**	31
3.1		Plant material	31
3.2		Preparation of the Extract from *Psidium guajava* Leaves	31
3.3		Extraction of compounds from different parts of the Guava plant (Arnon D I, 1949)	32
	3.3.1	Chemicals and materials required	32
	3.3.2	Apparatus and Glass wares required	32
	3.3.3	Procedure	33

3.4	Preparation of Hot Extract	33
3.5	Preparation of Normal Extract	33
3.6	Estimation of Flavanoids by Aluminium Chloride method	34
3.6.1	Principle	34
3.6.2	Reagents	34
3.6.3	Procedure	34
3.7	α-Amylase inhibitory assay	35
3.7.1	Preparation of Enzyme working standard	35
3.7.2	Principle- DNS Method	35
3.7.3	Reagents required	36
3.7.4	Flow of procedure for testing α – amylase inhibiting activity	37
3.8	Free Radical Scavenging Activity of Extracts	38
3.8.1	Ferric Ion Reducing Antioxidant Power Assay (FRAP)	38
3.8.1.1	Principle	38
3.8.1.2	Reagents	38
3.8.1.3	Procedure	39
3.9	Investigation of antidiabetic effects of α- Amylase inhibitor from *Psidium guajava* on alloxan-induced diabetic rats	40

	3.9.1	Animals and treatments	40
	3.9.2	Induction of diabetes	40
	3.9.3	Decoction preparation	40
	3.9.4	Assessment of antidiabetic activity	41
	3.9.5	Statistical Analysis	41
4.		**Results and Discussion**	42
4.1		*In vitro* alpha amylase inhibition	42
4.2		Estimation of Flavonoids by Aluminium Chloride Method	44
4.3		Antioxidant Assays	48
	4.3.1	Ferric Ion Reducing/Antioxidant Power Assay (FRAP) Assay	48
4.4		Investigation of antidiabetic effects of α- Amylase inhibitor from *Psidium guajava* on alloxan-induced diabetic rats	51
	4.4.1	Body Weight	51
	4.4.2	Blood Glucose	53
5.		**Conclusion**	57
6.		**References**	58

ABSTRACT

Diabetes mellitus is a metabolic disorder in the endocrine system. This dreadful disease is found in all parts of the world and is becoming a serious threat to mankind health. There are lots of chemical agents available to control and to treat diabetic patients, but total recovery from diabetes has not been reported up to this date. Alternative to these synthetic agents, antidiabetics of plant origin provide a potential source of hypoglycaemic drugs and are widely used in several traditional systems of medicine to prevent diabetes. Important constituents for the inhibitory activity against alpha-amylase are mainly polyphenolic, terpenoidal and flavonoid compounds. Earlier studies indicated that the leaves of *Psidium guajava* possessed α- Amylase inhibitory activity (98%). The presence of flavonoids such as apigenin-7-O- glucoside, genistein, genistein-p-coumarate, quercetin-7-O-malonate, 24-hydroxy guavanoic acid and betulinic acid in the leaves of *Psidium guajava* were responsible for the α-amylase inhibitory activity. The antidiabetic/antihyperglyceamic effect of the aqueous extract of the leaves of *Psidium guajava* was examined in alloxan induced diabetic albino Wistar rats. Oral administration of the aqueous leaf extracts (250 and 500 mg/kg) for a period of 21 days resulted in significant reduction in blood glucose in diabetic rats. Body weight showed significant increase after treatment with aqueous leaf extracts when compared with the control. The effects of the extracts at both dose levels are quiet comparable with Acarbose (a standard drug used to treat diabetes mellitus). Further, the antioxidant capacity of guava leaf extract was confirmed by FRAP assay. The results from the present study suggested that aqueous leaf extracts of *Psidium guajava* possess antidiabetic effects in diabetic rats and potential antioxidant effects. There is a need for further clinical studies to establish a rational therapy with traditional herbal preparations, especially for the leaves from *Psidium guajava L.*

LIST OF TABLES

TABLE NO.	TITLE OF TABLE	PAGE NO.
1.1	Scientific Classification of *Psidium guajava L.*	3
4.1.1	Percentage Inhibition of Porcine Pancreatic α- Amylase by Extracts Obtained from Different Extraction Procedures	42
4.2.1	Standard Curve for Rutin	45
4.2.2	Estimation of Total Flavonoids Content in test samples	46
4.2.3	Estimation of Total Flavonoids Content in Hot Extract	46
4.2.4	Estimation of Total Flavonoids Content in Normal Extract	47
4.3.1	Ferric Ion Reducing/Antioxidant Power Assay (FRAP) of *Psidium guajava* leaf Extract	48
4.4.1	Effect of aqueous leaf extract of P.guajava and Acarbose on Body Weight (g) in Alloxan induced diabetic rats	51
4.4.2	Effect of aqueous leaf extract of P.guajava and Acarbose on Blood level concentration (mg/dl) in Alloxan induced diabetic rats.	55

LIST OF FIGURES

FIGURE NO.	TITLE OF FIGURE	PAGE NO.
2.9.1	The mechanism of alloxan-induced reactive oxygen species generation in B cells of rat pancreas. GKa, GKi – glucokinase active and inactive, respectively; $HA^{\cdot}$ – Alloxan radicals; $[Ca^{2+}]_i$ – intracellular calcium concentration.	27
4.1.1	Percentage Inhibition of Porcine Pancreatic α- Amylase by Extracts Obtained from Different Extraction Procedures	43
4.2.1	Standard Curve for Rutin	45
4.3.1	Ferric Ion Reducing/Antioxidant Power Assay (FRAP) of *Psidium guajava* leaf Extract	49
4.4.1	Histogram showing changes in body weight following treatments with *Psidium guajava* leaf extracts and Acarbose in Alloxan induced diabetic rats	52
4.4.2	Histogram showing changes in blood glucose level following treatments with *Psidium guajava* leaf extracts and Acarbose in Alloxan induced diabetic rats	56

INTRODUCTION

CHAPTER 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. Diabetes mellitus (DM) is a group of ailments characterized by abnormal carbohydrate, lipid, and protein metabolism resulting from insufficient action of insulin. This results primarily in elevated fasting and postprandial blood glucose levels. If abnormal metabolism does not return to normalcy and continues for a protracted period of time, it leads to hyperglycemia (glucose rate on an empty stomach higher than 1.2 g/l in plasma blood and confirmed in at least two occasions) that in due course turns into a syndrome called diabetes mellitus. This disorder is characterized by polyuria (frequent and abundant urines), glycosuria (presence of glucose in urines). DM is associated with premature death, and disability such as nephropathy, retinopathy, neuropathy, microvascular or macrovascular disease and cardiovascular diseases. It has now become an epidemic with a worldwide incidence of 5% in the general population. The number of adults with diabetes in the world will rise from 135 million in 1995 to 300 million in the year 2025 (Torben H, 2002). The countries with the largest number of diabetic people in the year 2025 will be India, China and United States (Ramachandran *et al.*, 2002). There are more than 30 million people with diabetes mellitus in India and the incidence is increasing (Shankar *et al.*, 2001). Also, there are many patients in the community with undiagnosed diabetes. Diabetes is being projected as the World's main disabler and killer in the next 25 years (Edwin *et al.*, 2006). Thus, currently there is a great interest in the prevention of DM. 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 mellitus is a chronic metabolic disease, which can be classified into type 1 diabetes (insulin-dependent diabetes mellitus or IDDM) and type 2 diabetes (non-insulin dependent diabetes mellitus or NIDDM). In type 1 diabetes, the cause is an absolute deficiency of insulin secretion. In the other, much more prevalent category, type 2 diabetes, this cause is a combination of resistance to insulin action and an inadequate compensatory insulin-secretory response (American Diabetes Association, 2005).

Type 1, insulin-dependent diabetes mellitus (IDDM) most often occurs in children and young adults. People with type 1 diabetes must take daily insulin injections to stay alive. Type 1 diabetes accounts for 5–10% of diabetes. Type 2, noninsulin-dependent diabetes mellitus (NIDDM), in which the body does not produce enough, or properly use, insulin, is the most common form of the disease, accounting for 90–95% of diabetes. Type 2 diabetes is nearing epidemic proportions, due to an increased number of elderly people, and a greater prevalence of obesity and sedentary lifestyles. As a very common chronic disease, diabetes is becoming the third "killer" of the health of mankind along with cancer, cardiovascular and cerebrovascular diseases because of its high prevalence, morbidity and mortality.

Currently, diabetes therapy is based on the use of hypoglycaemics (sulfonamides, biguanides, insulin), on hygieno-diet measures and exercises. If the injections of insulin or other products make it possible diabetic to remain in life, they cannot, however, make it possible to face the many abrupt fluctuations of the insulin rate which the organization needs. Moreover the diabetes requires a life long treatment, which the patients have of the evil to support. In the search of means of fighting, people recognized and used the medicinal properties of many cultivated or wild plants to fight the disease. It is estimated that more than 200 species of plants exhibit hypoglycemic properties. In natural system of medicine many plants have been claimed, to be useful in the treatment of diabetes mellitus.

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 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.

TABLE 1.1: Scientific Classification of *Psidium guajava L.*

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

Psidium guajava L is a fruit-bearing tree commonly known as guava, which belongs to the family Myrtaceae. The French call it goyave or goyavier; the Dutch, guyaba, goeajaaba; the Surinamese, guave or goejaba; and the Portuguese, goiaba or goaibeira. Hawaiians call it guava or kuawa. In Guam, it is abas. In Malaya, it is generally known either as guava or jambu batu.

Guava is native to the Caribbean, Central America and northern South America. Guava grows nearly throughout India up to 1500m in height and is cultivated commercially in almost all states, the total estimated area being 50,000 hectares. The important guava growing states in India are Uttar Pradesh, Bihar, Maharashtra, Assam, West Bengal and Andhra Pradesh. Cultivated varieties grow about 10m in height and produce fruits within 4 years. Wild trees grow up to 20m high and are well branched. The tree can be easily identified by its distinctive thin, smooth, copper-colored bark that flakes off, showing a greenish layer beneath. Guava trees have spread widely throughout the tropics because they thrive in variety of soils, propagate easily and bear fruits quickly. The fruits are enjoyed by birds and monkeys, which disperse guava seeds and cause spontaneous dumps of guava saplings to grow throughout the rainforest (Wealth of India, 2003).

Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, leucocyanidin, ellagic acid, amritoside, beta-sitosterol, uvaol, oleanolic acid, ursolic acid, vitamins, fibre and fatty acids. Guava fruit is higher in vitamin C than citrus fruits (80 mg of vitamin C in 100g of fruit) and contains appreciable amount of vitamin A as well. Guava fruit are also a good source of pectin. Psidium guajava L, belonging to the Myrtaceae family, has been reported to have anti-diarrheal, hepatoprotective, hypoglycemic, lipid lowering, anti-bacterial and antioxidant activities. This study aims at finding new affordable therapies, able to normalize glycaemia and provide scientific evidence of the effectiveness of the traditional use of plants having antidiabetic effect.

Antioxidants act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Health problems such as heart disease, macular degeneration, diabetes, cancer etc are all contributed by oxidative damage. Antioxidants are substances or nutrients in our foods which can prevent or slow the oxidative damage to our body. When our body cells use oxygen, they naturally produce free radicals (byproducts) which can cause damage. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells.

Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols. Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells.

Alloxan and streptozotocin are widely used to induce experimental diabetes in animals. The mechanism of their action in B cells of the pancreas has been intensively investigated and now is quite well understood. The cytotoxic action of both these diabetogenic agents is mediated by reactive oxygen species; however, the source of their generation is different in the case of alloxan and streptozotocin. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of reactive oxygen species with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of B cells. Streptozotocin enters the B cell *via* a glucose transporter (GLUT2) and causes alkylation of DNA. DNA damage induces activation of poly ADP-ribosylation, a process that is more important for the diabetogenicity of streptozotocin than DNA damage itself. Poly ADP-ribosylation leads to depletion of cellular NAD+ and ATP. Enhanced ATP dephosphorylation after streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action, B cells undergo the destruction by necrosis (Szkudelski T, 2001).

1.1OBJECTIVES

The present investigation was initiated in ***Psidium guajava*** with the following objectives:

1. **To study the potential of α- Amylase inhibitor from *Psidium guajava* leaves in inhibiting porcine pancreatic α- Amylase.**
2. **To investigate the antidiabetic effects of α- Amylase inhibitor from the extracts of *Psidium guajava* leaves on alloxan-induced diabetic albino rats.**
3. **To estimate the amount of total flavonoids content responsible for α- Amylase inhibition.**
4. **To investigate *in vitro* antioxidant properties of *Psidium guajava* leaves.**

CHAPTER 2

REVIEW OF LITERATURE

2.1 Diabetes

The term diabetes was coined by Aretaeus of Cappadocia. It is derived from the Greek word diabainein 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'. 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 a chronic disorder in metabolism of carbohydrates, proteins, and fat due to absolute or relative deficiency of insulin secretion with/without varying degree of insulin resistance (Barar FSK 2000; Devlin MT 1997). The cost of treating diabetes and associated complications exceeds $ 100 billion per year. The complications are far less common and less severe in people who have well-controlled blood sugar level (Andrew JK, 2000). Acute complications include diabetic ketoacidosis, nonketotic hyperosmolar coma, and diabetic coma. In case of chronic complication, chronic elevation of blood glucose level leads to damage to blood vessels. In diabetes, the resultant problems are grouped under "microvascular disease"

(due to damage to small blood vessels) and "macrovascular disease" (due to damage to the arteries) (Andrew JK, 2000). Microvascular disease leads to retinopathy, neuropathy and nephropathy (nephropathy leads to anaemia) (Halder *et al.*, 2003; Merlin *et al.*, 2005). Macrovascular disease leads to cardiovascular disease, mainly by accelerating atherosclerosis. These disorders include: (1) Coronary artery disease, leading to myocardial infarction (heart attack) or angina, (2) Stroke (mainly ischemic type), (3) Peripheral vascular disease, which contributes to intermittent claudication (exertion-related foot pain) as well as diabetic foot (Andrew JK, 2000).

2.2 Pathophysiology

Most of the food we eat is broken down into simple sugar called glucose. This glucose is the main source of fuel to get energy for the body. After digestion, the glucose reaches our blood stream where it is available for body cells to utilize for energy, but insulin is needed for the glucose to get into cells. Insulin is a hormone secreted by the pancreas to transport glucose from blood into different cells of the body. If the pancreas does not produce enough insulin or the produced insulin does not work properly, the glucose cannot enter the body cells. So glucose stays in the blood cells, which makes the blood sugar level high. Diabetes is initially characterized by a loss of glucose homeostasis. The major effects of insulin on glucose, fatty acid, and amino acid metabolism and on ion flux are initiated by the attachment of the insulin molecule to a specific insulin receptor on the cell surface. This hormone receptor interaction is reversible, and the insulin molecule is not chemically altered during this contact. The hormone receptor complex is then internalized by an endocytotic mechanism. Insulin molecule eventually is metabolized, and the insulin receptor is recycled into the membrane for reusage. Thus, the body loses its main source of fuel for energy even though the blood contains high amount of glucose (Andrew JK, 2000; Edwin *et al.*, 2006). The insulin resistance mainly happens in cell membrane where glucose is not transported to the cells for oxidation. Since glucose is not metabolized, high amount of glucose circulates in the blood. To keep the normal level of glucose in the blood, the kidney removes the extra sugar from the blood and excretes it in the urine. Since body does not utilize glucose, the body is under constant impression of hunger and that is why

diabetics feel increased appetite (polyphagia) and eat more frequently. Symptoms of insulin resistance include a decreased stimulation of muscle glycogen synthesis, defects in glycogen synthesis activity, hexokinase activity and glucose uptake (Alam *et al.*, 2003).

2.3 Types of Diabetes

The three main types of diabetes are

2.3.1 Type 1 Diabetes

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 should be noted that there is no known preventive measure that can be taken against type 1 diabetes. Diet and exercise cannot reverse or prevent type 1diabetes. Sensitivity and responsiveness to insulin are usually normal, especially in the early stages. 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.

2.3.2 Type 2 Diabetes

It 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 ketoacidosis 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.

2.3.3 Gestational Diabetes

Some women develop gestational diabetes late in pregnancy. Although this form of diabetes usually disappears after the birth of the baby, women who have had gestational diabetes have a 20 to 50% chance of developing type 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 most 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.

2.3.4 Other specific forms, these include

- Genetic defects of β-cell function, e.g. MODY syndromes
- Genetic defects in insulin action e.g. leprechaunism
- Diseases of the exocrine pancreas, e.g. pancreatitis
- Secondary to endocrinopathies, e.g. acromegaly
- Drug- or chemical-induced, e.g. glucocorticoids
- Infections, e.g. congenital rubella
- Uncommon forms of immune-mediated diabetes, e.g. 'Stiff Man' syndrome
- Other genetic syndromes associated with diabetes, e.g. Down's syndrome (Lokesh *et al.*, 2006).

2.4 Treatments of diabetes

Regardless of the type of diabetes, patients are required to control their blood glucose with medications and/or by adhering to an exercise program and a dietary plan.

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 meals times.

Type 1 treatment must be continued indefinitely. The average glucose levels 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 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.

2.4.1 Mechanism and present drugs for therapy of diabetes mellitus

The present treatment of diabetes is focused on controlling and lowering blood glucose to a normal level. The mechanisms of both western medicines and the Chinese traditional medicines to lower blood glucose are:

- to stimulate β-cell of pancreatic islet to release insulin (sulfonylureas and meglitinides);
- to resist the hormones which rise blood glucose;
- to increase the number or rise the appetency and sensitivity of insulin receptor site to insulin;
- to decrease the leading-out of glycogen (biguanides and alpha-glucosidase inhibitors);
- to enhance the use of glucose in the tissue and organ (thiazolidinediones);
- to clear away free radicals, resist lipid peroxidation and correct the metabolic disorder of lipid and protein;
- to improve microcirculation in the body.

Based on the above-mentioned mechanisms, the drugs clinically used to treat diabetes can be mainly divided into insulin, insulin-secretagogues, insulin sensitivity improvement factor, insulin-like growth factor, aldose reductase inhibitor, α-glucosidase inhibitors and protein glycation inhibitor, almost all of which are chemical and biochemical drugs. The effect of these drugs is only aimed to lower the level of blood glucose. Moreover, in most cases, side-effect such as hypoglycemia, lactic acid intoxication and gastrointestinal upset appear after patients took these medicines.

The drugs commonly used in clinic to treat or control diabetes are the following:

- *Insulin*: There are many kinds of preparations
- *Sulfonylureas* (*SU*): Tolbutamide (D860, Orinase), Glibenclamide (Glyburide, HB419, Micronase, Daonil), Gliclazide (Diamicron), Glibenese (Minidiab), Glurenorm (Gliquidone), Glutril (Glibornuride) and Glimepiride, and so on
- *Biguanide* (*BG*): Phenformin (Phenethyldiguanidi Hydrochloridum, Diabenide, DBI), Dimethylbiguanide (FluamineMetformin, Diaformin, Diabex, Mellitin, Obin, Melbine, Metformin, Hydrochloride, Glucophage, DMBG)
- *α-Glucosidase inhibitors* (*α-GDI*): Glucobay (Acarbose), Voglibose, Miglitol, Emiglitate, Glyset, Precose
- *Aldose reductase inhibitor* (*ARI*): Tolrestat, Alredase, Epslstat, Kinedak, Imirestat, Opolrestat
- *Thiazolidinediones* (*TZD*): Troglitazone, Rosigitazone, Pioglitazone, Englitazone
- *Carbamoylmethyl benzoic acid* (*CMBA*): Repaglinide
- *Insulin-like growth factor* (*IGF*): IGF-1
- *Others*: Dichloroacetic acid

These treatments have their own drawbacks, ranging from the developing of resistance and adverse effects to lack of responsiveness in large segment of patients population. Sulfonylureas lose effectiveness for 44% of patients within six years. Also, these treatments are associated with side effects or even toxic effects (e.g., thiazolidinediones may cause liver toxicity; sulphonylureas might worsen heart disease, lower the glucose below the normal range and increase the body weight gain; bloating, flatulence, diarrhea and abdominal discomfort and pain are the major complaints with glucosidase inhibitors) (Michael *et al.*, 2005; Dey *et al.*, 2002; De Fronzo RA, 1999). According to literature, two-thirds of medications prescribed for use in children have not been proven safe or effective for this patient population (Michael *et al.*, 2005). Moreover, none of these glucose-lowering agents adequately controls the hyperlipidemia that frequently met with the disease (Derek LR, 2001).

The limitations of currently-available oral antidiabetic agents either in terms of efficacy/safety coupled with the emergence of the disease into a global epidemy have encouraged a concerted effort to discover drugs that can manage type 2 diabetes more efficiently (Ranjan *et al.*, 2002). Also, with increasing incidence of diabetes mellitus in rural population throughout the world and due to adverse effects of synthetic medicine, there is a clear need for development of indigenous, inexpensive botanical sources for anti-diabetic crude or purified drugs (Venkatesh *et al.*, 2003).

2.5 Natural treatments for diabetes

Diabetes mellitus is a heterogeneous metabolic disorder as old as mankind and its incidence is considered to be high (4–5%) all over the world (Pickup and William, 1997). The use of medicinal plants for the treatment of diabetes mellitus dates back from the Ebers papyrus of about 1550 B.C. A multitude of herbs spices and other plant materials have been described for the treatment of diabetes throughout the world (Kesari et al., 2005; Gupta et al., 2005; Ivorra et al., 1989; Marles and Fransworth, 1995). The medicinal plants might provide a useful source of new oral hypoglycemic compounds for development of pharmaceutical entities or as a dietary adjunct to existing therapies (Bailey and Day, 1989). Few of the plants used for the treatment of diabetes have received scientific or medical scrutiny and even the WHO expert committee on diabetes recommends that this area warrant further attention (WHO, 1980).

Plants-based products have been popular all over the world for the centuries. In diabetes, some herbal alternatives are proven to provide symptomatic relief and assist in the prevention of the secondary complications of the disease. Some herbs have also been proven to help in the regeneration of ß-cells and in overcoming resistance. In addition to maintaining normal blood sugar level, some herbs are also reported to possess antioxidant activity and cholesterol-lowering action. The management of type 2 diabetes mellitus (NIDDM) is possible with the drugs that can lower the blood sugar level in one hand and restore the liver glycogen level on the other. In modern system of medicine, there is no drug, which is reported to posses both of these properties (Shrabana *et al.*, 2003). However, the hypoglycemic effect of some herbal extracts have been

confirmed in human and animal models of type 2 diabetes and conventional drugs have been derived from the active molecules of these medicinal plants. Metformin, a less toxic biguanides and potent oral glucose-lowering agent, was developed from *Galega officianalis* and used to treat diabetes (Daniel *et al.*, 2001). Out of dozens of oral medications for diabetes, only one medication (metformin) is approved for use in children and it has been originated from herbs (Michael *et al.*, 2005)

The NAPRALERT database lists over 1200 species of plants representing 725 genera in 183 families extending from the marine algae and fungi with antidiabetic activity. Over half of these have been used ethnopharmacologically in traditional medicine as antidiabetics, and some 50% of these traditional remedies have been studied experimentally (Marles et al., 1996). The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed. Furthermore, an increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extraction and development of several drugs and chemotherapeutics from these plants as well as from traditionally used herbal remedies. Among these plants, some like *Allium cepa* (Onion), *Allium sativum* (Garlic), *Syzygium cumini* (Syn.), *Eugenia jambolana*; (black plum), *Momordica charantia* (bitter gourd), *Gymnema 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). These plants have been identified to display anti-diabetic potential. α-amylase inhibitors or 'Starch Blockers' have been extensively studied in these plants. The α- amylase inhibitor may be proteinaceous or non-proteinaceous in nature.

2.6 Guava and Diabetes

Extensive literature survey revealed that guava, acclaimed as 'poor man's apple of the tropics', has a long history of traditional use for a wide range of diseases. Guava (Psidium guajava L.) is widely cultivated and its fruit is popular in Asia. The leaves and the bark of guava tree have a long history of medicinal uses. Guava was also used as hypoglycaemic in folk medicine. The leaves and skin of the fruit have greater effects. Guava tea, the infusion of dried guava fruit and leaves has recently become popular as a drink in Taiwan. In India, decoction of the leaves and bark of guava is used to cure diarrhea, dysentery, vomiting and sore throats, and to regulate menstrual cycles. The tribes of Amazon use leaf decoction for mouth sores, bleeding gums, as douche for vaginal discharge and to tighten and tone up vaginal walls after labor. Guava is cultivated throughout the tropics. Commercially, the fruit is consumed raw or used in making jams, jellies, pastes, and juice. Guava leaves are official in Dutch Pharmacopoeia. They are also an extent source of fiber, potassium and vitamin A. It contains important phytoconstituents such as tannins (Tanaka et al., 1992), triterpenes (Meckes et al., 1996; Begum et al., 2002), and flavonoid: quercetin (Lozoya et al., 1994), pentacyclic triterpenoid: guajanoic acid, saponins, carotenoids, lectins

2.6.1 Phytochemistry

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. It has demonstrated antibacterial and anti-diarrheal effects and is able to relax the intestinal smooth muscle and inhibit bowel contractions. Guava has antioxidant properties attributed to polyphenols found in its leaves. A high percentage of vitamin C, carotene, vit B1, B2, B6, free sugars (glucose, fructose and sucrose) has been reported to be present in these fruits. The bark of guava tree contains considerable amounts of tannins (11-27%), and hence is used for tanning and dyeing purposes. Leucocyanidin, luectic acid, ellagic acid and amritoside have been isolated from the stem bark. Five constituents, including one new

pentacyclic triterpenoid: guajanoic acid and four known compounds beta-sitosterol, uvaol, oleanolic acid and ursolic acid, have been recently isolated from the leaves of *P.guajava.*

2.6.2 Biological Activity and Clinical Research

The long history of guava use has led modern-day researchers to study guava extracts. Its traditional use against diarrhea, gastroenteritis and other digestive complaints has been validated in numerous clinical studies. Bark and leave extracts were shown to have in vitro toxic action against numerous bacteria. Guava fruit and leaf showed antioxidant and free radical scavenging capacity (Hui-Yin Chen *et al.*, 2007).

Earlier studies indicated that the leaves of *Psidium guajava* possessed α- Amylase inhibitory activity (98%). The presence of flavanoids such as apigenin-7-o- glucoside is responsible for the inhibition of α-amylase activity (Karthic K *et al.*, 2008). The compounds responsible for inhibitory activity in guava leaves were isolated and identified to be genistein, genistein-p-coumarate, quercetin-7-o-malonate, 24-hydroxy guavanoic acid and betulinic acid through earlier studies (Mangalathillam Ratish *et al.*, 2010).

Although the fruit of *P. guajava* is known to contain free sugars yet the fruit juice showed hypoglycaemic effect in alloxan treated mice and diabetic volunteers. The extract of the whole plant of *P. guajava* excluding roots was reported to be devoid of any antibacterial, antifungal, antiviral, antifertility, hypoglycaemic, diuretic and anti-inflammatory activities. The leaves of *P. guajava* inhibit the increase of plasma sugar level in alloxan induced diabetic rats, during glucose tolerance test. Several flavonoids, glycosides, terpenoids, *etc.* have been shown to possess antidiabetic properties.

During various episodes of screening of medicinal plants, extract from *P.guajava* leaves exhibited significant inhibitory effect on the protein tyrosine phosphatase 1B (PTP1B). Significant blood glucose lowering effects of the extract were observed after intraperitoneal injection of the extract at a dose of 10mg/kg in both 1- and 3- month- old Lepr(db)/Lepr(db) mice (Oh *et al.*, 2005).

In the study undertaken to investigate the hypoglycemic and hypotensive effects of *P.guajava* leaf aqueous extract in rats, it showed hypoglycemic activity. The hypoglycemic effect of plant extract was examined in normal and diabetic rats, using streptozotocin (STZ) - induced diabetes mellitus model (Ojewole, 2005). A glycoprotein with the molecular weight of 50,000–100,000 was also identified as active component for anti-diabetes (Basnet *et al.*, 1995).

Maruyama *et al.* (1985) reported that the butanol soluble fraction prepared from 50% ethanol extract from *Psidium guajava* leaves inhibited the increase of the plasma sugar level in alloxan-induced diabetic rats and decreased plasma glucose level in the glucose tolerance test. Hypoglycemic effect in alloxan-treated mice and diabetic volunteers exerted by the fruit extract from *Psidium guajava* has also been reported (Cheng and Yang, 1983).

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).

2.7 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 (Vitamins C and E, β Carotene, Uric acid) defenses; yet these 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 and 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 decoctions of guava leaf for long for the 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 antioxidant 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 considerably a highly nutritious fruit because it contains a high level of ascorbic acid (50-300 mg/100 g fresh weight), which is 3-6 times higher than in oranges. Reddish 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 specifically bread 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.*, 2003), 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 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 objective was to perform FRAP assay to estimate antioxidant activities in guava leaf powder extracts.

The antioxidant related compounds have been isolated from methanol extracts of the leaves of Guava (*Psidium guajava* L) The isolated compounds possessed significant antioxidant activity that was revealed by a DPPH free radical scavenging assay which provides a scientific basis for the use of this plant as traditional medicine. The isolated compound was found to be quercetin, quercetin-3-*O*-glucopyranoside and Morin. (Suganya Tachakittirungrod et al., 2008)

The guava fruit contains relatively high antioxidant activity than other citrus fruits because of the presence of polyphenolics and ascorbic acid. It also has high primary, but low secondary antioxidant potential. Storage at 4° C has the effect of increasing ascorbic acid content and the non-peeled fruit has higher total phenol and ascorbic acid contents compared to the peeled fruit. The length and width of the guava seeds were also monitored over a period of 17 weeks to define specific stages of fruit ripening (Lim Yau Yan et al., 2006).

2.8 Laboratory Animal

A laboratory rat is a rat of the species *Rattus norvegicus* which is bred and kept for scientific research. Laboratory rats have served as an important animal model for research in psychology, medicine, and other field.

Laboratory rats share origins with their cousins in domestication, the fancy rats. In 18th century Europe, wild Brown rats ran rampant and this infestation fueled the industry of rat-catching. Rat-catchers would not only make money by trapping the rodents, but also by turning around and selling them for food, or more importantly, for rat-baiting. Rat-baiting was a popular, but brutal blood sport which involved filling a pit with rats and timing how long it took for a terrier to kill them all. Over time, breeding the rats for these contests produced variations in color, notably the albino and hooded varieties. The first time one of these albino mutants was brought into a laboratory for a study was in 1828, in an experiment on fasting. Over the next 30 years rats would be used for several more experiments and eventually the laboratory rat became the first animal domesticated for purely scientific reasons.

Over the years, rats have been used in many experimental studies, which have added to our understanding of genetics, diseases, the effects of drugs, and other topics in health and medicine. Laboratory rats have also proved valuable in psychological studies of learning and other mental processes. The historical importance of this species to scientific research is reflected by the amount of literature on it, roughly 50% more than that on mice.

Domestic rats differ from wild rats in many ways. They are calmer and less likely to bite. they can tolerate greater crowding, they breed earlier and produce more offspring, and their brains, livers, kidneys, adrenal glands, and hearts are smaller. Scientists have bred many strains or "lines" of rats specifically for experimentation. Most are derived from the albino Wistar rat, which is still widely used. Other common strains are the Sprague Dawley, Fischer, Holtzman albino strains, the Long-Evans, and Lister black hooded rats. Inbred strains are also available but are not as commonly used as inbred mice. Rat strains are generally not transgenic, or genetically modified, because the gene knockout and embryonic stem cell techniques that work in mice are relatively difficult in rats. This has disadvantaged many investigators, who regard many aspects of behavior and physiology in rats as more relevant to humans and easier to observe than in mice and who wish to trace their observations to underlying genes. As a result, many have been forced to study questions in mice that might be better pursued in rats. In October 2003, however, researchers succeeded in cloning two laboratory rats by nuclear transfer. So rats may begin to see more use as genetic research subjects. Much of the genome of *Rattus norvegicus* has been sequenced.

2.8.1 Strains

A *strain*, in reference to rodents, is a group in which all members are genetically identical. In rats, this is accomplished through inbreeding. By having this kind of population, it is possible to conduct experiments on the roles of genes, or conduct experiments that exclude variations in genetics as a factor. The opposite, out bred strains, are used when identical genotypes are unnecessary or a random population is required, and are more defined as *stocks* as opposed to *strains*.

Wistar rat

Wistar rats are an out bred strain of albino rats belonging to the species *Rattus norvegicus*. This strain was developed at the Wistar Institute in 1906 for use in biological and medical research, and is notably the first rat strain developed to serve as a model organism at a time when laboratories primarily used *Mus musculus*, or the common House mouse. More than half of all laboratory rat strains are descended from the original colony established by physiologist Henry Donaldson, scientific administrator Milton J. Greenman, and genetic researcher/embryologist Helen Dean King. The Wistar rat is currently one of the most popular rat strains used for laboratory research.

2.9 Induction of diabetes in rats

The induction of experimental diabetes in the rat using chemicals which selectively destroy pancreatic B cells is very convenient and simple to use. The most usual substances to induce diabetes in the rat are alloxan and streptozotocin. The understanding of changes in B cells of the pancreas as well as in the whole organism after alloxan or streptozotocin treatment is essential for using these compounds as diabetogenic agents. The metabolic disturbances in alloxan- and streptozotocin- treated rats were described recently by Szkudelski *et al.* (1998).

2.9.1 The mechanism of alloxan action

Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 5, 6-dioxyuracil) was first described by Brugnatelli in 1818. Wöhler and Liebig used the name "alloxan" and described its synthesis by uric acid oxidation. The diabetogenic properties of this drug were reported many years later by Dunn, Sheehan and McLethie (1943), who studied the effect of its administration in rabbits and reported a specific necrosis of pancreatic islets. Since then, alloxan diabetes has been commonly utilized as an animal model of insulin-dependent diabetes mellitus (IDDM).

Alloxan exerts its diabetogenic action when it is administered parenterally: intravenously, intraperitoneally or subcutaneously. The dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status. Human islets are considerably more resistant to alloxan than those of the rat and mouse. The most frequently used intravenous dose of this drug to induce diabetes in rats is 65 mg/kg b.w. When alloxan is given intraperitonealy or subcutaneously its effective dose must be 2-3 times higher. The intraperitoneal dose below 150 mg/kg b.w. may be insufficient for inducing diabetes in the rat. Fasted animals are more susceptible to alloxan (Szkudelski *et al.* 1998), whereas increased blood glucose provides partial protection (Szkudelski *et al.* 1998).

The mechanism of alloxan action has been intensively studied, predominantly *in vitro*, and is now characterized quite well. Using isolated islets and perfused rat pancreas it was demonstrated that alloxan evokes a sudden rise in insulin secretion in the presence or absence of glucose. This phenomenon appeared just after alloxan treatment and was not observed after repetitive exposure of islets to this diabetogenic agent. The sudden rise in blood insulin concentration was also observed *in vivo* just after alloxan injection to rats (Szkudelski *et al.* 1998). Alloxan-induced insulin release is, however, of short duration and is followed by complete suppression of the islet response to glucose, even when high concentrations (16.6 mM) of this sugar were used.

Alloxan is a hydrophilic and unstable substance. Its half-life at neutral pH and 37° C is about 1.5 min and is longer at lower temperatures. On the other hand, when a diabetogenic dose is used, the time of alloxan decomposition is sufficient to allow it to reach the pancreas in amounts that are deleterious.

The action of alloxan in the pancreas is preceded by its rapid uptake by the B cells. Rapid uptake by insulin-secreting cells has been proposed to be one of the important features determining alloxan diabetogenicity. Another aspect concerns the formation of reactive oxygen species. A similar uptake of alloxan also takes place in the liver. However, the liver and other tissues are more resistant to reactive oxygen species in comparison to pancreatic B cells and this resistance protects them against alloxan toxicity. The formation of reactive oxygen species is

preceded by alloxan reduction. In B cells of the pancreas its reduction occurs in the presence of different reducing agents. Since alloxan exhibits a high affinity to the SH-containing cellular compounds, reduced glutathione (GSH), cysteine and protein-bound sulfhydryl groups (including SH-containing enzymes) are very susceptible to its action. However, other reducing agents such as ascorbate may also participate in this reduction. They proposed that one of the SH-containing compounds essential for proper glucose-induced insulin secretion is glucokinase (EC 2.7.1.2), being very vulnerable to alloxan. Alloxan reacts with two -SH groups in the sugar-binding side of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme. Glucose can protect glucokinase against the inactivation hindering the access of alloxan to the -SH groups of the enzyme.

Dialuric acid is formed as a result of alloxan reduction. It is then re-oxidized back to alloxan establishing a redox cycle for the generation of superoxide radicals (Munday 1988). The reaction between alloxan and dialuric acid is a process in which intermediate alloxan radicals ($HA^{\bullet}$) and an unidentified "compound 305" (maximum absorption at 305 nm) is formed. The latter appears when alloxan is reduced by GSH. Superoxide radicals are able to liberate ferric ions from ferritin and reduce them to ferrous ions. Fe^{3+} can also be reduced by alloxan radicals. Moreover, superoxide radicals undergo dismutation to hydrogen peroxide:

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\,H^{+} \rightarrow H_2O_2 + O_2$$

This reaction may occur spontaneously or may be catalyzed by superoxide dismutase (EC 1.15.1.1)

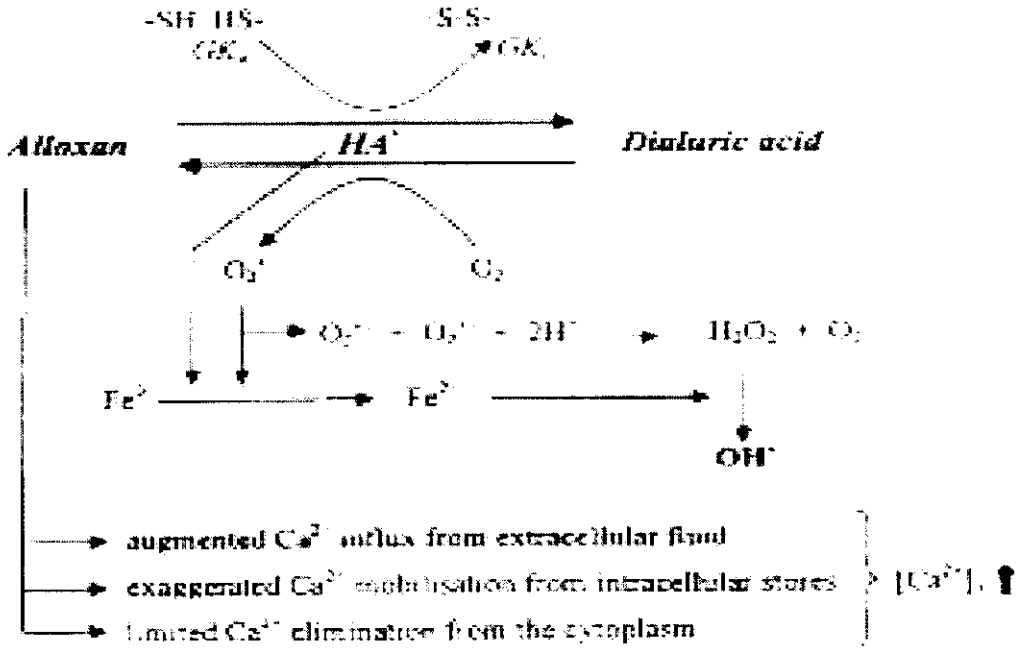


Fig 2.9.1: The mechanism of alloxan-induced reactive oxygen species generation in B cells of rat pancreas. GKa, GKi – glucokinase active and inactive, respectively; HA· – Alloxan radicals; $[Ca^{2+}]_i$ – intracellular calcium concentration.

One of the targets of the reactive oxygen species is DNA of pancreatic islets. Its fragmentation takes place in B cells exposed to alloxan. DNA damage stimulates poly ADP-ribosylation, a process participating in DNA repair. Some inhibitors of poly ADP-ribosylation can partially restrict alloxan toxicity. This effect is, however, suggested to be due to their ability to scavenge free radicals rather than to a restriction of poly ADP-ribosylation initiated by alloxan. Superoxide dismutase, catalase (EC 1.11.1.6) and non-enzymatic scavengers of hydroxyl radicals were also found to protect against alloxan toxicity. Therefore, chemicals rendering anti-oxidative properties and inhibiting poly ADP-ribosylation can attenuate alloxan toxicity.

It has been argued that glucose counteracts alloxan cytotoxicity *in vitro* and *in vivo*. This ability, however, is not only the result of the protection of glucokinase. The protective effect of glucose against necrotic death of B cells may be due to interaction of the sugar with the glucose transporter GLUT2 resulting in limited alloxan uptake. In the presence of Fe2+ and hydrogen peroxide, highly reactive hydroxyl radicals are then formed according to the Fenton reaction (Fig. 1):

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

The action of hydroxyl radicals following alloxan treatment was demonstrated *in vitro* and *in vivo*.

It has been proposed that the action of glucose is also related to its metabolism and to the increased generation of reducing equivalents (NADH and NADPH) accelerating the recirculation of glutathione. GSH is known to provide protection against free radicals. It may thus divert hydrogen peroxide from the pathway leading to the formation of hydroxyl radicals.

$$GSSG + 2\,NADPH \rightarrow 2\,GSH + 2\,NADP^+$$
$$H_2O_2 + 2\,GSH \rightarrow GSSG + 2\,H_2O$$

Moreover, the *in vitro* generation of hydroxyl radicals in the presence of alloxan strongly depends on GSH concentration. GSH in low concentrations potentiated the formation of these radicals, whereas the oxygen consumption, autoxidation of dialuric acid and formation of hydroxyl radicals were significantly inhibited in higher concentrations. GSH at high concentrations can also inhibit $HA^{\bullet}$ generation and directly neutralize hydroxyl radicals. Thiyl radicals ($GS^{\bullet}$) formed in this reaction are then converted to GSSG:

$$GSH + OH^{\bullet} \rightarrow GS^{\bullet} + H_2O$$
$$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$$

Indeed, in rat islets incubated with alloxan the GSH content and GSH/GSSG ratio were decreased, whereas glucose evoked the opposite effect.

In the *in vivo* experiment, glucose given to rats 20 min prior to alloxan partially restricted alloxan induced increase in the activity of glutathione peroxidase (EC 1.11.1.9) and mitigated the drop of liver nonprotein -SH groups (especially reduced glutathione) (Szkudelski *et al.* 2005). The protective action of this sugar is, however, strongly glucose and alloxan dose-dependent.

It has been proposed that disturbances in intracellular calcium homeostasis constitute an important step in the diabetogenic action of alloxan. This concept was confirmed by *in vitro* and *in vivo* experiments demonstrating that alloxan elevates cytosolic free Ca^{2+} concentration in pancreatic B cells. This effect arises from several events: alloxan-induced calcium influx from extracellular fluid, exaggerated calcium mobilization from intracellular stores and its limited elimination from the cytoplasm. The calcium influx may result from the ability of alloxan to depolarize pancreatic B cells. Depolarization of the cell membrane opens voltage-dependent calcium channels and enhances calcium entry into cells. Alloxan was also found to exert a stimulatory effect on mitochondrial Ca^{2+} efflux with simultaneous inhibitory action on Ca^{2+} uptake by mitochondria. The restriction of calcium removal from the cells due to alloxan-induced inhibition of liver plasma membrane Ca^{2+} -ATPase was also reported. The effect of alloxan on intracellular calcium concentration seems to be mediated, at least partially, by H_2O_2 since hydrogen peroxide itself exerts a similar effect on calcium concentration in B cells.

Thus, the previously mentioned sudden rise in insulin release from B cells treated with alloxan may be one of the effects of alloxan-induced augmentation in cytosolic Ca^{2+} concentration. The exaggerated concentration of this ion contributes to supraphysiological insulin release and, together with reactive oxygen species, causes damage of pancreatic B cells.

The results of experiments with calcium channel antagonists have confirmed the important role of cytosolic calcium in the cytotoxic action of alloxan. Pretreatment of rats with verapamil prevented the alloxan-induced increase in B cell Ca^{2+} concentration and abolished the stimulatory effect of alloxan on insulin release. The calcium channel antagonists (verapamil and diltiazem) also suppressed hyperglycemia and the onset of alloxan diabetes in rats. Summing up, the toxic action of alloxan on pancreatic B cells are the sum of several processes such as

oxidation of essential -SH groups, inhibition of glucokinase, generation of free radicals and disturbances in intracellular calcium homeostasis.

Many investigators suggested that the selectivity of alloxan action is not quite satisfactory. Recent experiments confirmed this objection. The diabetogenic dose of alloxan was found to decrease -SH groups accompanied by a simultaneous rise in glutathione peroxidase activity in the rat liver two minutes after its administration (Szkudelski *et al.* 2005). At the same time, the blood insulin concentration rose dramatically. This exaggerated insulinemia did not evoke, however, any significant reduction of blood glucose suggesting impaired peripheral insulin sensitivity in the short time after alloxan treatment (Szkudelski *et al.* 2005). It was also observed that alloxan intensified basal and epinephrine-induced lipolysis in isolated rat adipocytes and insulin failed to restrict this effect.

Thus, using alloxan to evoke diabetes, animals should be examined after proper period of time to minimize side effects of alloxan action. It should also be emphasized that the range of the diabetogenic dose of alloxan is quite narrow and even light overdosing may be generally toxic causing the loss of many animals. This loss is most likely due to kidney tubular cell necrotic toxicity, in particular when too high doses of alloxan are administered.

MATERIALS AND METHODS

CHAPTER 3

MATERIALS & METHODS

3.1 Plant material

The leaves – tender, young leaves of *P.guajava* were collected from the orchard, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu and authenticated by Dr.Balamohan.

3.2 Preparation of the Extract from *Psidium guajava* Leaves

The crude inhibitor was prepared in the powder form from the fresh leaves sample. The leaves of guava plant were taken and they are washed with distilled water three times until the dust and other particles were removed and were sun dried for some time. The leaves were further dried at 40 °c for 12 hrs in hot air oven. All samples were then ground into fine powder using mixture grinder. The crude powder was stored at -20 °c for further use.

The crude powder was used for two methods of extraction.

Method 1

In this method the dried crude powder at concentrations of 5g and 10g were mixed in 50 ml of distilled water in two different conical flasks. The mixture was subjected to 12 hr shaking in a rotary shaker. Then the filtrate was collected by filtering through Whatman No. 2 filter paper and the filtrate was dried in hot air oven. The powder was scrapped from the petriplate and used for assay.

Method 2

Samples of the dried crude powder (20 g) were extracted with boiling water (500 ml) for 5 min. The extracts were filtered through Whatman No. 2 filter paper and the filtrates were freeze-dried.

3.3 Extraction of compounds from different parts of the Guava plant (Arnon D I, 1949)

3.3.1 Chemicals and materials required

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

3.3.2 Apparatus and Glass wares required

- Separating funnel, 500ml
- Beaker, 100ml
- Erlenmeyer flask
- Mortar and pestle
- Glass rod

3.3.3 Procedure

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

- Using a pestle and mortar 5g of the guava leaf were ground with 22 ml acetone and 3 ml petroleum ether.
- The extract was filtered using filter paper.
- The filtrate was poured into a separating funnel and mixed with 20 ml of petroleum ether and 20 ml of 10% aqueous NaCl solution.
- 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 equal volume of water. It is named as organic phase 2.
- The extracts were collected and stored at 4° c till further use.

3.4 Preparation of Hot Extract

In a clean, dry 250 ml beaker about 5 g of dried guava leaf powder is weighed and dissolved in 50 ml of distilled water. The beaker is placed on a hot plate and boiled to 100° C with constant stirring for about 10 mins. The hot extract is then cooled in running tap water; filtered using whatmann filter paper and the filtrate is used for α-amylase inhibitory assay and other experimental procedures.

3.5 Preparation of Normal Extract

In a clean, dry 250 ml conical flask about 5 g of dried guava leaf powder is weighed and dissolved in 50 ml of distilled water. The beaker is placed in an orbital shaker for 12 hours at 37° C room temperature. The extract is the centrifuged at 10,000 rpm for 10 mins and the supernatant is collected and used for α-amylase inhibitory assay and other experimental procedures.

3.6 Estimation of Flavanoids by Aluminium Chloride method

3.6.1 Principle

Flavonoids present in the extract formed a charge transfer complex with several heavy metals to give a characteristic color. In this reaction, the high electropositive nature of aluminium (Al^{3+}, Aluminium chloride) attracts the atomic nuclei of the aromatic rings in the flavonoid through the μ- electrons (Flavonoids) and creates a charge transfer resonance hybrid. This hybrid is highly stable in the aqueous medium, which then interacted with sodium nitrite in an alkaline medium to form a pink colored complex i.e. spectro photometrically measured at 510 nm.

3.6.2 Reagents

- 5% sodium nitrite (5% $NaNO_2$)

  Dissolve 5 g of Sodium nitrite in 100 ml of distilled water.

- 10% Aluminium Chloride (10% $AlCl_3.6H_2O$)

  Dissolve 10 g of Aluminium chloride in 100 ml of distilled water.

- 1 M NaOH

  Dissolve 4 g of sodium hydroxide in 100 ml of distilled water.

3.6.3 Procedure

- Pipette out 0.5, 1.0, 1.5, 2.0, 2.5, ml (concentration varying from 50 μg to 250 μg) of the standard solution into a series of test tubes.
- Pipette out 0.1 ml of the sample into a test tube
- To all the tubes, including the blank, distilled water was added to make it upto 2.5 ml.
- To all the tubes, add 75 μl of 5% $NaNO_2$ and incubate at room temperature for 5 minutes.
- Add 150 μl of 10% $AlCl_3$ and incubate at room temperature for 6 minutes.
- Then add 0.5 ml of 1 M NaOH, mix well and the pink color formed was spectro photometrically measured at 510 nm.

3.7 α-Amylase inhibitory assay

α- Amylase inhibitory activity of the extract was analyzed by the method of Bernfeld P (1955) with a minor modification as described below. One hundred µl of the test extract was allowed to react with 100 µl of the porcine pancreatic α-amylase (6 U), 200 µl of water and 200 µl of 20mM of phosphate buffer (pH 6.9) in a test tube. After incubating for 20 mins, 200 µl of 1% starch solution was added. The same was performed for the control in another test tube where 100 µl of the extract was replaced by the same quantity of buffer. After incubating for 15 mins, 1000 µl of Dinitrosalicylic acid reagent was added to both control and tests. The tubes were then kept in boiling water bath for 10 mins. The absorbance was measured colorimetrically at 540 nm.

3.7.1 Preparation of Enzyme working standard

The **porcine pancreatic α- Amylase powder (Sigma A-3176)** was used as the source of enzyme. The enzyme working standard was prepared by dissolving 25 mg of powder in 100 ml of 20mM Phosphate buffer pH 6.9 containing 6.7mM Sodium chloride. This is used for assay.

3.7.2 Principle- DNS Method

The method of Bernfeld (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:

Aldehyde group [-CHO] $\underset{\longleftarrow}{\xrightarrow{\text{Oxidation}}}$ **Carboxyl group [-COOH]**

3, 5- Dinitrosalicylic acid $\underset{\longleftarrow}{\xrightarrow{\text{Reduction}}}$ **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.

3.7.3 Reagents required

- **20mM Phosphate buffer** pH 6.9 containing 6.7 mM Sodium chloride.
- **1% Starch solution** (Freshly prepared)
- **Dinitrosalicylic acid reagent (DNS Reagent)**
  - Dissolve by stirring 1g dinitrosalicylic acid; 200mg crystalline phenol and 50mg sodium sulphite in 100ml 1% NaOH. Store at 4 °c.
  - 40% Rochelle salt solution [Sodium potassium tartarate].

3.7.4 Flow of procedure for testing α – amylase inhibiting activity

100 µl of amylase +100 µl of extract

200 µl water

↓

200 µl of 20mM phosphate buffer

↓ **20 min at 37° C**

200 µl 1% of starch solution

↓ **15 min at 37° C**

1000 µl of DNS reagent

↓ **10 min at 100° C**

Absorbance at 540 nm

The absorbance was recorded at 540nm using a spectrophotometer and the percentage inhibition of α-amylase was calculated.

Inhibition % = control-test / control x 100.

The inhibition of α-amylase was tested with varying concentration of crude leaf extracts and their IC50 values were then calculated.

3.8 Free Radical Scavenging Activity of Extracts

3.8.1Ferric Ion Reducing Antioxidant Power Assay (FRAP)

3.8.1.1 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. This ion then conjugated with the ferricyanide ion to form a Prussian blue colored product. The blue color 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.

Fe^{3+} + Antioxidant → Fe^{2+} + Oxidized Antioxidant,

Fe^{2+} + $Fe(CN)_6^{3-}$ → $Fe[Fe(CN)_6]^-$

3.8.1.2 Reagents

- **Sample preparation**

In a clean dry conical flask, weigh 5 g of dried leaves (fresh leaves were air dried in the incubator at 37° C for two days) and add 50 ml of ethanol. 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 50 mg of the dried content was dissolved in a 50 ml standard flask and made up to the mark with distilled water (1 ml = 1 mg). From this 1:10 dilution was performed and 2ml of the extract corresponded to 100 µg was used for the experimental analysis.

- **0.2 M Phosphate buffer (pH= 6.6)**

Weigh 35.6 g/ L of Disodium hydrogen phosphate (A) and 31.2 g/L of Sodium dihydrogen phosphate (B). Mix 26.5 ml of (A) and 73.5 ml of (B) and made upto 200 ml with distilled water and adjusted to pH 6.6.

- **1% Potassium ferric cyanide**

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

- **10 % TCA**

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

- **0.1% Ferric Chloride**

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

3.8.1.3 Procedure

- Two ml (concentration varying from 100 to 500 µg) of the extract was pipetted out into a series of test tubes.
- Two ml of Phosphate buffer (pH 6.6) and two ml of 1% Potassium Ferricyanide was added to all the tubes.
- To a "Blank" tube, 4 ml of Phosphate buffer (pH 6.6) and 2 ml of 1% Potassium Ferricyanide were added.
- All the tubes were boiled at 50°C for 20 minutes.
- The reaction was arrested by adding 2 ml of 10% TCA in all the tubes.
- The tubes were centrifuged at 3000g for 10 minutes and 4 ml of supernatant was pipetted out in a separate tube.
- To this 0.8 ml of 0.1% $FeCl_3$ was added.
- The blue colour formed was colorimetrically read at 700 nm and an increase in the OD reading showed an increased antioxidant activity in the leaf extract.

3.9 Investigation of antidiabetic effects of α- Amylase inhibitor from *Psidium guajava* on alloxan-induced diabetic rats

3.9.1 Animals and treatments

The experiment was conducted in Animal House; PSG Institute of Medical Science & Research, Coimbatore. Experiment was carried out with Male/Female Albino Wistar rats weighing 120–250 g. The rats were housed in polypropylene cages and maintained under standard conditions (12 h light–dark cycles at 25° C). During the experimental period, rats were fed with standard rat pellet and water *ad libitum*. The research was conducted in accordance with the internationally accepted guidelines for Laboratory animal use and care. The study was approved by the Institutional Animal Ethics Committee PSGIMSR, Coimbatore, India **(Approval No: 158/99/CPCSEA; Dated 19th Jan 2010)**.

3.9.2 Induction of diabetes

Diabetes was induced in overnight fasted adult Wistar strain albino rats. A single dose of freshly prepared Alloxan (Sigma-Aldrich Chemical Pvt Limited, Bangalore, India) in 0.05 M sodium citrate buffer, pH 4.5, was immediately injected intraperitoneally **(120 mg/kg b.w.)** in a vehicle volume of 0.5 ml solution of sodium citrate buffer with 40 U Insulin syringe. Three hours after induction animals started to receive 5% glucose solution *ad libitum* to avoid hypoglycemic shock. The diabetic state was assessed by measuring the non fasting serum glucose concentration 72 h after alloxan treatment. Only animals with blood glucose values above 180mg/dl were considered diabetic and used in the experiment.

3.9.3 Decoction preparation

Decoctions were prepared as follows: 5g and 10g dried guava leaf powder respectively for 250 and 500 mg dose were boiled in 100 ml of distilled water for 10 minutes. This preparation was filtered and the filtrate administered to the experimental animals. All preparations were administered to rats through oral route using Venflon (5 ml/rat).

3.9.4 Assessment of antidiabetic activity

In the experiment, a total of 36 rats (12 normal; 24 Alloxan treated diabetic surviving rats were used. Animals were divided into 6 groups of 6 animals each and treated as follows. The study was conducted for 21 days in these experimental rats.

Group I: Normal control rats received vehicle (distilled water only 4 ml/kg) daily for 21 days;
Group II: Normal rats administered *Psidium guajava* aqueous extract (both 250 mg/kg and 500 mg/kg; 2 animals each);
Group III: Diabetic control rats administered drinking water daily for 21 days;
Group IV: Diabetic rats administered *Psidium guajava* aqueous extract (250 mg/kg);
Group V: Diabetic rats administered *Psidium guajava* aqueous extract (500 mg/kg);
Group VI: Diabetic rats received 10 mg/kg of the reference drug Acarbose (synthetic hypoglycaemic agent).

Diabetic rats were administrated with crude amylase inhibitors given as per dose fixed and compared to that with standard drug Acarbose. Blood glucose levels were determined at variable interval of time (0, 90 min, 1 Day, 1st week, 2nd week, 3rd week) and peak blood glucose observed. Body weight was taken during the experimental period (0, 90 min, 1 Day, 1st week, 2nd week, 3rd week) with the help of single pan balance. Blood samples were collected by end tail vein cutting method and blood glucose level was estimated by using one touch glucometer for regular checkup with the help of glucose strips.

3.9.5 Statistical Analysis

All values were presented as the means ± SD. Statistical comparisons of the differences were performed using one way analysis of variance (One-way ANOVA). P values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSIONS

CHAPTER 4

RESULTS AND DISCUSSION

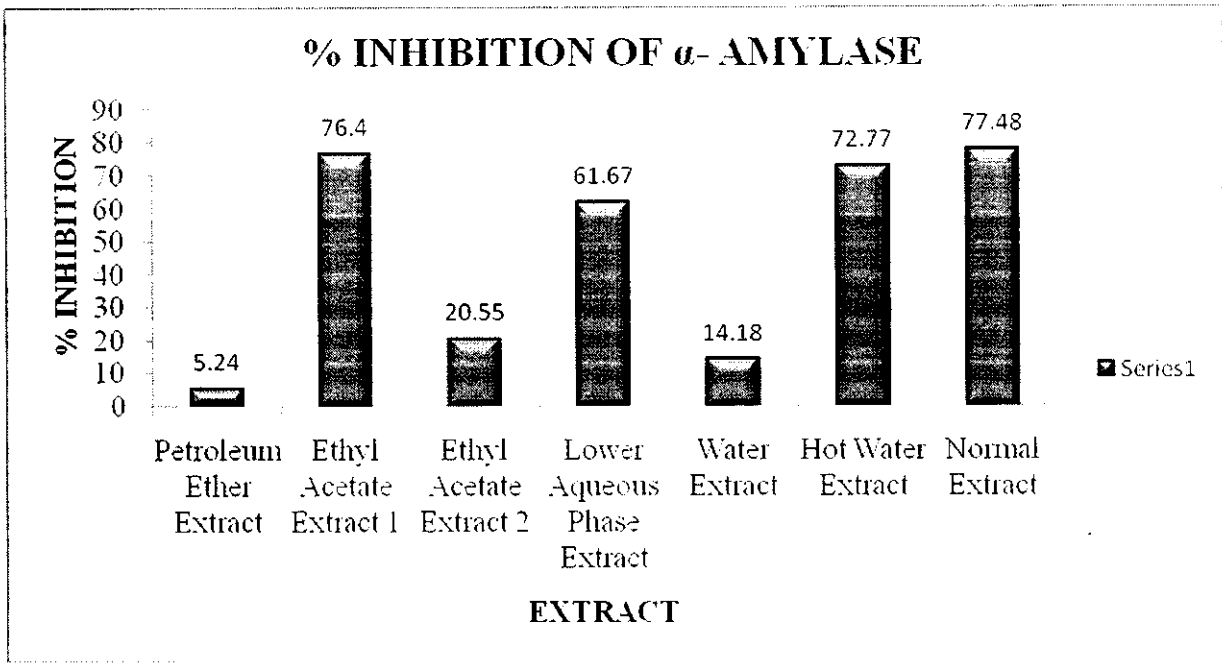
4.1 *In vitro* alpha amylase inhibition

The inhibitor extract obtained from the *Psidium guajava* leaves using different extraction procedures were used for testing for α- amylase inhibitory activity. The method used was given by Bernfeld and the inhibitory activity checked using DNS (3, 5- dinitrosalicylic acid) method using 1% starch as substrate. The enzyme used was porcine pancreatic α- amylase (PPA). The absorbance reading measured from the assay and the % of α- amylase inhibition by the extracts from *Psidium guajava* leaves are shown in Table 4.1.1 and Fig. 4.1.1.

TABLE 4.1.1: Percentage Inhibition of Porcine Pancreatic α- Amylase by Extracts Obtained from Different Extraction Procedures

S No	Extract	Absorbance at 540 nm			Inhibition Percentage (%)		
		Control	Test 1	Test 2	Test 1	Test 2	Mean
1	Petroleum Ether Extract	3.361	3.185	3.185	5.24	5.24	5.24
2	Ethyl Acetate Extract 1	3.326	0.781	0.789	**76.52**	**76.28**	**76.40**
3	Ethyl Acetate Extract 2	3.187	2.532	2.532	20.55	20.55	20.55
4	Lower Aqueous Phase Extract	2.819	1.074	1.087	**61.90**	**61.44**	**61.67**
5	Water Extract	3.187	2.709	2.761	14.99	13.37	14.18
6	Hot Water Extract	1.809	0.416	0.569	**77.00**	**68.55**	**72.77**
7	Normal Extract	1.925	0.526	0.341	**72.67**	**82.29**	**77.48**

FIG 4.1.1: Percentage Inhibition of Porcine Pancreatic α- Amylase by Extracts Obtained from Different Extraction Procedures



The results of the assay implied that the normal aqueous leaf extract of Psidium guajava showed a maximum of 77.48% of α- amylase inhibition activity. This was followed by the Ethyl Acetate extract which showed 76.4% inhibition; followed by the hot water extract which showed 72.77% 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 60% 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. 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. This clearly signifies that the aqueous leaf extracts of *Psidium guajava* has α-amylase inhibitory activity and it can be potentially used as an anti-diabetic drug for effective treatment of diabetes.

4.2 Estimation of Flavonoids by Aluminium Chloride Method

Plants constitute an important source of active natural products, which differ widely in terms of structure and biological properties. They have a remarkable role in the traditional medicine in different countries. The protective effects of plant products are due to the presence of several components, which have distinct mechanisms of action; some of them are enzymes and proteins and others are low molecular weight compounds such as vitamins, carotenoids, flavonoids (Zhang and Wang 2002), anthocyanins and other phenolic compounds (Sanchez-Moreno et al. 1998). Flavonoids are nearly ubiquitous in plants and are low molecular weight compounds composed of a three-ring structure with various substitutions. Flavonoids (more than 8000) constitute the largest and most important group of polyphenolic compounds in plants.

These flavonoids exhibit both antidiabetic and antioxidant activities. The antidiabetic and antioxidant activities of *Psidium guajava* are accounted due to the presence of these low molecular weight compounds. These compounds are non-proteinaceous in nature and do not get degraded by gastric enzymes and they get easily absorbed. The inhibitory activity exhibited by these compounds was noncompetitive in nature. So we are interested in estimating the amount of flavonoids present in one gram of dried leaf powder.

Flavonoids were estimated using Aluminium chloride method. In the presence of sodium nitrite in alkaline medium these flavonoids from the extract form a stable charge transfer complex with Al^{3+} from aluminum chloride to give pink color. The change in color was spectro photometrically measured at 510 nm. To estimate the exact amount of flavonoids present in the extract Standard α- amylase inhibitor Rutin was used. The standard curve for Rutin was used to calculate the exact amount of flavonoids in the extracts obtained from *Psidium guajava* leaves.

The formula used to calculate the flavonoid content in the extracts is as follows:

$$\frac{\textbf{Absorbance value of Test}}{\textbf{Absorbance value of Standard}} * \textbf{(Conc. of Std)} * \frac{\textbf{Made up volume}}{\textbf{Sample volume}}$$

TABLE 4.2.1: Standard Curve for Rutin

S No	Amt of Std Rutin (ml)	Conc. of Std Rutin (µg/ml)	Amt of Dist H_2O (ml)	Amt of 5% $NaNO_2$ (µl)	Amt of 10% AlCl3 (µl)	Amt of 1M NaOH (ml)	Absorbance At 510nm
1	0.5	50	2.0	75	150	0.5	0.153
2	1.0	100	1.5	75	150	0.5	0.270
3	1.5	150	1.0	75	150	0.5	0.371
4	2.0	200	0.5	75	150	0.5	0.583
5	2.5	250	0.0	75	150	0.5	0.712

FIG 4.2.1: Standard Curve for Rutin

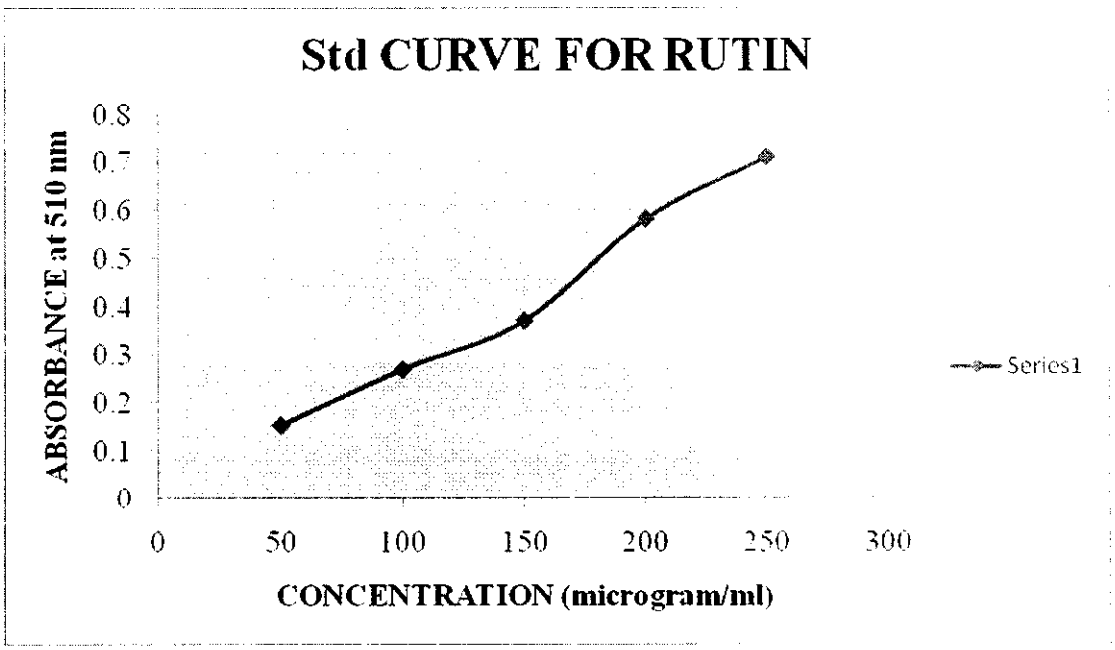


TABLE 4.2.2: Estimation of Total Flavonoids Content in test samples

S No	Test		Amt of Dist H_2O (ml)	Amt of 5% $NaNO_2$ (µl)	Amt of 10% AlCl3 (µl)	Amt of 1M NaOH (ml)	Absorbance At 510nm	
	Hot Extract	Normal Extract					Hot Extract	Normal Extract
1	0.1	0.1	2.4	75	150	0.5	0.319	0.150
2	0.1	0.1	2.4	75	150	0.5	0.326	0.174
3	0.1	0.1	2.4	75	150	0.5	0.343	0.161
Mean							**0.329**	**0.161**

TABLE 4.2.3: Estimation of Total Flavonoids Content in Hot Extract

S No	Conc. of Std Rutin (µg/ml)	Absorbance at 510 nm		Total Flavonoid Content	
		For Std Rutin	For Test	In µg/g	In mg/g
1	50	0.153	0.329	53758.17	53.76
2	100	0.270		60925.93	60.93
3	150	0.371		66509.43	66.51
4	200	0.583		56432.25	56.43
5	250	0.712		57759.84	57.76
Mean				**59077.12**	**59.078**

TABLE 4.2.4: Estimation of Total Flavonoids Content in Normal Extract

S No	Conc. of Std Rutin (µg/ml)	Absorbance at 510 nm		Total Flavonoid Content	
		For Std Rutin	For Test	In µg/g	In mg/g
1	50	0.153	0.161	26307.19	26.31
2	100	0.270		29814.82	29.81
3	150	0.371		32547.17	32.55
4	200	0.583		27615.78	27.62
5	250	0.712		28265.45	28.27
Mean				28910.08	28.91

The results from the table show that the hot extract of Psidium guajava contains around 59.078 mg of flavonoids per gram of dried guava leaf powder. Similarly the normal extract contains around 28.91 mg of flavonoids per gram of dried guava leaf powder. The hot extract contains more amounts of flavonoids than the normal extract due to the disruption of cells by heat treatment. Therefore hot extracts account for good antidiabetic and antioxidant activities due to the excess amount of flavonoids present.

4.3 Antioxidant Assays

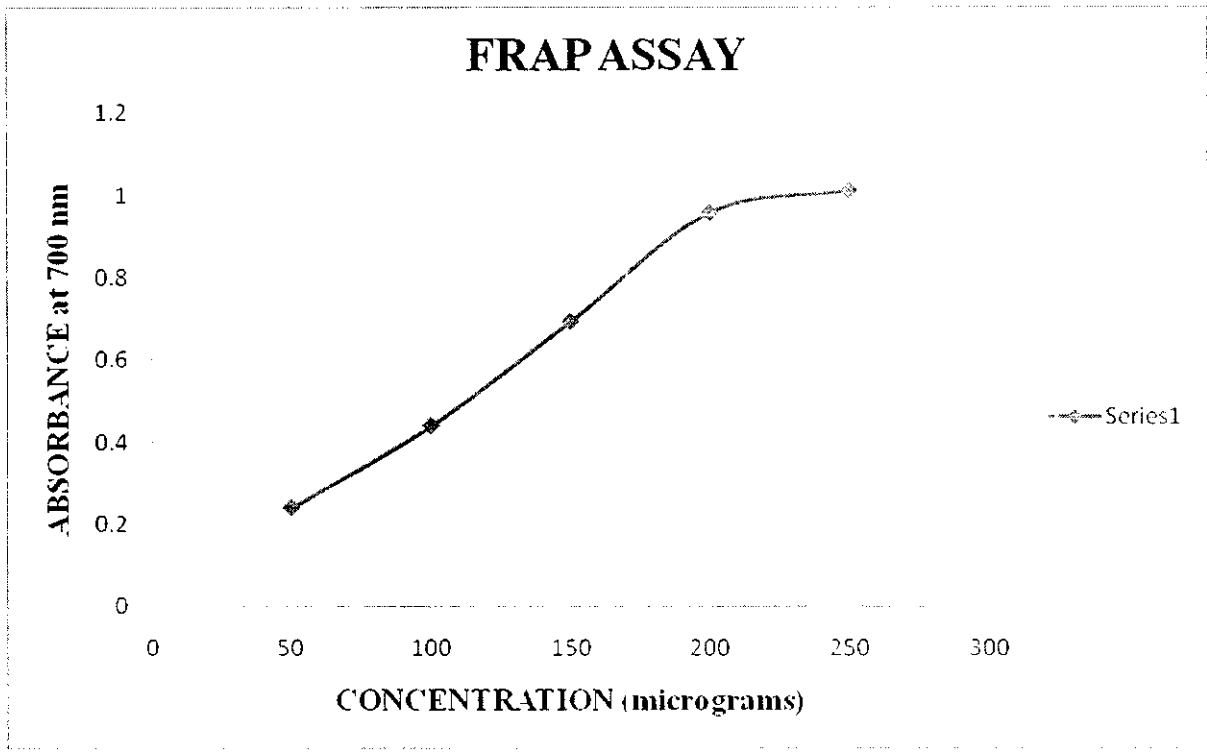
4.3.1 Ferric Ion Reducing/Antioxidant Power Assay (FRAP) Assay

The antioxidant activities of extracts from guava was determined by using the 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 minutes incubation at 50° C with 10% TCA. The tubes were centrifuged at 3000g for 10 minutes and 4 ml of supernatant was treated with 0.8 ml 0.1% $FeCl_3$ was added. The blue color developed [Ferrous tripyridyltriazine complex] was colorimetrically read at 700 nm (Kriengsak *et al.*, 2006). The absorbance reading measured from the assay is shown in the Table 4.2.1 and Fig 4.2.1.

TABLE 4.3.1: Ferric Ion Reducing/Antioxidant Power Assay (FRAP) of *Psidium guajava* leaf Extract

S No	Concentration (µg/ml)	Psidium guajava		
		Test 1	Test 2	Mean
1	50	0.262	0.218	**0.240**
2	100	0.446	0.433	**0.440**
3	150	0.714	0.672	**0.693**
4	200	0.974	0.940	**0.957**
5	250	1.013	1.007	**1.010**

FIG 4.3.1: Ferric Ion Reducing/Antioxidant Power Assay (FRAP) of *Psidium guajava* leaf Extract



The results showed that the increase in absorbance values was due to reducing capacity of antioxidants present in the leaf extracts of guava. The absorbance increases as a result of a color change to Prussian blue as the radical was scavenged by antioxidant through donation of hydrogen ion to form stable complex [Ferrous tripyridyltriazine complex].

Recently, there has been considerable interest in preventive medicine through the quest for natural antioxidants from plant material. Various phytochemical components, such as flavonoids, phenolic acids and carotenoids, are known to be responsible for the antioxidant capacity of plants. However, the effectiveness of flavonoids as effective antioxidants is dependent upon the environment. A number of factors may influence the behavior of flavonoids and may result in alterations to their efficacy as antioxidants. The antioxidant activity of flavonoids may be reduced by the autoxidation of flavonoids, catalyzed by transition metals to produce superoxide anion. The latter dismutates to generate hydrogen peroxide and form hydroxyl radicals via a Fenton reaction in the presence of transition metals (Canada, Giannella, Nguyen, & Mason, 1990). Most plant polyphenol compounds possess both antioxidant and prooxidant properties, depending on concentration and environmental factors (Cao, Sofic, & Prior, 1997).

Guava leaf extracts showed potential antioxidant activity and can be used to extend shelf life of food stuffs, to reduce wastage and nutritional losses by inhibition and delaying oxidation. Increased intake of guava leaf extracts is therefore good for our health.

4.4 Investigation of antidiabetic effects of α- Amylase inhibitor from *Psidium guajava* on alloxan-induced diabetic rats

4.4.1 Body Weight

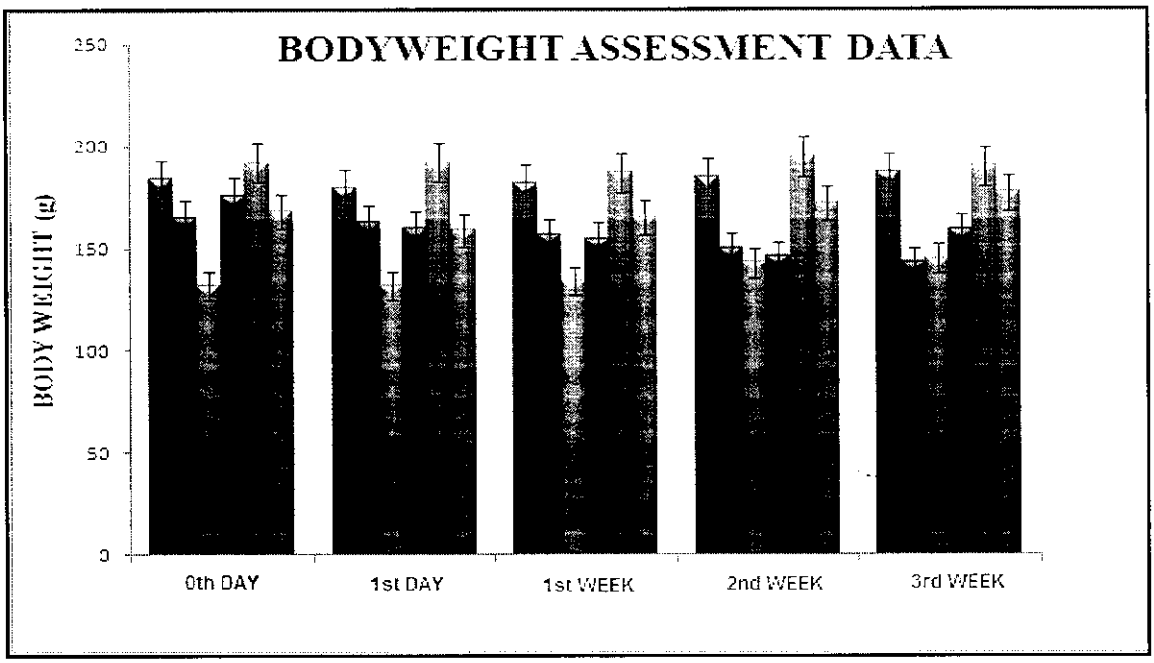
Diabetes is characterized by weight loss and it was also seen in this study. Alloxan administration brought about marked reduction in body weight of rats. This reduction was found to be statistically significant ($p<0.05$) when compared with normal control group. These reduced body weights were found to be increased when compared to their respective diabetic control group and this increase was found to be statistically significant in rats treated with *Psidium guajava* leaf extracts ($p<0.05$) (Table 4.4.1 & Fig 4.4.1). Percent increase in body weight was 9.5% for *Psidium guajava* treated animals.

As far as the relative efficacy in increasing or maintaining body weight is concerned, *Psidium guajava* treated group showed minimum increase in percentage body weight and comparable to Acarbose treated group.

TABLE 4.4.1: Effect of aqueous leaf extract of P.guajava and Acarbose on Body Weight (g) in Alloxan induced diabetic rats

S NO	GROUPS	TREATMENT PERIOD (BODY WEIGHT (g)				
		0^{th} DAY	1^{st} DAY	1^{st} WEEK	2^{nd} WEEK	3^{rd} WEEK
1	Normal Control	184	180	182	185	187
2	Diabetic Control	165	163	157	150	143
3	Dia + P.guajava	132	132	134	143	[illegible]
4	Dia + 250 mg/kg P.guajava	176	160	155	146	159
5	Dia + 500 mg/kg P.guajava	192	192	187	195	190
6	Dia + Acarbose	168	159	165	172	177

FIG 4.4.1: Histogram showing changes in body weight following treatments with *Psidium guajava* leaf extracts and Acarbose in Alloxan induced diabetic rats



4.4.2 Blood Glucose

Alloxan causes selective destruction of β cells of islets of pancreas and brings an increase in blood glucose levels. It is evident from the present investigation that alloxan administration at the dose of 120 mg/kg b.w. causes significant diabetogenic response in albino rats. Blood glucose levels in diabetic control rats treated with *Psidium guajava* and Acarbose were raised nearly to 3 folds as compared to their respective normal control group rats on 7th day after treatment. Interestingly, the increase in glucose levels in diabetic control groups was found to be highly statistically significant ($p<0.001$) when compared to their respective normal control groups. These raised levels of blood glucose in diabetic rats were declined sharply after oral feeding of aqueous extract of leaves of *Psidium guajava*. When comparisons were made between diabetic and drug treated animals, blood glucose levels were found to be declined sharply from 454.25-361.00 mg/dl for 250 mg/kg and 257.50-195.25 mg/dl for 500 mg/kg on 3rd week after oral feeding of *Psidium guajava* leaf extract respectively. Acarbose treatment also decreased blood glucose levels on 3rd week (from 472.14-282.18 mg/dl). This decline in blood glucose levels of drug treated groups when compared with their respective diabetic control group was found to be highly statistically significant ($p<0.001$) (Table 4.4.2 & Fig 4.4.2).

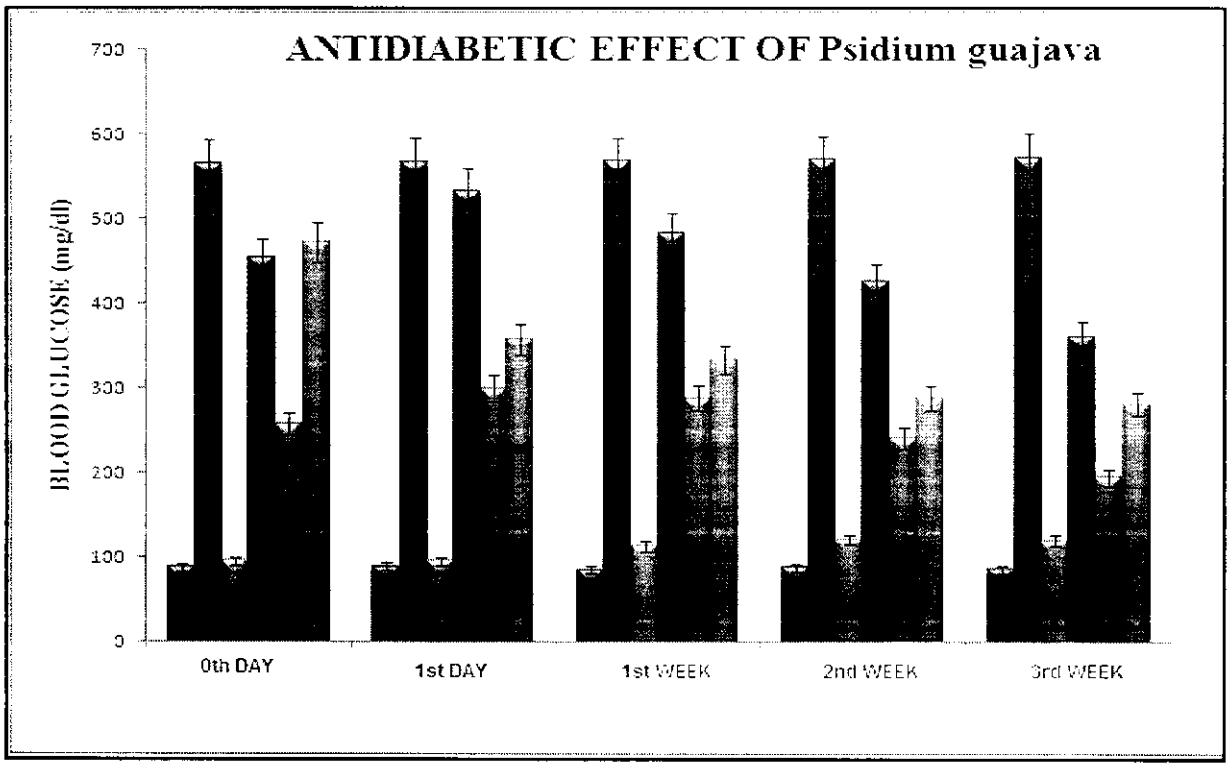
Hence if decline in blood glucose levels is to be the only indices, then treatment with aqueous extract of leaves of *Psidium guajava* and Acarbose has proved highly effective in causing significant antihyperglycemic response in this strain of rats. A comparative account of the antihyperglycemic activity of *Psidium guajava* and Acarbose is well displayed in the present study. The reduction of sugar after administering *Psidium guajava* was 32.24% for 250 mg/kg of extract, 34.92% for 500 mg/kg of extract and 40.23% for Acarbose respectively. It is evident from these results that reduction in blood glucose levels brought by 2 different dose of the herbal extract from *Psidium guajava* is quiet comparable with reduction brought about by Acarbose. As far as the relative efficacy is concerned, *Psidium guajava* exhibited little less antihyperglycemic activity than Acarbose in this study.

Our data showed that freshly prepared aqueous leaf extracts of *P.guajava* attenuates hyperglycemia in alloxan induced diabetic rat model. In agreement with our present results, several investigators (Prasad *et al.*, Kazi Rafiq *et al.*, 2009; Gutierrez *et al.*, 2008; Lin, 1964; Shen *et al.*, 2008) have reported that different parts of *P. guajava* including its leaf extract have an antihyperglycemic effect and also stimulate glucose utilization in liver tissues in STZ-induced diabetic rats. Tannins, flavonoids, pentacyclic triterpenoids, guaijaverin, quercetin and other chemical compounds present in the plant are speculated to account for the observed hypoglycemic effects of the leaf extract (Ojewole, 2005; Wang *et al.*, 2005). Based on our present experimental data, it is possible to provide herbal therapy against diabetes disease through aqueous leaf extract of *Psidium guajava* by their antihyperglycemic effect and/or miscellaneous antioxidant compounds present in the extracts.

TABLE 4.4.2: Effect of aqueous leaf extract of P.guajava and Acarbose on Blood level concentration (mg/dl) in Alloxan induced diabetic rats

S NO	GROUPS	TREATMENT PERIOD (BLOOD GLUCOSE LEVEL (mg/dl)				
		0^{th} DAY	1^{st} DAY	1^{st} WEEK	2^{nd} WEEK	3^{rd} WEEK
1	Normal Control	88 ± 4.2	89.3 ± 3.9	85.3 ± 4	88 ± 5.1	86 ± 4
2	Diabetic Control	565.2 ± 2.7	567.4 ± 3	568.7 ± 2.8	570.2 ± 1.4	572.5 ± 3
3	Dia + P.guajava	95.25 ± 14.31	95.50 ± 12.58	113.50 ± 15.67	121.75 ± 15.67	
4	Dia + 250 mg/kg P.guajava	454.25 ± 163.51	532.75 ± 85.02	483.00 ± 44.63	426.00 ± 113.40	361.00 ± 172.37
5	Dia + 500 mg/kg P.guajava	257.50 ± 45.24	300.00 ± 87.69	289. 25 ± 179.23	241.25 ± 180.40	195.25 ± 116.30
6	Dia + Acarbose	472.14 ± 19.87	358.39 ± 14.51	334.56 ± 17.55	289.68 ± 18.45	282.18 ± 18.45

FIG 4.4.2: Histogram showing changes in blood glucose level following treatments with *Psidium guajava* leaf extracts and Acarbose in Alloxan induced diabetic rats



CONCLUSION

CHAPTER 5

CONCLUSION

Inhibitors of carbohydrate-hydrolyzing enzyme play an important role to control post parandial blood glucose levels in diabetic patients. Alpha-amylase inhibitors from natural sources like medicinal plants offer an attractive 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 potent, reversible, non-competitive α-amylase inhibitors from the aqueous extracts of *Psidium guajava* leaves. The results obtained from the animal studies to prove the antidiabetic effect of the leaf extracts suggest that these natural amylase inhibitors from food-grade sources offers an attractive therapeutic approach to the treatment of post prandial hyperglycemia by decreasing glucose release from starch. The results also showed lowering of blood glucose level and increase in body weight in the leaf extracts treated diabetic animals is statistically significant compared to their respective control animals. The antidiabetic effect of the aqueous leaf extracts of Psidium guajava is quiet comparable to the effect of Acarbose (a standard drug used to treat diabetes mellitus). From this study, we can conclusively state that aqueous leaf extracts of *Psidium guajava* has beneficial effects on blood glucose levels. Guava leaf extracts also shown potential antioxidant activities and it is evident from our results. Further pharmacological and biochemical investigations are underway to elucidate the exact mechanism of antidiabetic effects of leaf extracts of this plant. **Future work is to investigate the antidiabetic effects of the compound identified from *Psidium guajava* on Type 2 (non-insulin-dependent) diabetic patients.**

REFERENCES

CHAPTER 6

REFERENCES

1. Alam K, Mahpara S (2003). "Role of Diet, Nutrients, Spices and Natural Products in Diabetes Mellitus". Pakistan J Nutr; Vol: 2, pp 1-12.
2. American Diabetes Association, (2005). "Diagnosis and classification of diabetes mellitus" Diabetes Care; Vol: 28, pp 37–42.
3. Andrew JK (2000). "Diabetes". Churchill living stone: New York.
4. Bailey, L.J., Day, C., (1989). "Traditional plant medicine as treatment for diabetes" Diabetes Care; Vol: 12, pp 553–564.
5. Barar FSK (2000). "Essentials of Pharmacotherapeutics". S.Chand and Company Ltd: New Delhi: 3rd Ed.
6. Basnet, P., Kadota, S., Pandey, R.R., Takahashi, T., Kojima, Y., Shimizu, M., Takata, Y., Kobayashi, M., Namba, T., (1995). "Screening of traditional medicines for their hypoglycemic activity in streptozotocin (STZ)-induced diabetic rats and a detailed study on Psidium guajava" Wakan Iyakugaku Zasshi; Vol: 12, pp 109–117.
7. Bernfeld P (1955) "Amylases, alpha and beta" Methods in Enzymology. (Ed.) S. P. Colowick and N. O. Kaplan, Academic press, Newyork, pp 149-158.
8. Chaudhury, R. R. and Vohora, S. B. (1970) "Plants with possible hypoglycaemic activity" in Advances in Research in Indian Medicine, pp 57.
9. Chen, H. Y., & Yen, G. C. (2007). "Antioxidant activity and free radical scavenging capacity of extracts from guava (Psidium guajava L leaves)" Food Chemistry; Vol: 101, pp 686–694.
10. Cheng JT, Yang RS. (1983). "Hypoglycemic effect of guava juice in mice and human subjects" Am J Chin Med; Vol: 11, pp 74-76.
11. Daniel SF, Norman RF (2001). "The Value of Plants Used in Traditional Medicine for Drug Discovery". Environ Health Perspect; Vol: 109, pp 69-75.
12. DeFronzo RA (1999). "Pharmacologic therapy for type 2 diabetes mellitus". Ann Intern Medc; Vol: 131, pp 281-303.

13. Derek LR (2001). "Current therapeutics algorithms for type 2 diabetes". Diabetes: Vol: 4, pp 38-49.
14. Devlin MT (1997). "Text book of Bio Chemistry". Wileyliss Inc: NewYork: 4th edn.
15. Dey L, Attele AS, Yuan CS (2002). "Alternative therapies for type 2 diabetes". Altern Med Rev; Vol: 7, pp 45-58.
16. Edwin E, Sheeja E, Gupta VB, Jain DC (2006). "Fight Diabetes the herbal way". Express Pharma review; Vol: 1, pp 41-2.
17. Gupta, R.K., Kesari, A.N., Murthy, P.S., Chandra, R., Tandon, V., Watal, G., (2005). "Hypoglycemic and Antidiabetic Effect of Ethanolic Extract of Leaves of Annona squamosa L. in Experimental Animals" Journal of Ethnopharmacology; Vol: 99, pp 75–81.
18. Halder N, Joshi S, Gupta SK (2003). "Lens aldose reductase inhibiting potential of some indigenous plants". J Ethnopharmacol; Vol: 86, pp 113–6.
19. Ivorra, M.D., Paya, M., Villar, A., (1989). "A review of natural products and plants as potential antidiabetic drugs". Journal of Ethnopharmacology Vol: 27, pp 243–275.
20. Ju-Wen Wua, Chiu-Lan Hsieh, Hsiao-Yun Wang, Hui-Yin Chen (2008) "Inhibitory effects of guava (Psidium guajava L.) Leaf extracts and its active compounds on the glycation process of protein" Food Chemistry, Vol: 113, pp 78–84.
21. Kamath J. V, Nair Rahul, Ashok kumar C. K, Mohana Lakshmi S. (2007) "Psidium guajava L: A review" International Journal of Green Pharmacy, Jan-Mar 2008.
22. Karthic K, Kirthiram K S, Sadasivam S & Thayumanavan B (2008) "Identification of α-amylase inhibitors from Syzygium cumini Linn seeds" Indian Journal of Experimental Biology; Vol: 46, pp 677-680.
23. Kazi Rafiq, Shamshad J. Sherajee, Akira Nishiyama, M. A. Sufiun and Mahbub Mostofa (2009) "Effects of indigenous medicinal plants of Bangladesh on blood glucose level and neuropathic pain in streptozotocin-induced diabetic rats". African Journal of Pharmacy and Pharmacology; Vol: 3 (12), pp: 636-642.
24. Kesari A.N, Gupta R.K, Watal G, (2005) "Hypoglycemic effects of Murraya koenigii on normal and alloxan diabetic rabbits". Journal of Ethnopharmacology; Vol: 97, pp 247–251.

25. Lokesh D, Amit SD (2006). "Diabetes mellitus- its possible pharmacological evaluation techniquesand naturotherapy". Int J Green Pharm; Vol: 1, pp 15-28.
26. Lozoya, X., Meckes, M., Abou-Zaid, M., Tortoriello, J., Nozzolillo, C., & Amason, J. T. (1994). "Quercetin glycosides in Psidium guajava Linn leaves and determination of spasmolytic principle". Archives Medical Research; Vol: 25, pp 11–15.
27. Mangalathillam Ratish, Krishnamurthi Poornima, Yuvraj Padhmavathy, Sankar Sadasivam, Balsamy Thayumanavan. (2009). "Inhibition of porcine pancreatic α-amylase by the extracts of Psidium guajava plant parts". Journal of Medicinal and Aromatic Plant Sciences; Vol: 31 (4), pp 312-315.
28. Marles, R.J., Fransworth, N.R., (1995). "Antidiabetic plants and their active constituents". Phytomedicine; Vol: 2, pp 137–189.
29. Marles RJ, Farnsworth N (1996). "Antidiabetic Plants and their Active Constituents: An update". Prot J Bot Med; Vol: 1, pp 85-135.
30. Maryuma Y, Matsuda H, Matsuda R, Kubo M, Hatano T, Okuda T.(1985). "Study on Psidium guajava L. (I). Antidiabetic effect and effective components of the leaf of Psidium guajava L. (Part I)". Shoyakugaku Zasshi; Vol: 39, pp 261-269.
31. Matthias F. Melzig und Ines Funke (2007) "Inhibitors of alpha-amylase from plants – a possibility to treat diabetes mellitus type II by phytotherapy" Wien Med Wochenschr; Vol: 157/13–14, pp 320–324.
32. Meckes, M., Calzada, F., Tortoriello, J., Gonzalez, J.L., Martinez, M., (1996). "Terpenoids isolated from Psidium guajava hexane extract with depressant activity on central nervous system". Phytotherapy Research; Vol: 10, pp 600–603.
33. Mehta K. C. (1982) "Indian herbal drugs in the treatment of diabetes", Current Med Pract, Vol. 26, pp. 195-199.
34. Merlin T, Con T, Richard M, George J (2005). "Anaemia in Diabetes: An Emerging Complication of Microvascular Disease". Curr Diab Rev; Vol: 1, pp 107-26.
35. Michael PK, Asim AB, Robert SB (2005). "The Utility of Oral Diabetes Medications in Type 2 Diabetes of the Young". Curr Diab Rev; Vol: 1, pp 83-92.
36. Oh WK, Lee CH, Lee MS, Bae EY, Sohn CB, Oh H, et al. (2005) "Antidiabetic effect of extracts from Psidium guajava" J Ethnopharmacol; Vol: 96, pp 411-415.

37. Ojewole, J.A.O. (2005). "Hypoglycemic and hypotensive effects of Psidium guajava Linn (Myrtaceae) leaf aqueous extract". Methods and Findings Exp. Clin. Pharmacol Vol 27(10), pp 689- 695.

38. Padhmavathy.Y, Poornima Murthi.K, Ratish.M. (2007) "Isolation and Identification of α- Amylase Inhibitor(s) from Guava Leaves" (UNPUBLISHED).

39. Pickup, J.C., William, G., (1997). "Epidemiology of diabetes mellitus" In: Textbook of Diabetes; vol. I, second ed. Blackwell, Oxford, pp 3.1–3.28.

40. Prashant K. Rai, Dolly Jaiswal, Shikha Mehta & Geeta Watal., (2009) "Anti-hyperglycaemic potential of Psidium guajava raw fruit peel" Indian J Med Res; Vol: 129, pp 561-565.

41. Rai PK, Singh SK, Kessari AN, Watal G. (2007). "Glycemic evaluation of Psidium guajava in rats" Indian J Med Res; Vol: 126, pp 224-227.

42. Ramachandran A, Snehalatha C, Vijay V (2002). "Burden of type 2 diabetes and its complications – The Indian scenario". Curr Sci; Vol: 83, pp 1471-76.

43. Ranjan C, Ramanujam R (2002). "Diabetes and insulin resistance associated disorders: Disease and the therapy". Curr Sci.; Vol: 83, pp 1533- 38.

44. S.K. Prasad, Alka Kulshreshtha and Taj N. Qureshi (2009), "Antidiabetic Activity of Some Herbal Plants in Streptozotocin Induced Diabetic Albino Rats" Pakistan Journal of Nutrition; Vol: 8(5), pp: 551-557.

45. Shankar P, Sundarka MK (2001). "Management of Type 2 Diabetes: Evidence Based Approach". J Indian Acad Clin Med; Vol: 2, pp 244- 50.

46. Shrabana C, Tuhin KB, Begum R, Liaquat A, Mosihuzzaman M, Nilufer N, Azad KAK, Biswapati M (2003). "Advanced studies on the hypoglycemic effect of Caesalpinia bonducella F. in type 1 and 2 diabetes in Long Evans rats". J Ethnopharmacol; Vol: 84, pp 41-46.

47. Shukla R, Sharma S. B, Puri D, Prabhu K. M, and Murthy P. S. (2000) "Medicinal Plants for Treatment of Diabetes Mellitus".

48. Szkudelski, T., (2001) "The Mechanism of Alloxan and Streptozotocin Action in B Cells of the Rat Pancreas" Physiol. Res; Vol: 50, pp 536-546.

49. Tanaka, T., Ishida, N., Ishimatsu, M., Nonaka, G., Nishioka, I.. (1992). "Tannins and related compounds. CXVI. Six new complex tannins, guajavins, psidinins and psiguavin from the bark of Psidium guajava L". Chemical and Pharmaceutical Bulletin; Vol: 40, pp 2092–2098.
50. Torben H (2002). "Genetics of Type 2 diabetes". Curr Sci; Vol: 83, pp 1477- 82.
51. Venkatesh S, Reddy GD, Reddy BM, Ramesh M, Apparao AVN (2003). "Antihyperglycemic activity of Carulluma asttenuate". Fitoterapia; Vol: 74, pp 274-277.
52. WHO expert committee on Diabetes mellitus, Technical reports series World Health Organisation, Geneva, 1980.