P-3402

IDENTIFICATION, ISOLATION AND PARTIAL CHARACTERIZATION OF PLANT BASED α-GLUCOSIDASE INHIBITOR(S)

A PROJECT REPORT

Submitted by

SAILESH.K (0710204039)

SINDHU MAHADEVAN (07102040246)

SUMETHA.K (0710204052)

VINI RAVINDRAN (0710204054)

In partial fulfillment for the award of the degree

of

Bachelor of Technology

In

BIO TECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE

(An Autonomous Institution affiliated to Anna University of Technology, Coimbatore)

APRIL: 2011

KUMARAGURU COLLEGE OF TECHNOLOGY
COIMBATORE 641049

BONAFIDE CERTIFICATE

Certified that this project report **"IDENTIFICATION, ISOLATION AND PARTIAL CHARACTERIZATION OF PLANT-BASED α-GLUCOSIDASE INHIBITIOR(S)"** is the bonafide work of **SAILESH K. (0710204039), SINDHU MAHADEVAN (0710204046), SUMETHA K. (0710204052) and VINI RAVINDRAN (0710204054)** who carried out the project work under my supervision.

SUPERVISOR

Dr. S. Sadasivam

DEAN (Biotechnology)

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore - 641049

HEAD OF THE DEPARTMENT

Dr. S. Sadasivam

DEAN (Biotechnology)

Department of Biotechnology

Kumaraguru College of Technology

Coimbatore - 641049

19/4/11

Internal Examiner

External Examiner

ACKNOWLEGDEMENT

We are extremely thankful to **Dr. S. Sadasivam**, Dean, Department of Biotechnology, Kumaraguru College of Technology, Coimbatore for his unflinching support and guidance throughout the course of the project.

We are extremely grateful to **Mr. T. Sathish Kumar**, Assistant Professor (Sr.G), Department of Biotechnology, for sparing time for us and giving us direction at every stage of the project. For explanation, day-to-day guidance and most of all for the kindness and patience, we would like to place on record our sincere thanks.

We express our sincere thanks to **Dr. R. Baskar,** Associate professor, Department of Biotechnology and to **Dr. S. Shanmugam,** Assistant Professor, Department of Biotechnology for having reviewed our project on time to time.

For their timely help and critical suggestions, we sincerely thank **Dr. P. Ramalingam**, Assistant professor, Department of Biotechnology and **Dr. V. Stephen Rapheal**, Assistant professor, Department of Biotechnology.

We would like to express our gratitude to Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Mumbai, for extending their lab facilities for the project proceedings.

We would also like to thank our project co-ordinator **Mr. M. Shanmuga prakash**, Assistant professor, Department of biotechnology for his constant support and encouragement.

We extend sincere thanks to all the **teaching and non-teaching staff** for their support and cooperation towards our project and shaping its successful completion.

Last but certainly not least, we express our gratitude to **our beloved family** for their faith in us and for their encouragement and cooperation.

SAILESH K.

SINDHU MAHADEVAN

SUMETHA K.

VINI RAVINDRAN

TABLE OF CONTENTS

ABSTRACT

ABSTRACT

Type II diabetes mellitus is a disease condition growing in prevalence across the world. Alpha glucosidase is an enzyme implicated in high post-prandial blood glucose level and thus has caught the attention of the scientific fraternity towards the possibility of its inhibition. Several synthetic competitive inhibitors- Acarbose, Miglitol and Voglibose are commercially available and prescribed either in combination with other oral hyperglycemic agents or insulin. The search for plant based alpha glucosidase inhibitors is now a thrust area in the search for a novel diabetes therapy. Plant pigments under the broad category of polyphenols that have been proven to have antioxidant properties, are seen as potential alpha glucosidase inhibitors. The mechanism through which anti-hyperglycemic effect is exerted include inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic cells, modulation of glucose release from the liver, activation of insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of intracellular signaling pathways and gene expression. Of the several plants assayed for alpha glucosidase inhibition the two plants Wax jambo (*Syzygium samarangense*) and Bilimbi (*Averrhoa bilimbi*) showed potential inhibition. Thin Layer Chromatography carried out revealed high phenolic acid content in *Syzygium samarangense* which can be associated with the alpha glucosidase inhibitory action. Preparative TLC elutes of the phenolic acid showed high inhibition of alpha glucosidase. The PTLC isolate of aqueous extract of *Syzygium samarangense* leaves with high phenolic acid content was analyzed with FTIR and LC-MS at IIT(Bombay). The following components were identified in the extract: 4-Hydroxybenzoic acid, Sailcyl alcohol derivative, Gallic alcohol, Pinobanksin-3-O-acetate, 5-Ferulolylquinic Acid.

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Diabetes - Etiology and prevalence

"Diabetes is a chronic disease, which occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. This leads to an increased concentration of glucose in the blood (hyperglycaemia)" (WHO). Carbohydrates constitute the principal energy source that powers bodily functions. Humans are capable of digesting some carbohydrates, in particular starch, being the most common in food and some disaccharides such as sucrose and convert them to simpler forms, most notably the monosaccharide glucose, the principal carbohydrate used by the body. The rest are passed on for processing by gut flora largely in the colon.

Insulin is released into the blood by beta cells (β-cells), of the Islets of Langerhans in the pancreas, in response to rising levels of blood glucose, typically after food intake. Insulin is used by most of the body's cells to absorb glucose from the blood for use as fuel, for conversion to other essential molecules, or for storage (in muscle cells and adipose cells). The role of insulin is critical in facilitating the entry of glucose into the cells upon which they are used to generate ATPs, the energy currency of a cell. The normal blood sugar level before a meal reads from 70-110 mg/dL with minor variations in the population. Functional or physiological hyperglycemia is a state of high blood glucose level that is usually recorded after a meal as digestion progresses. Cellular uptake of glucose, facilitated by insulin usually returns the blood sugar levels to the normal range after about two hours after the meal. If the amount of insulin available is insufficient, if cells respond poorly to the produced insulin (insulin insensitivity or resistance), or if the insulin itself is defective, then glucose will not have its usual effect as a result of which glucose is not absorbed properly by those body cells that require it and nor is it stored appropriately in the liver and muscles. The net effect is persistent high levels of blood glucose, poor protein synthesis, and other metabolic derangements. The cells, devoid of the principal energy source, are subject to starvation thus

causing damage to organs and organ systems. When the glucose concentration in the blood is raised beyond its renal threshold (about 10 mmol/L, although this may be altered in certain conditions, such as pregnancy), reabsorption of glucose in the proximal renal tubuli is incomplete, and part of the glucose remains in the urine (glycosuria). This increases the osmotic pressure of the urine and inhibits re-absorption of water by the kidney, resulting in increased urine production (polyuria) and increased fluid loss (Cooke DW et. al., 2008) Lost blood volume will be replaced osmotically from water held in body cells and other body compartments, causing dehydration and increased thirst. Diabetes is initially managed by increasing exercise and dietary modification. Initially diabetes can be managed by lifestyle modifications and dietary restrictions, but as the condition progresses, medications may be needed.

Diabetes affects the quality of life and productivity of a large percentage of the Indian population. Statistics suggest that India is poised to be the diabetes capital of the world in the next 20 years (Times of India Health Statistics, 2010). The International Diabetes Federation estimates that the number of diabetic patients in India has more than doubled from 19 million in 1995 to 40.9 million in 2007. It is projected to increase to 69.9 million by 2025. Currently, up to 11 per cent of India's urban population and 3 per cent of rural population above the age of 15 has diabetes. Globally, diabetes affects 246 million people, which is about 6 per cent of the total adult population. It is the fourth leading cause of death by disease and over three million deaths worldwide, every year are tied directly to diabetes.

1.2 CLASSIFICATION OF DIABETES

1.2.1 Type 1 Diabetes Mellitus

Type 1 Diabetes Mellitus is often referred to as insulin-dependent diabetes mellitus or juvenile diabetes and is typically diagnosed before the age of 35, especially during adolescence. In this case, some or all of the insulin producing β-cells of the pancreas are destroyed by the body's own immune system-only about 5 to 10 per cent of diabetes patients suffer from type 1 diabetes mellitus. Administering insulin through injection is required in patients of Type 1 diabetes mellitus.

1.2.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus was erstwhile referred to as non-insulin dependent diabetes mellitus. Type 2 diabetes mellitus is usually diagnosed in patients who have crossed the age of 40 though its incidence in young adults is on the rise. About 90-95% of diabetes patients are afflicted with Type 2 diabetes mellitus. The issue of insulin resistance is associated with type 2 diabetes mellitus. Wherein the pancreatic cells produce insulin at levels that would otherwise suffice, but the failure of the body cells to respond to insulin in blood necessitates heightened insulin production by the β-cells. Initially, the pancreas produce insulin in greater amount so as to compensate the insensitivity of the cells but this is not long-lasting as the β-cells wear out. Thus, the insulin levels fall short of the requirement and consequently glucose uptake by cells is adversely affected. Insulin resistance in muscle and adipose cells lead to lower uptake and thus low storage of glucose.

1.2.3 Gestational Diabetes

Gestational diabetes is associated with pregnancy, often in women who have never had high blood glucose levels. Gestational diabetes mellitus (GDM) resembles type 2 diabetes in several respects, involving a combination of relatively inadequate insulin secretion and responsiveness. It occurs in about 2%–5% of all pregnancies and may improve or disappear after delivery.

1.3 SECONDARY COMPLICATIONS ASSOCIATED WITH DIABETES

1.3.1 SHORT TERM COMPLICATIONS

1.3.1.1 Diabetic ketoacidosis

Diabetic ketoacidosis (DKA) is an acute and dangerous complication that is a medical emergency. Low insulin levels cause the liver to turn to fat (lipolysis) for want of an energy source (i.e., ketosis). Elevated levels of ketone bodies, when sustained in the blood, decrease the blood's pH leading to DKA. The patient in DKA is typically dehydrated and breathing rapidly and deeply. Abdominal pain is common and may be severe. The level of consciousness is typically normal until late in the process, when lethargy may progress to coma. Ketoacidosis can easily become severe enough to cause hypotension, shock, and death.

1.3.1.2 Hyperglycemia hyper-osmolar state

Hyper-osmolar non-ketotic state (HNS) is an acute complication sharing many symptoms with DKA. In case of very high (usually considered to be above 300 mg/dl) blood glucose levels, water is osmotically drawn out of cells into the blood and the kidneys eventually begin to dump glucose into the urine. This results in loss of water and an increase in blood osmolarity. If fluid is not replaced, the osmotic effect of high glucose levels, combined with the loss of water and electrolytes, will cause the body's cells become progressively dehydrated. Urgent medical attention is necessary, commonly beginning with fluid volume replacement. Lethargy may ultimately progress to a coma, though this is more common in type 2 diabetes than type1 diabetes.

1.3.1.3 Hypoglycemia

Hypoglycemia, or abnormally low blood glucose, is an acute complication of several diabetes treatments. The patient may become agitated, sweaty, weak, and have symptoms of feelings akin to dread and immobilized panic. Consciousness can be altered or even lost in extreme cases, leading to coma, seizures, or even brain damage and death. In patients with diabetes, this may be caused by several factors, such as too much or incorrectly timed insulin, too much or incorrectly timed exercise (exercise decreases insulin requirements) or insufficient food (specifically glucose containing carbohydrates). In most cases, hypoglycemia is treated with sugary drinks or food. In severe cases, an injection of glucagon (a hormone with effects largely opposite to those of insulin) or an intravenous infusion of dextrose is used for treatment.

1.3.2 LONG TERM COMPLICATIONS

1.3.2.1 Arteriosclerosis

It is the narrowing of arteries of the extremities due to calcium deposits that is characterized by the narrowing and stiffening of these blood vessels. This diminishes the blood flow to the arms and legs, the effect of which is mostly seen in the feet.

1.3.2.2 Diabeteic Microangiopathy

The damage to small blood vessels leads to a microangiopathy, which refers to the thickening of the basement membrane of small blood vessels and capillaries of various organs and tissues such as skin, skeletal muscle etc. Such thickening may also be observed in other non-vascular tissues, which can cause several other complications depending on the tissue that is affected.

1.3.2.3 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a disorder of the heart muscle in people with diabetes. It can lead to inability of the heart to circulate blood through the body effectively, a state known as heart failure, with accumulation of fluid in the lungs (pulmonary edema) or legs (peripheral edema). Most heart failure in people with diabetes results from coronary artery disease, and diabetic cardiomyopathy is only said to exist if there is no coronary artery disease to explain the heart muscle disorder.

1.3.2.4 Diabetic nephropathy

Diabetic nephropathy is the condition in which damage to the kidneys occurs due to diabetes, arising due to thickening of the basement membrane of the glomerular capillaries. A particular type of real lesion is observed in diabetic nephropathy and can lead to chronic renal failure, eventually requiring dialysis. Diabetes mellitus is the most common cause of adult kidney failure worldwide in the developed world.

1.3.2.5 Diabetic neuropathy

Decreased blood flow and high blood glucose contribute to temporary or permanent damage to nerve tissue, resulting in a condition termed as Diabetic neuropathy. An abnormal and decreased sensation, starting at the feet but potentially in other nerves, later often fingers and hands is the most noticeable symptom. Initial tingling sensations later pave way for continuous and severe pain in subsequent stages.

1.3.2.6 Diabetic retinopathy

Retinopathy is the most common of the long term complications of diabetes due to the growth of friable and poor-quality new blood vessels in the retina that leak proteins and blood onto the retina, as well as macular edema (swelling of the macula), which can lead to severe vision loss or blindness. Retinal damage (from microangiopathy) makes it the most common cause of blindness among non-elderly adults suffering from diabetes.

1.3.2.7 Infections

Diabetic patients show increased susceptibility to infectious diseases such as pneumonia, tuberculosis, diabetic ulcers etc. which is attributed to poor blood supply and hyperglycemia and reduced cellular immunity.

1.3.2.8 Heart Disease and Stroke

Patients with high blood glucose level over long time durations are more prone to heart disease than their non- diabetic counterparts in the population. Heart attack, chest pain or angina, high blood pressure, stroke etc. are the common consequences of uncontrolled hyperglycemia. 'Silent Heart Attacks' are characteristic to diabetics since the damage in the nervous tissue causes partial loss of the sensation of pain and thus the patient does not feel the chest pain due to an impending heart attack and thus remains unaware of it.

1.4 TYPE II DIABETES-TREATMENT AND DRUG TARGETS

1.4.1 Sulphonylureas

Sulphonylurea (alternatively, sulfonylurea) derivatives are a class of antidiabetic drugs that are used in the management of diabetes mellitus type 2. There are three generations into which the sulphonylureas fall- First, Second and Third.

Mechanism of action

Sulfonylureas bind to an ATP-dependent K^+ (KATP) channel on the cell membrane of pancreatic beta cells. This inhibits a tonic, hyperpolarizing efflux of potassium, thus causing the electric potential over the membrane to become more positive. This depolarization opens

voltage-gated Ca2+ channels. The rise in intracellular calcium leads to increased fusion of insulin granulae with the cell membrane, and therefore increased secretion of (pro)insulin.

1.4.2 Meglitinides

Meglitinides, or "Glinides", are a class of drugs used treat diabetes type 2.

Mechanism of action

They bind to an ATP-dependent K^+ (KATP) channel on the cell membrane of pancreatic beta cells in a similar manner to sulfonylureas but at a separate binding site (Rendell et. al.)

1.4.3 Biguanides

Biguanide can refer to a molecule, or to a class of drugs based upon this molecule. Biguanides can function as oral antihyperglycemic drugs used for diabetes mellitus or prediabetes treatment. Some of the common biguanides are Metformin, Phenformin, Proguanil and Buformin.

1.4.3.1 Mechanism of action

The mechanism of action of biguanides is not fully understood. Their therapeutic uses derive from their tendency to reduce gluconeogenesis in the liver and, as a result, reduce the level of glucose in the blood. Biguanides also tend to make the cells of the body more willing to absorb glucose already present in the blood stream, thus reducing the level of glucose in the plasma.

1.4.4 Thiazolidinediones

The thiazolidinediones, also known as glitazones, are a class of medications used in the treatment of diabetes mellitus type 2.

Mechanism of action

Thiazolidinediones or TZDs act by binding to PPARs (peroxisome proliferator-activated receptors), a group of receptor molecules inside the cell nucleus,

specifically PPARγ (gamma). When activated, the receptor migrates to the DNA, activating transcription of a number of specific genes. TZDs also increase the synthesis of certain proteins involved in fat and glucose metabolism, which reduces levels of certain types of lipids, and circulating free fatty acids. TZDs generally decrease triglycerides and increase high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

1.5 ENZYMES INVOLVED IN CARBOHYDRATE DIGESTION

All carbohydrates absorbed in the small intestine must be hydrolyzed to monosaccharides prior to absorption. The digestion of starch begins with the action of salivary alpha-amylase, although their activity is slight in comparison with that made by pancreatic amylase in the small intestine. Amylase hydrolyzes starch to alpha-dextrin, which are then digested by gluco-amylase (alpha-dextrinases) to maltose and maltotriose. The products of digestion of alpha-amylase and alpha-dextrinase, along with dietary disaccharides are hydrolyzed to their corresponding monosaccharides by enzymes (maltase, isomaltase, sucrase and lactase) present in the brush border of small intestine. In the typical Western diet, digestion and absorption of carbohydrates is fast and takes place usually in the upper small intestine. However, when the diet contains carbohydrates not easily digestible, digestion and absorption take place mainly in the ileal portion of the intestine.

1.6 SIGNIFICANCE OF α-GLUCOSIDASE

α-Glucosidase (EC 3.2.1.20) is a group of enzymes categorized under hydrolytic enzymes that acts upon alpha- 1, 4-glycosidic linkage. Maltase which is an enzyme that acts upon and cleaves maltose is considered an equivalent of α-Glucosidase.

Inhibition of α-Glucosidase can delay and significantly reduce the intestinal absorption of glucose, since lesser complex carbohydrates are broken down to glucose and other monosaccharides. This is a solution to the problem of high post-prandial blood glucose levels, i.e. high blood glucose levels two hours after a meal.

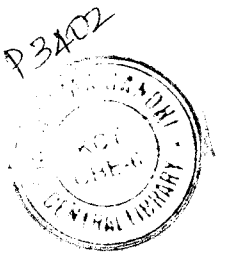
1.6.1 Alpha-Glucosidase inhibition using conventional drugs

1. **Acarbose**: Acarbose inhibits enzymes (glycoside hydrolases) needed to digest carbohydrates specifically, alpha-glucosidase enzymes in the brush border of the small intestines and pancreatic alpha-amylase. Pancreatic alpha-amylase hydrolyzes complex starches to while intestinal alpha-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine.
2. **Miglitol**: It is primarily used to achieve greater glycemic control in diabetes mellitus type 2, and works on the same principle as acarbose i.e. inhibition of glycoside hydrolases. As against the case with acarbose, miglitol is systemically absorbed but is not metabolized and gets excreted by the kidneys.
3. **Voglibose**: Though based on the same principle as the other two drugs in this category, voglibose has a better side effect profile than the former two. But acarbose scores on efficiency.

α -Glucosidase inhibitors may be used to achieve greater glycemic control particularly with reference to post prandial blod glucose levels. They can be used as monotherapy along with strict dietary regulation or in conjunction with conventional therapy.

1.7 OBJECTIVES

- To screen a few plants for the presence of α-glucosidase inhibitors.
- To identify the chemical constituents present in the plant extract with TLC.
- To derive the structure of the inhibiting compounds through FTIR and LCMS,.



LITERATURE REVIEW

CHAPTER 2

REVIEW OF LITERATURE

2.1 INTRODUCTION

Alpha glucosidases are exo type alpha glucosidic O-linkage hydrolases. Releasing D,α-glucose (S.Chiba et al,1983) from non reducing end of the substrate. The hydrolytic reactions occur by splitting of anomeric carbon of glycosyl residue and glycosidic oxygen and thus releases alpha glucose. Glucosyl residue is replaced by proton from water or acceptor, that is an exchange reaction glucosyl residue and the proton.

2.1.1 Substrate specificity

Various types of alpha glucosidases are found in plants, microbes and plant tissues. Their substrate specificities are diverse; it can be synthetic alpha glucosides, oligosaccharides or even alpha glucans, like soluble starch and glycogen. There are three catalytic subsites. These sites have different affinities (K.Hiromi et al 1973) to malto-oligosaccharides, the second having the highest affinity (4.82kcal/mol), followed by A1 (.79kcal/mol), and A3 (.19kcal/mol).

2.1.2 Structure

Glucosidases have (β/α) barrel fold structure (P.M.Coutinho et al,1994). Around twenty different amino acid sequences have been reported. Alpha glucosidases isolated from *Sacchromyces carlsbergensis* (S.h.Hong and J. Mamur,gene, 1986), *B.cereus* are similar but different from mammals, fungus and plants. Four conservative alpha glucosidases can be broadly classified into two families based on the primary structure, family I and family II. These seem to have evolved independently by converged evolution from ancestral proteins. Four regions of alpha glucosidase is conserved in *S.Carlsbergensis*, *B.cereus*, *Bacillus* (M.Nakano et al ,1994) species and insects (M.Snyder and N.Davidson, 1986). Mammals (L.H.Hoefsloot et al 1990, F.Greean et al, 1987, G.Chandrasena et al,1994, A.Kimura et al, 1992), plants (K.Watanabe et al ,1990, M.Sugimoto and Y.Suzuki,1996, B.K.Tibbot and

R.W.Skadsen 1996), fungus (H.Matsui,1997) *Candida tsukubaensis*, and *Mucor javanicus* completely lack regions 1,3,and 4, while *A.Niger* and *A.Oryzae* are devoid of region 1.

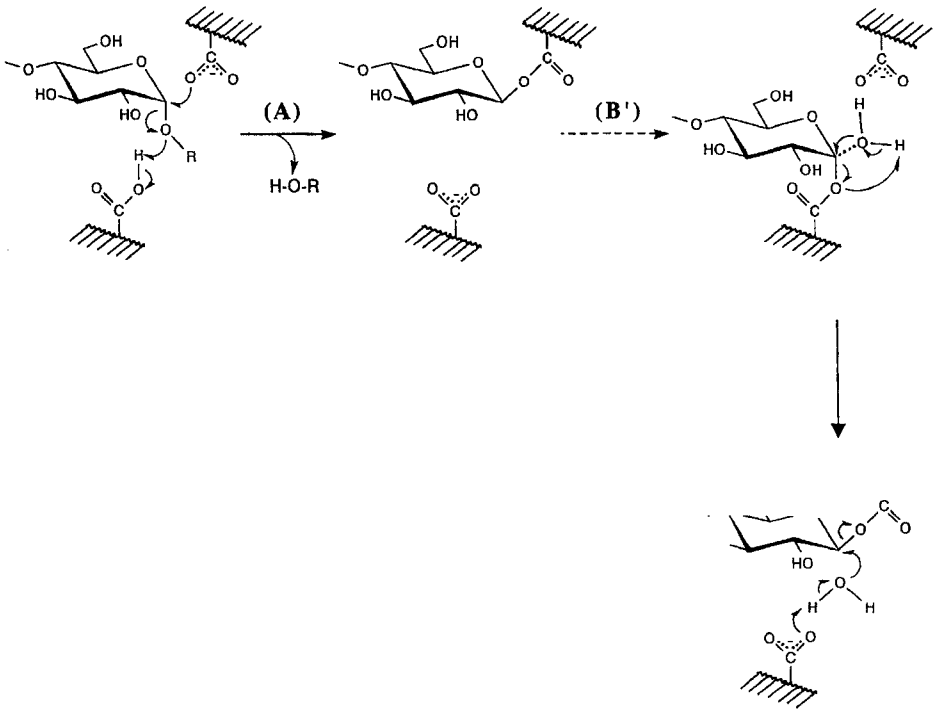
The essential amino acid directly involved in catalytic reaction with chemical modification conduritol B epoxide is Asp in region 2 (M. M. P. Hermans et al 1991 and S. Iwanami, 1995). It is hypothesised that Asp might be the amino acid in the catalytic site in all the glucosidases. The other amino acids have not become apparent yet. It is proposed that the catalytic amino acid in region 3 and 4 of family II glucosidases might be Glu or Asp. It is also proposed that these might be the catalytic sites in other regions of family II.

2.1.3 Catalytic Reaction Mechanism

Two mechanisms are proposed for alpha glucosidases: nucleophilic displacement mechanism and oxocarbenium ion mechanism. In the first mechanism two catalytic groups carboxyl –COOH and carboxylate, COO^- cleave glycosidic linkage cooperatively by electrophilic and nucleophilic attacks against glycosyl oxygen and anomeric carbon atoms respectively resulting in a covalent β-glucosyl enzyme complex by asinle displacement involving anomeric inversion. This is followed by replacement of β -carboxy acetal bond by hydroxyl ion of water to stop hydrolysis retaining the anomer of product by double displacement.

In Oxocarbenium ion intermediate mechanism the two catalytic groups carboxylate and carboxyl groups act cooperatively in the hydrolytic reaction. Carboxylate group is known to promote oxocarbenium ion and stabilise the intermediate, while the carboxyl groups attack the oxygen of glycosidic linkage. This is followed by nucleophilic displacement by hydroxyl ions of water to oxycarbenium ion is allowed to complete the reaction.

Fig 2.1 Nucleophilic Double Displacement Mechanism in Hydrolytic Reaction of x-Glucosidic Linkage.



2.2 POLYPHENOLS

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. Polyphenols are secondary metabolites of plants and are generally involved in defence against ultraviolet radiation or aggression by pathogens.3 In food, polyphenols may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability. Epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases.

2.2.1 Structure and Classes of Polyphenols

More than 8,000 polyphenolic compounds have been identified in various plant species. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist. Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans.

2.2.2 Phenolic acids:

Phenolic acids are found abundantly in foods and divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid. The hydroxybenzoic acid content of edible plants is generally low, with the exception of certain red fruits, black radish and onions, which can have concentrations of several tens of milligrams per kilogram fresh weight The hydroxycinnamic acids are more common than hydroxybenzoic acids and consist chiefly consist of *p*-coumaric, caffeic, ferulic and sinapic acids.

2.2.3 Flavonoids

Flavonoids comprise the most studied group of polyphenols. This group has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle. More than 4,000 varieties of flavonoids have been identified, many of which are responsible for the attractive colours of the flowers, fruits and leaves.8 Based on the variation in the type of heterocycle involved, flavonoids may be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones. Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Quercetin, myricetin, catechins etc., are some most common flavonoids.

2.2.4 Stilbenes

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Occurrence of stilbenes in the human diet is quite low. Most stilbenes in plants act as antifungal phytoalexins, compounds that are synthesized only in response to infection or injury. One of the best studied, naturally occurring polyphenol stilbene is resveratrol (3,4',5-trihydroxystilbene), found largely in grapes. A product of grapes, red wine also contains significant amount of resveratrol.

2.2.5 Lignans

Lignans are diphenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues. Several lignans, such as secoisolariciresinol, are considered to be phytoestrogens.

2.3 OCCURRENCE AND CONTENT

Distribution of phenolics in plants at the tissue, cellular and sub cellular levels is not uniform. Insoluble phenolics are found in cell walls, while soluble phenolics are present within the plant cell vacuoles.(G.Chandrasena et al,1994) .Certain polyphenols like quercetin are found in all plant products; fruit, vegetables, cereals, fruit juices, tea, wine, infusions etc., whereas flavanones and isoflavones are specific to particular foods. In most cases, foods contain complex mixtures of polyphenols. The outer layers of plants contain higher levels of phenolics than those located in their inner parts.(A.Kimura et al, 1992) Numerous factors affect the polyphenol content of plants, these include degree of ripeness at the time of harvest, environmental factors, processing and storage. Polyphenolic content of the foods are greatly affected by environmental factors as well as edaphic factors like soil type, sun exposure, rainfall etc. The degree of ripeness considerably affects the concentrations and proportions of various polyphenols (K.Watanabe, 1990). In general, it has been observed that phenolic acid content decreases during ripening, whereas anthocyanin concentrations increase. Many polyphenols, especially phenolic acids, are directly involved in the response of plants to different types of stress: they contribute to healing by lignifications of damaged areas possess antimicrobial properties, and their concentrations may increase after infection (M.Sugimoto and Y.Suzuki, 1996). Another factor that directly affects the polyphenol content of the foods is storage. Studies have proved that polyphenolic content of the foods

change on storage, the reason is easy oxidation of these polyphenols (B.K.Tibbot and and R.w.Skadsen,1996). Oxidation reactions result in the formation of more or less polymerized substances, which lead to changes in the quality of foods, particularly in colour and organoleptic characteristics . Cold storage, in contrast, has slight effect on the content of polyphenols in apples, pears or onions (M.Nakano, 1994). Cooking also has a major effect on concentration of polyphenols.

2.4 BIOAVAILABILITY

Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways. Bioavailability of each and every polyphenol differs however there is no relation between the quantity of polyphenols in food and their bioavailability in human body. Generally, aglycones can be absorbed from the small intestine; however most polyphenols are present in food in the form of esters, glycosides or polymers that cannot be absorbed in native form (A.A.James, 1989). Before absorption, these compounds must be hydrolyzed by intestinal enzymes or by colonic microflora. During the course of the absorption, polyphenols undergo extensive modification; in fact they are conjugated in the intestinal cells and later in the liver by methylation, sulfation and/or glucuronidation (K.Ohashi, 1996). As a consequence, the forms reaching the blood and tissues are different from those present in food and it is very difficult to identify all the metabolites and to evaluate their biological activity (A.Kimura et al, 1992). Importantly it is the chemical structure of polyphenols and not its concentration that determines the rate and extent of absorption and the nature of the metabolites circulating in the plasma. The most common polyphenols in our diet are not necessarily those showing highest concentration of active metabolites in target tissues; consequently the biological properties of polyphenols greatly differ from one polyphenol to another. Evidence, although indirect, of their absorption through the gut barrier is given by the increase in the antioxidant capacity of the plasma after the consumption of polyphenol rich foods (M. M. P. Hermans et al, 1991 and M. M. P. Hermans et al, 1995). Polyphenols also differs in their site of absorption in humans. Some of the polyphenols are well absorbed in the gastro-intestinal tract while others in intestine or other part of the digestive tract. In foods, all flavonoids except flavanols exist in glycosylated forms. The fate of glycosides in the stomach is not clear yet. Most of the glycosides probably resist acid hydrolysis in the stomach and thus arrive intact in the intestine where only aglycones and few glucosides can be absorbed. It was suggested that glucosides could be

transported into enterocytes by the sodium dependent glucose transporter SGLT1, and then hydrolyzed by a cytosolic alpha-glucosidase (K.Watanabe et al, 1990). Proanthocyanidins differ from most of other plant polyphenols because of their polymeric nature and high molecular weight. This particular feature should limit their absorption through the gut barrier, and oligomers larger than trimers are unlikely to be absorbed in the small intestine in their native forms. It was observed that the hydroxycinnamic acids, when ingested in the free form, are rapidly absorbed by the small intestine and are conjugated as the flavonoids (Crespy V, et al, 2002).However these compounds are naturally esterified in plant products and esterification impairs their absorption because intestinal mucosa, liver and plasma do not possess esterases capable of hydrolyzing chlorogenic acid to release caffeic acid, and hydrolysis can be performed only by the microflora present in colon. Though most of the poyphenols get absorbed in gastrointestinal tract and intestine but there are some poyphenols which are not absorbed in these locations. These polyphenols reach the colon, where microflora hydrolyze glycosides into aglycones and extensively metabolize these aglycones into various aromatic acids (Halliwell B et al, 2000). Aglycones are split by the opening of the heterocycle at different points depending on their chemical structure, and thus produce different acids that are further metabolized to derivatives of benzoic acid. After absorption, polyphenols go to several conjugation processes. These processes mainly include methylation, sulfation and glucuronidation, representing a metabolic detoxication process, common to many xenobiotics, that facilitates their biliary and urinary elimination by increasing their hydrophilicity. It is important to identify the circulating metabolites, including the nature and the positions of the conjugating groups on the polyphenol structure, because the positions can affect the biological properties of the conjugates. Polyphenol metabolites circulate in the blood bound to proteins; in particular albumin represents the primary protein responsible for the binding. Albumin plays an important role in bioavailability of polyphenols. The affinity of polyphenols for albumin varies according to their chemical structure binding to albumin may have consequences for the rate of clearance of metabolites and for their delivery to cells and tissues. It is possible that the cellular uptake of metabolites is proportional to their unbound concentration. Finally, it is still unclear if the polyphenols have to be in the free form to exert their biological activity, or the albumin-bound polyphenols can exert some biological activity (Price K.R, et al 1997 and Falany C.N, 1997). Accumulation of polyphenols in the tissues is the most important phase of polyphenol metabolism because this is the concentration which is biologically active for exerting the

effects of polyphenols. Studies have shown that the polyphenols are able to penetrate tissues, particularly those in which they are metabolized such as intestine and liver. Excretion of polyphenols with their derivatives occurs through urine and bile. It has been observed that the extensively conjugated metabolites are more likely to be eliminated in bile, whereas small conjugates, such as monosulfates, are preferentially excreted in urine. Amount of metabolites excreted in urine is roughly correlated with maximum plasma concentrations. Urinary excretion percentage is quite high for flavanones from citrus fruit and decreases from isoflavones to flavonols. Thus the health beneficial effects of the polyphenols depend upon both the intake and bioavailability.

2.5 ANTIDIABETIC ACTIVITY OF POLYPHENOLS

- Inhibition of digestion and absorption.
- Protection of β-cell glucotoxicity.
- Suppression of glucose release from liver storage
- Improvement of glucose in peripheral tissues.

Impairment in glucose metabolism leads to physiological imbalance with the onset of the hyperglycemia and subsequently diabetes mellitus. There are two main categories of diabetes; type-1 and type-2. Studies have shown that several physiological parameters of the body get altered in the diabetic conditions. Long term effects of diabetes include progressive development of specific complements such as retinopathy, which affects eyes and lead to blindness; nephropathy in which the renal functions are altered or disturbed and neuropathy which is associated with the risks of amputations, foot ulcers and features of autonomic disturbance including sexual dysfunctions. Numerous studies report the antidiabetic effects of polyphenols. Tea catechins have been investigated for their anti-diabetic potential.(Dangles O,,2 et al 2001 and Dufour C, et al 2007).Polyphenols may affect glycemia through different mechanisms,This suggests that these effects are due to an inhibition of glucosidase in the gut mucosa. The inhibition of intestinal glycosidases and glucose transporter by polyphenols has been studied.I(Matsui T, et al 1956).Individual polyphenols, such as (+)catechin, (-)epicatechin, (-)epigallocatechin,epicatechin gallate, isoflavones from soyabeans, tannic acid, glycyrrhizin from licorice root, chlorogenic acid and saponins also decrease S-Glut-1 mediated intestinal transport of glucose. Saponins additionally delay the transfer of glucose from stomach to the small intestine. Resveratrol

has also been reported to act as an anti-diabetic agent. Many mechanisms have been proposed to explain the anti-diabetic action of this stilbene, modulation of SIRT1 is one of them which improves whole-body glucose homeostasis and insulin sensitivity.(Pandey KB, et al ,2009 and Pandey KB et al 2009). Resveratrol inhibits diabetes-induced changes in the kidney (diabetic nephropathy). A possible mechanism was thought to be related to the inhibition of K + ATP and K + V channel in beta cells.(Renaud S et al,1992) Onion polyphenols, especially quercetin is known to possess strong anti diabetic activity. A recent study shows that quercetin has ability to protect the alterations in diabetic patients during oxidative stress. Quercetin significantly protected the lipid peroxidation nd inhibition antioxidant system in diabetics.. Ferulic acid (FA) is another polyphenol very abundant in vegetables and maize bran. Several lines of evidence have shown that FA acts as a potent anti-diabetic agent by acting at many levels.

2.6 DIFFERENT PLANT SOURCES

2.6.1 *Laurus Nobilis* (Bay leaf)

The Lauraceae family comprises over 2,500 species which occur within the subtropics and tropics of Eastern Asia, South and North America. Most species possess aromatic roots, stems and fruits. One of the most well-known and most frequently used plants from this family is *Laurus nobilis* L., also called bay laurel. (Biljana Kaurinovic ..et al, 2010). As a medicinal plant, Bay leaves and fruits have been employed against rheumatism, skin rashes, and earaches. In addition, it has been used as a stomachic, astringent, carminative, diaphoretic, stimulant, emetic, emmenagogue, abortifacient, and insect repellent. .

In previous phytochemical investigations on *L.nobilis* leaves and fruits, different groups of chemicals were isolated: flavones (apigenin and luteolin) , flavonols (kaempferol, myricetin, and quercitin) , sesquiterpene lactones , alkaloids , glycosylated flavonoids , and monoterpene and germacrane alcohols . Roots and leaves are a source of sesquiterpene lactones, and two distinct chemical types were found containing laurenobiolide and costunolide as major compounds, respectively . Sesquiterpene lactones identified in bay leaf were found to have different pharmacological properties including inhibitory effects on NO production (anti-inflammatory) , inhibitory effects on alcohol absorption , and enhancement of liver glutathione S-transferase (GST) activity .

2.6.2 *Phyllanthus Niruri* (Kizharnelli)

Phyllanthus niruri also known as "Chanca pledra" belongs to the family Euphorbiaceae. A lot of researchers who worked on *Phyllanthus niruri* confirmed that it has hypoglycaemic properties.. It is an excellent remedy of jaundice and infective hepatitis. It is effective in jaundice in children. The plant is of medicinal importance for numerous ailments like dysentery, diuretics, kidney stones, influenza, antibacterial, antihyperglycaemic and antiviral.

P. niruri has been the subject of much phytochemical studies since the mid 1960s. Different classes of organic compounds with various medical interest have been reported, the major being the lignans, tannins, polyphenols, alkaloids, flavonoids,terpenoids and steroids .The following chemical constituents have been isolated from *P niruri*.

2.6.2.1 Chemical Composition

- Lignans (phyllanthin, phylnirurin, hydroxyniranthin, lintetralin, nirurin, phyltetralin, hypophyllanthin, isolintetralin, niranthin, nirurinetin, phyltetrin, hydroxylignans, kinokinina, Nirtetralin, nirphylin, isolariciresinoltrimethyl ether, seco-4-hydroxylintetralin).
- Terpenes (cymene, limonene).
- Triterpenes (lupeol acetate, lupeol).
- Flavonoids (astragalin, quercetin, quercitrin, isoquercitrin, nirutinetin, nirurinetin, kaempferol-4-0-a-L-rhamnoside, eriodictyol-7-a-L-rhamnoside, phyllantus, physetinglucoside, isoquercetin,rutin, nirurin, FG1, FG2, physetin-41-0-b-D-glucoside).
- Lipids (ricinoleic acid, linoleic acid, dotriacontanoic acid).
- Benzenoids (phyllester, methyl-salicylate, 4-methoxy-norsecurinine).
- Steroids (beta-sitosterol, estradiol, 24-isopropil-cholesterol).
- Alkanes (triacontanal, tricontanol).
- Alkaloids (phyllanthin, nirurin).
- Pyrrolizidine alkaloids (norsecurinine, 4-methoxy-norsecurinine, nor-ent-securinine).
- Indolizidine Alkaloids (nirurin, phyllanthin, phyllochrysine).

- Methyl-salicylate
- Tannins
- Vitamin C

2.6.3 *Coccinia Indica* (Ivy gourd)

Coccinia indica Wight and Arnold (Cucurbitaceae) commonly known as 'Ivy gourd' and 'Kundru' in Hindi is a perennial tendril climber, available in wild and cultivated form. It is the native of Central Africa, India and Asia and distributed naturally in China, Tropical Asia, India, Australia and Africa (Syed Zeenat Shaheen, 2008).. Every part of this plant is valuable in medicine for ring worm, psoriasis, small pox, scabies (Perry, 1980) and other itchy skin eruptions and ulcers. *C. indica* has antidiabetic hypoglycemic, anti-inflammatory, analgesic hepatoprotective antioxidant, antilitihic), and antimutagenic activities.

2.6.3.1 Chemical Composition of the fruit

Alkaloids, Steroids, Tannins, Saponins, Ellagic acid, phenols, Glycosides, Triterpenoids, Flavonoids.

2.6.4 *Syzgium samanaragenes* (Wax apple)

The plant belongs to the species Myrtaceae, native to Philippines, India, Indonesia, Malaysia and Samoa, and widely cultivated in the tropics (K. M. Moneruzzaman, 2006). English common names include wax apple, love apple, java apple, Royal Apple, bell fruit, Jamaican Apple, water apple, mountain apple, cloud apple, wax jambu, rose apple, and bell fruit The plant has been reported to have antibacterial (Santos, 1981), anti-diabetic .

2.6.4.1 Chemical Composition

Compounds previously isolated from this plant include mearnsitrin, 2_-C-methyl-5_-O-galloylmyricetin-3-O-α-lrhamnopyranoside, desmethoxymatteucinol ,4_,6_-dihydroxy-2_-methoxy-3_,5_-dimethylchalcone, methyl 3-epi-betulinate, oleanolic acid, jacoumaric acid, ursolic acid, arjunolic acid), samarangenin A and samarangenin. (Evangeline C. Amor)

2.6.5 *Brassica Nigra* (Mustard)

When taken internally, the seeds are laxative, mainly because of the mucilage they produce, but only small doses are advised as they may inflame the stomach. The stimulating, diaphoretic action can also be utilized for fevers, colds and influenza. But this well-known herb has its primary medicinal use as a stimulating external application. The rubefacient action causes a mild irritation to the skin, stimulating the circulation in that area to relieve muscular and skeletal pain. An infusion or poultice of Mustard will aid in cases of bronchitis and pleurisy, and it is often taken as a tea, or ground and sprinkled into a bath.

2.6.5.1 Chemical Compsition

The seeds are high in essential oils as well as plant sterols (E.A.Hussein, 2007) such as brassicasterol, campesterol , sitosterol, avenasterol and stigmasterol. They also contain sinigrin, myrosin, erucic, eicosenoic, oleic and palmitic acids.Mustard seeds contain flavonoid antioxidants such as carotenes, zeaxanthin and lutein. In addition, the seeds have small amount of vitamin anti-oxidants such as vitamin A, C and vitamin K. The seeds are an excellent source of tocopherol-γ. Vitamin E is a powerful lipid soluble antioxidant, required for maintaining the integrity of cell membrane of mucus membranes and skin by protecting it from harmful oxygen free radicals. Mustards are rich source of many health benefiting minerals. Calcium, manganese, copper, iron, selenium and zinc are some of the minerals especially concentrated in these seeds.

2.6.6 *Piper nigrum* (Pepper)

Black pepper (*Piper nigrum*) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. Peppercorns are often categorised under a label describing their region or port of origin. Two well-known types come from India's Malabar Coast: Malabar pepper and Tellicherry pepper. Black Pepper (or perhaps long pepper) was believed to cure illness such asconstipation, diarrhea, Limonene, Safrole, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches (Nobuji et al , 1986).

2.6.6.1 Chemical composition

The fruit contains volatile oil, piperine and a resin.The essential oil is composed of various chemical constituents and includes the following; a-thujone, a-pinene, camphene, sabinene, b-pinene, a-phellandrene, myrcene, limonene, caryophyllene, b-farnesene, b-bisabolene, linalool and terpinen-4-ol.

3.6.7 *Averhoa Bilimbi* (Irunbampuli)

The bilimbi, *Averrhoa bilimbi,* L., (Oxalidaceae), is closely allied to the carambola but quite different in appearance, manner of fruiting, flavor and uses. The only strictly English names are "cucumber tree" and "tree sorrel", bestowed by the British in colonial times. "Bilimbi" is the common name in India and has become widely used. In Malaya, it is called *belimbing asam, belimbing buloh, b'ling,* or *billing-billing.* In Indonesia, it is *belimbing besu, balimbing, blimbing,* or *blimbing wuluh;* in Thailand, it is *taling pling,* or *kaling pring.* n the Philippines, the leaves are applied as a paste or poulticed on itches, swellings of mumps and rheumatism, and on skin eruptions. Elsewhere, they are applied on bites of poisonous creatures. Malayans take the leaves fresh or fermented as a treatment for venereal disease. A leaf infusion is a remedy for coughs and is taken after childbirth as a tonic. A leaf decoction is taken to relieve rectal inflammation. A flower infusion is said to be effective against coughs and thrush.

3.6.7.1 Chemical Composition

Phytochemical screening of fruit extracts yielded flavonoids, saponins and triterpenoids but no alkaloids.

3.6.8 *Piper betel* (Betel Leaf)

The Betel (*Piper betle*) is the leaf of a vine belonging to the Piperaceae family, which includes pepper and Kava. It is valued both as a mild stimulant and for its medicinal properties.

The betel plant is an evergreen and perennial creeper, with glossy heart-shaped leaves and white catkin. The betel plant originated from South and South East Asia (India, Bangladesh and Sri Lanka).In India, betel is used to cure worms. According to

traditional Ayurvedic medicine, chewing areca nut and betel leaf is a remedy for bad breath. They are also said to have aphrodisiac properties.

In Malaysia they are used to treat headaches, arthritis and joint pain. In the Philippines, Thailand, Indonesia and China they are used to relieve toothache. In the Philippines, they are used specifically as a stimulant and was believed to strengthen the teeth and gums. In Indonesia they are drunk as an infusion and used as an antibiotic. They are also used in an infusion to cure indigestion, as a topical cure for constipation, as a decongestant and as an aid to lactation. In Indonesia, betel is also used to cure nosebleeds.

3.6.8.1 Chemical Composition

The active compounds isolated from leaf and other parts are hydroxychavicol, hydroxylchavicol acetate, allypyrocatechol, chavibetol, piperbetol, methylpiperbetol, piperol A and piperol B. Phenol-rich leaves of *P. Betle* show high antioxidant activities.(Nikhil kumar et al, 2010).

3.6.*9 Capsicum annum* (Red Capsicum)

Red capsicums, commonly known as red peppers, are not classified as a vegetable. They are actually a fruit, and belong to the Solanaceae family. The Solanaceae family includes tomatoes, petunias, tobacco and potatoes. In addition to their known nutritional value, the red pigments in Capsicum (chile pepper) are important as sources of non-toxic red dyes; the red pigments are added to many processed foods and cosmetics to enhance their appearance. Certain varieties of *Capsicum annuum* can be "extracted' to isolate red-coloured xanthophylls, an important economical source of red pigments that can replace carcinogenic synthetic red dyes.

3.6.9.1 Chemical composition

The active components in the extracts were carotenoids, glycosides glycolipids and the pungent compounds of the Capsicum fruit are called capsaicinoids (capsaicin and its analogs. The following five have been reported as the major components of most Capsicum species: capsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin. (Richard Cantrill, 2008).

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT MATERIALS

Different parts of the plant such as leafs, seeds were collected from Kerala, Coimbatore and all of them were botanically identified by Tamil Nadu Agricultural University (TNAU) (Table 3.1. and Fig 3.1)

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

SCIENTIFIC NAME	COMMON NAME	PART(S) UNDER INVESTIGATION
Laurus nobilis	Bay leaves	Leaf (Sun dried)
Piper betel	Betel leaves	Leaf (Sun dried)
Brasica nigra	Mustard	Seeds (dried)
Capsicum annum	Capsicum (red)	Fruit (sun dried)
Syzgium samarangenese	Wax jambu	Leaf (Sun dried)
Averrhoa bilimbi	Irubanbuli	Leaf (Sun dried)
Coccinia indica	Ivy gourd	Fruit
Piper nigrum	Pepper	Seeds (dried)
Phyllanthus niuri	Kizharnelli	Leaf (sun dried)

Fig 3.1 Plants selected for investigation

Laurus nobilis

Piper betel

Phyllanthus niuri

Brasica nigra

Capsicum annum

Syzgium samarangenese

Averrhoa bilimbi

Coccinia indica

Piper nigrum

3.2 LIST OF APPARATUS REQUIRED FOR THE EXPERIMENTS

- Centrifuge
- Test tubes
- Mortar and pestle
- Pipettes
- Spectrophotometer – **Beckman coulter DU 530** model.
- Beakers

3.3 SAMPLE PREPARATION

3.3.1 Procedure

Aqueous extract of the plants were prepared fresh as follows:

- Five grams of the fresh dried and powdered samples were taken.
- Then fresh distilled water was taken and boiled to 80 degree centigrade.
- Using mortar and pestle, the plant material were ground with 40ml of boiled distilled water.
- Later the homogenate was centrifuged at 10,000 RPM for 10 minutes.
- Supernatant was collected separately and used as a source of inhibitor for the alpha Glucosidase assay.

3.4 ENZYME PREPARATION

Pure alpha Glucosidase enzyme was purchased from SIGMA- ALDRICH.

Source	:	***Saccaromyces cervisiae*** **(Recombinant)**
Product code	:	G0660
Activity	:	45.6 U/mg
Temperature	:	Stable below 50 degree centigrade. ()
Packet quantity	:	16 mg

About 1 mg of the enzyme was weighed in an electronic balance. The enzyme was diluted with 45.6 ml of phosphate buffer (pH- 6.9) to get 0.2 units enzyme activity.

3.5 ALPHA- GLUCOSIDASE ASSAY:

3.5.1 Principle

P-Nitrophenyl + α-D-Glucoside $\xrightarrow{\text{α- Glucosidase}}$ **α-D-Glucose + p-Nitrophenol**

3.5.2 Conditions

Temperature = 37°C

pH = 6.9,

A_{400nm}, Light path = 1 cm

3.5.3 Method (Sigma Aldrich Assay)

Spectrophotometric Stop Rate Determination

3.5.4 Reagents Required

- 0.1M Potassium Phosphate Buffer, pH 6.9 at(Prepare 100 mL in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Adjust to pH 6.9 at 37°C)
- 5 mM p-Nitrophenyl- α-D-Glucoside Solution (PNP-Glucoside) (Prepare 5 mL in deionized water using p-Nitrophenyl α-D-Glucopyranoside)
- 200 mM Sodium Carbonate Solution, (NaCarb) (Prepare 50 mL in deionized water using Sodium Carbonate, Anhydrous)
- α-Glucosidase Enzyme Solution (Prepare a solution containing 0.2 unit/mL of α-Glucosidase in phosphate buffer.)

3.5.5 Procedure

Three tubes were taken and marked as blank, sample and test. The tubes were added with the reaction mixture as follows:-

Table 3.2 Alpha- Glucosidase assay:

Reagents (in ml)	**B lank** (in ml)	**Test** (in ml)
Buffer (0.1M phosphate buffer)	0.9	0.8
Enzyme (0.1M phosphate buffer containing α Glucosidase with activity 0.2U/ml)	-	0.1
Substrate	0.1	0.1
Incubation at 37^0C for 15 mins		
Stopping Solution (0.2M Sodium Carbonate)	3	3

Record the A_{410nm} for both the Test and blank using a suitable spectrophotometer

3.5.6 Calculation

A= ECL

A= Absorbance at 405 nm

E=Molar ellipticity of p-Nitrophenyl ($1.85*10^5$)

C= Concentration

L= Path length (1 cm)

Enzyme assay was carried out to check the activity of the enzyme.

3.6 α Glucosidase Inhibitor Assay:

3.6.1 Principle

α-Glucosidase inhibitors are saccharide that act as competitive inhibitors of α-Glucosidase enzymes in the brush border of the small intestines. α-Glucosidase hydrolyzes complex carbohydrates to glucose and other monosaccharides in the small intestine. Inhibition of **α-Glucosidase** reduces the rate of digestion of carbohydrates. Less glucose is absorbed because the carbohydrates are not broken down into glucose molecules.

3.6.2 Reagents Required

- p- Nitrophenyl α- d- Glucoside (5 mM)
- Purified α – D- Glucosidase enzyme
- Sodium Carbonate (200mM)
- Potassium Phosphate Buffer (pH 6.9) (0.1M)

3.6.3 Procedure

Four test tubes were labeled accordingly as control(C), blank (B), Inhibitor control(IC), Inhibitor test (IT). Reaction mixtures were added to the tube as follows

Table 3.3 α Glucosidase Inhibitor Assay:

Reagents (in ml)	**B (in ml)**	**C (in ml)**	**IC (in ml)**	**IT (in ml)**
Buffer	0.9	0.8	0.7	0.6
Enzyme	-	0.1	-	0.1
Inhibitor extract	-	-	0.2	0.2
Pre-incubation for 10 minutes at 37 ° c				
Substrate	0.1	0.1	0.1	0.1
Incubation for15 minutes at 37 ° c				

Record the A400nm for both the Test and blank using a suitable spectrophotometer.

3.6.4 Calculation

$$\% \text{ inhibition} = \frac{(IC-IT) * 100}{IC}$$

IC - Inhibitor Control

IT – Inhibitor Test

3.7 AMMONIUM SULPHATE PRECIPITATION: (Thai.J Pharm sci ,2010)

3.7.1 Principle

Ammonium Sulfate Precipitation is a classic first step to fractionate proteins by causing perturbations in the solvent with respect to ionic strength. The solubility of the protein varies according to the ionic strength of the solution, and the salt concentration. At low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. ionic strength) called as salting in. As the salt concentration is increased further, the solubility of the protein will begin to decrease. At sufficiently high salt concentration, the protein will be completely precipitated out (salting out). As more choices of inexpensive and quality resins are commercially available for precipitation, steps are typically limited to one or two initial cuts in the beginning of purification or simply used to concentrate the proteins. The major advantage to ammonium sulphate precipitation is that it easily causes the reversible precipitation of the protein and is non-denaturing to the protein structure.

3.7.2 Materials required

- Inhibitor sample solution
- Ammonium sulfate salt
- Ice pack

3.7.3 Procedure

- Measure the volume of the protein solution, pour it in to a beaker with a magnet bar and place it in an ice bath or at 4°C on a stirrer.
- Calculate the required amount of ammonium sulphate from the standard table.
- Start stirring the solution and add salt to it in small portions, allow salt to dissolve before adding the next portion. When all the salt has been added, take beaker off stirrer and leave it at 4°C for some time, ideally overnight.
- As a rule, you can store protein this way for a very long time. This is true for the majority of proteins, but not for all of them.

3.8 SEPARATION OF COMPOUNDS BY THIN LAYER CHROMATOGRAPHY (TLC): (Egon Stahl, 1988)

3.8.1 Introduction

Thin layer chromatography (TLC) called planar chromatography – are, like all chromatographic techniques, based on a multistage distribution process. This process involves a suitable adsorbent (the stationary phase), solvents or solvent mixtures (the mobile phase or eluent), and the sample molecules. For thin layer chromatography, the adsorbent is coated as a thin layer onto a suitable support (e.g. glass plate, polyester or aluminium sheet). On this layer, the substance mixture is separated by elution with a suitable solvent. The principle of TLC is known for more than 100 years now [1].The real break-through of TLC as an analytical method, however, came about 35 years ago as a consequence of the pioneering work of Egon Stahl[2].After sometime of stagnation thin layer chromatography has gained increasing importance as an analytical separation technique, which is probably due the effects of instrumentalisation and automatisation[3].At the same time the applicability of thin layer chromatography was enhanced by the development of new adsorbents and supports.

3.8.2. Principle

In paper chromatography, the stationary phase is a specially manufactured porous paper. The samples are added to one end of the sheet of paper and dipped into the liquid or mobile phase. The solvent is drawn through the paper by capillary action and the molecules are distributed by partition between the mobile and stationary phase. The partition coefficient, k, similar to the distribution coefficient for extraction, is the equilibrium constant for the distribution of molecules between the mobile phase and the stationary phase. This equilibrium separates the components. Different inks and dyes, depending on their molecular structures and interactions with the paper and mobile phase, will adhere to the paper more or less than the other compounds allowing a quick and efficient separation. TLC works on the same principles. In thin-layer chromatography, the stationary phase is a polar absorbent, usually finely ground alumina or silica particles. This absorbent is coated on a glass slide or plastic sheet creating a thin layer of the particular stationary phase. Almost all mixtures of solvents can be used as the mobile phase. By manipulating the mobile phase, organic compounds can be separated.

3.8.3 Materials required

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

3.8.4 Procedure

- Prepare developing chamber by outfitting beaker with a folded piece of filter paper and a watch glass for a lid. Add developing solvent to chamber to a depth of no more than 1/2 cm.
- Obtain a pre-cut alumina coated TLC plate being careful to only hold by edges. Lightly draw a pencil line approximately 1 cm from the end of the plate. With a pencil, lightly mark for each sample.
- Draw up some of the standard solution with a micropipette. Hold this pipette vertically over the plate. Aim the pipette over the pencil line. Touch the tip of the micropipette onto the surface of the alumina-coated plate to dispense an approximately one mm-sized spot of the solution. If the spot is not visible, make another application of the solution on top of the original spot. The spot should be small and concentrated.
- Allow solvent in spot to evaporate. Place "spotted" TLC plate in developing chamber being sure that pencil line with spots is above the level of the solvent in the developing chamber. Light pencil line micropipette with solution 1 mm spot.
- Allow developing solvent to migrate approximately ¾ up the length of the TLC plate. Remove plate from chamber and immediately mark the position to which the solvent rose.
- Allow the plate to air dry. The spots are visible but will fade with time. Circle the spots with pencil. Calculate the RF's for each spot. Staple TLC to data sheet. Draw a scale model of TLC plate in notebook.

3.8.5 Solvent system used for separation of compounds

- Chloroform: ethanol , 9:1
- Ethyl acetate : Ethanol : Water , 5:1:5

3.8.6 Preparative Thin Layer Chromatography

The silica gel thickness applied on the plate was 150μm. The conditions and the procedure are same as TLC.

3.9 FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY

3.9.1 Principle

An FT-IR Spectrometer is an instrument, which acquires broadband NIR to FIR spectra. Unlike a dispersive instrument, i.e. grating monochromator or spectrograph, an FT-IR Spectrometer collects all wavelengths simultaneously. This feature is called the Multiplex or Felgett Advantage. But for the purists, an FT-IR (Fourier Transform Infrared) is a method of obtaining infrared spectra by first collecting an interferogram of a sample signal using an interferometer, and then performing a Fourier Transform (FT) on the interferogram to obtain the spectrum. An FT-IR Spectrometer collects and digitizes the interferogram, performs the FT function, and displays the spectrum. Therefore, IR spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With intuitive software algorithms, infrared is an excellent tool for quantitative analysis.

3.9.2 Procedure

Inhibitor samples were sent to IIT Bombay for FTIR investigation.

3.10 LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

3.10.1 Principle

Liquid chromatography–mass spectrometry (**LC-MS**, or alternatively **HPLC-MS**) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications, which has very high sensitivity and selectivity. Generally, its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture)

3.10.2 Procedure

Inhibitor samples were sent to IIT Bombay for LCMS.

RESULTS AND DISCUSSIONS

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 INTRODUCTION

This chapter deals with detailed cross-referenced data and interpretation and discussion of the obtained data with relevant comparisons from literature. Data from initial screening of plants for alpha-glucosidase inhibition, Dixon plots, IC50 values, ammonium sulphate precipitation, TLC, PTLC, up to the final characterisation step including FTIR and LC-MS spectra has been discussed.

4.2 α- GLUCOSIDASE INHIBITION ASSAY:

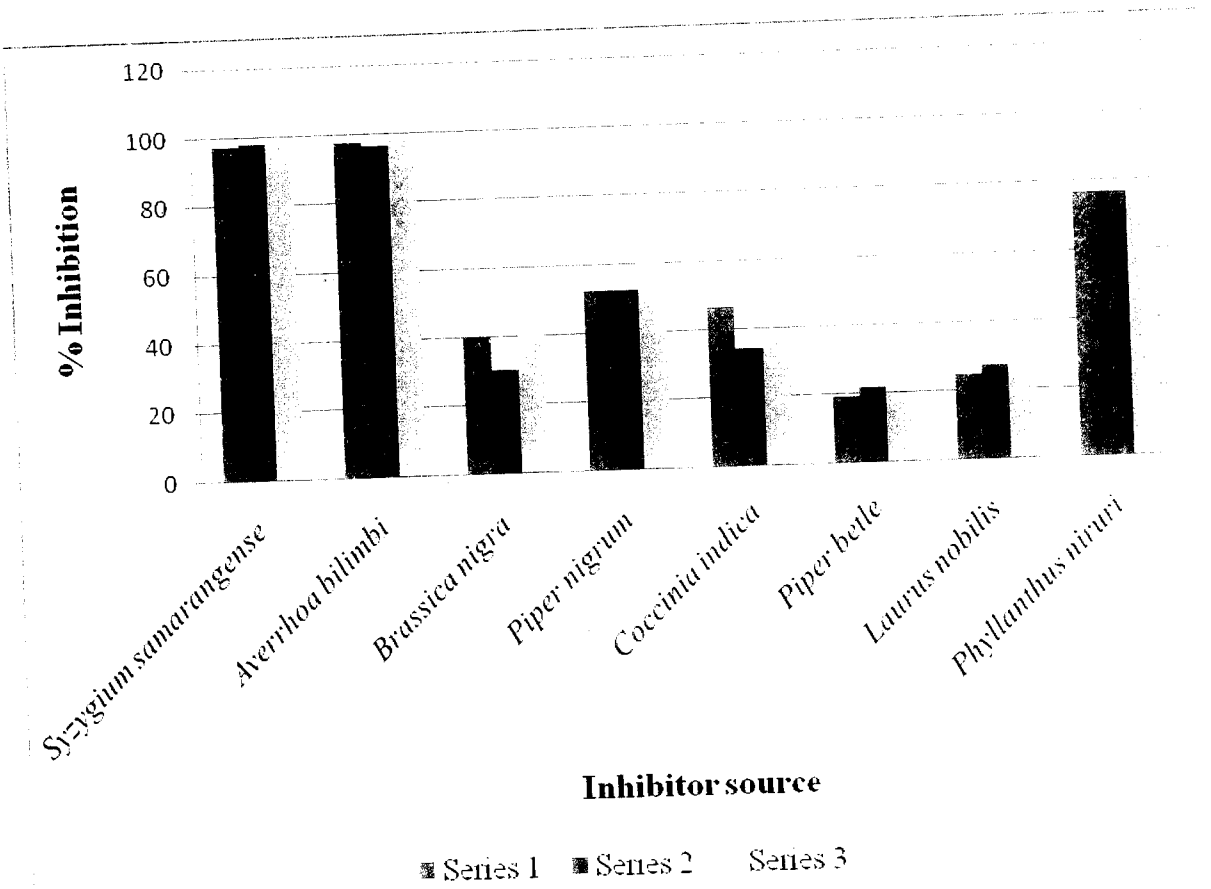
The α- glucosidase inhibitory assay were performed for for the various medicinal plants which were found to have anti-diabetic property. The recombinant α-Glucosidase (pure enzyme) from *Saccharomyces cerevisiae* (pure enzyme) in lyophilised powder form was employed for the inhibitory assay. The inhibitory assay was performed by PNPG method. Totally 9 medicinal plants namely *Syzygium samarangense* (Wax apple), *Averrhoa bilimbi*, *Phyllanthus niruri* (keezharnelli), *Brassica nigra* (Mustard-seeds), *Coccinia indica* (Ivy gourd - fruit), *Piper nigrum* (Black pepper - seeds), *Capsicum annuum* (Red capsicum - fruit), *Laurus Nobili* (bay leaf), and *Piper betle* (betel leaves) were screened, out of which 3 showed significant inhibition against α- Glucosidase.

The results of the assay implies that the aqueous extract of ***Syzygium samarangense*** (Wax apple) leaves showed the maximum inhibition of **97.62%** and this was followed by the aqueous leaf extract of ***Averrhoa bilimbi*** (Bilumbi) which recorded an inhibition of **97.33%.** Since *Syzygium samarangense* and *Averrhoa bilimbi* showed significant inhibition against α-Glucosidase these two plants were taken for further studies. The inhibition showed by the various plant extracts are tabulated in Table 4.1 and in Fig 4.1.

Table 4.1. % Inhibition showed by various plant extracts

PLANT NAME	% INHIBITION
Syzygium samarangense (Leaf)	97.62
Averrhoa bilimbi (Leaf)	97.33
Phyllanthus niruri (Leaf)	76.68
Brassica nigra (seeds)	41.67
Coccinia indica	39.84
Piper nigrum (seeds)	55.83
Capsicum annuum	38.34
Laurus nobilis (Leaf)	25.72
Piper betle	20.37

Fig 4.1 Comparison of % inhibition showed by various medicinal plant extracts



Syzgium aromaticum, a commonly used spice and which finds use as folk medicine for diabetes has been reported to have alpha-glucosidase inhibition with an IC50 value 10(-3) M 10mg/ml by Toda et al. (2000) ***Syzgium cumini L*** has been reported to have alpha-amylase inhibitory activity by Karthic et al. (2008) and several other observations have concluded the same. Thus, it can be inferred that the ***Syzgium*** family has a good potential as natural inhibitors of dietary enzymes. This is in agreement with the results obtained in this study which shows highest inhibition in ***Syzgium samarangenese*** reported to be 97.62%. Literature does not show comparable results for ***Syzgium samarangenese*** indicating that it may be a hitherto un-investigated and efficient plant source for alpha glucosidase inhibition.

Brassica nigra was reported to have zero alpha-glucosidase inhibition in the results reported by Ahmad Gholamhoseinian et al (2008), in a comparable assay but using acarbose showing 51% inhibition as control. With a control lacking enzyme and having only the inhibitor, the results obtained for ***Brassica nigra*** showed 41.67% inhibition, the two sets of data are in reasonable concurrence.

Piper nigrum was reported to have 5+ 0.3% in the same research paper (acarbose showing 51% inhibition used as control) as against 55.83% reported in Table 4.1 proving the data conclusive over comparable assays.

Averrhoa bilimbi and ***Coccinia indica*** have not been investigated for alpha-glucosidase inhibitory property, though they are proven to have anti-hyperglycemic action (Polyxeni Alexiou et al 2000) and ***Averrhoa bilimbi*** showed a statistically significant inhibitory activity with good precision in triplicates, hence indicating that the anti-hyperglycemic effect might be due to alpha glucosidase inhibtion. ***Coccinia indica*** though known to be anti-diabetic may exhert its anti-hyperglycemic effect via alternate mechanisms since the alpha-glucosidase inhibition percentage was found to be relatively insignificant over a series of repeated assays.

4.3 DIXON PLOT

Dose dependent study was carried out for the 4 plants which showed significant inhibition against α-Glucosidase. Dixon plot was plotted (i.e) with varying extract volume on x axis and their respective % inhibition on y axis. The Dixon plot was plotted so as to compare the IC_{50} values of inhibition (IC_{50} values as shown in Table 4.6 and Fig 4.6). IC_{50} refers to the concentration of the inhibitor required to inhibit 50% activity of the enzyme. The results are presented in Tables 4.2 to 4.5 and in Fig 4.2 to 4.5

Table 4.2 Inhibitor assay for *Syzygium samarangense*

Extract Volume (in µl)	% Inhibition
10	30.24
25	65.56
50	96.52
75	97.35
100	97.82

Fig 4.2 Extract volume vs % Inhibition of *Syzygium samarangense*

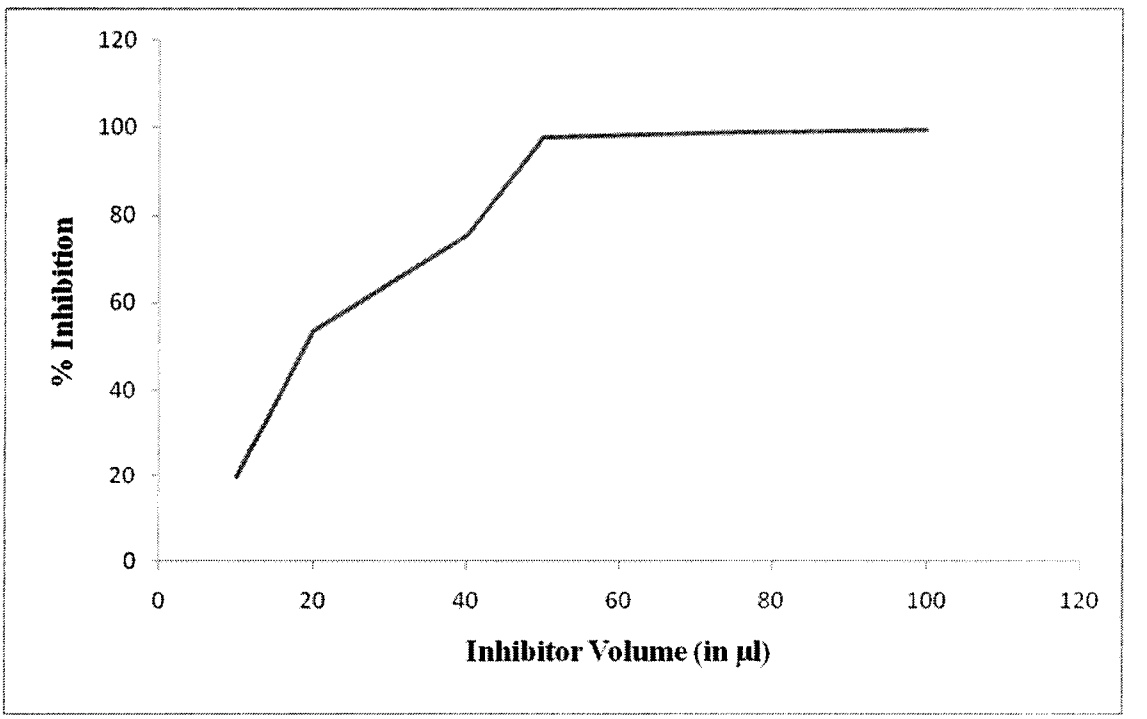


Table 4.3 Inhibitor assay for *Averrhoa bilimbi*

Extract Volume (in µl)	% Inhibition
25	20.56
50	40.24
75	65.52
100	97.35
150	97.85

Fig 4.3 Extract volume vs % Inhibition of *Averrhoa bilimbi*

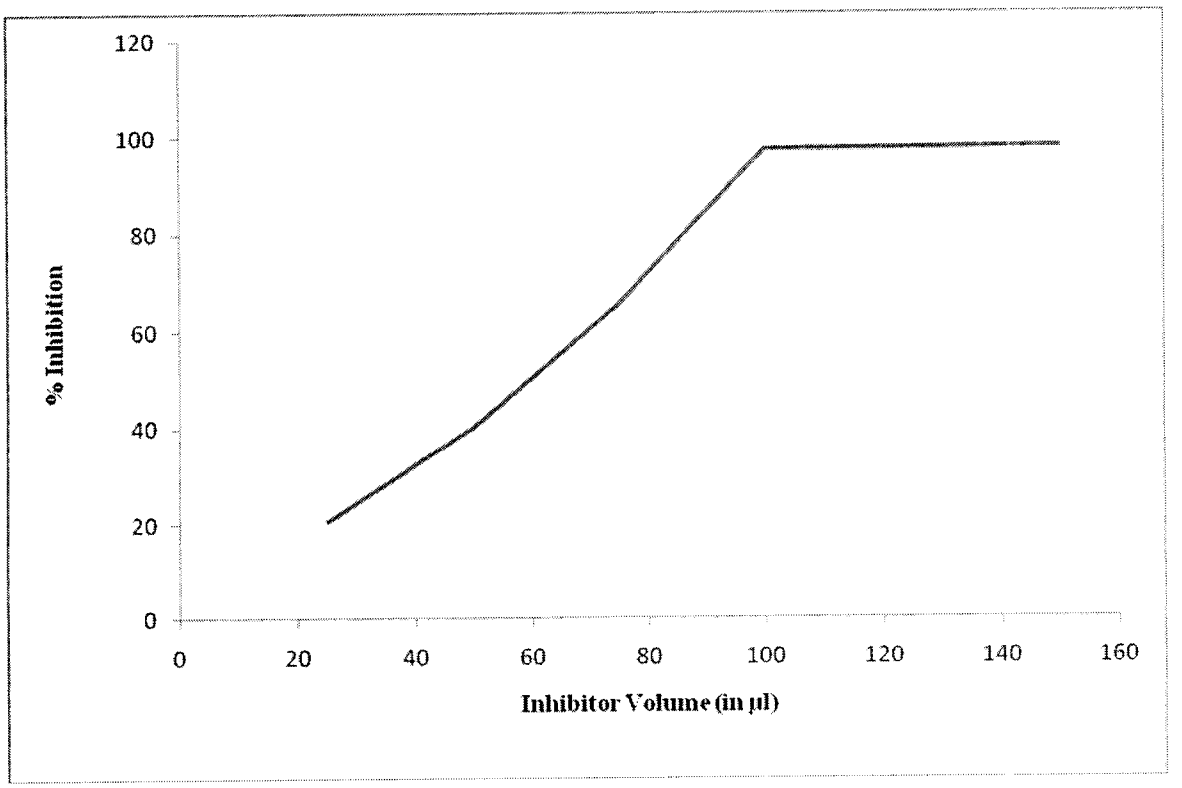


Table 4.4 Inhibitor assay for *Piper nigrum*

Extract volume (in µl)	% Inhibition
50	No inhibition
100	15.67
150	30.12
200	55.31
250	60.72
300	62.43

Fig 4.4 Extract volume vs. % Inhibition of *Piper nigrum*

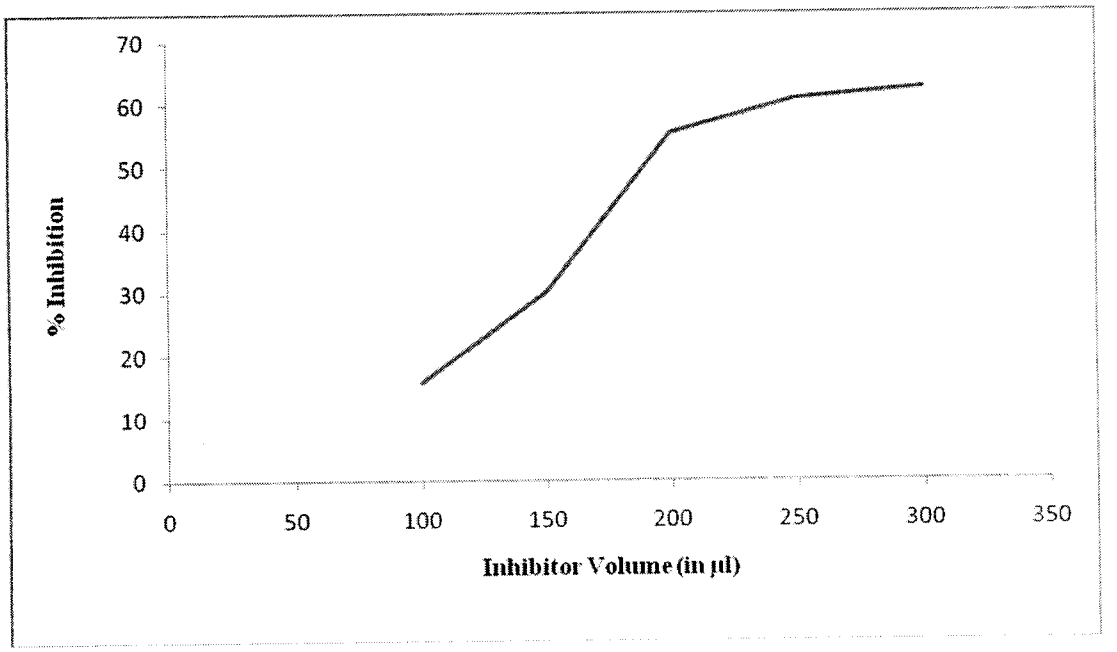


Table 4.5 Inhibitor assay for *Brassica nigra*

Extract volume (in µl)	% Inhibition
50	40.35
100	55.75
150	62
200	76.68
250	78
300	82.23

Fig 4.5 Extract volume vs. % Inhibition of *Brassica nigra*

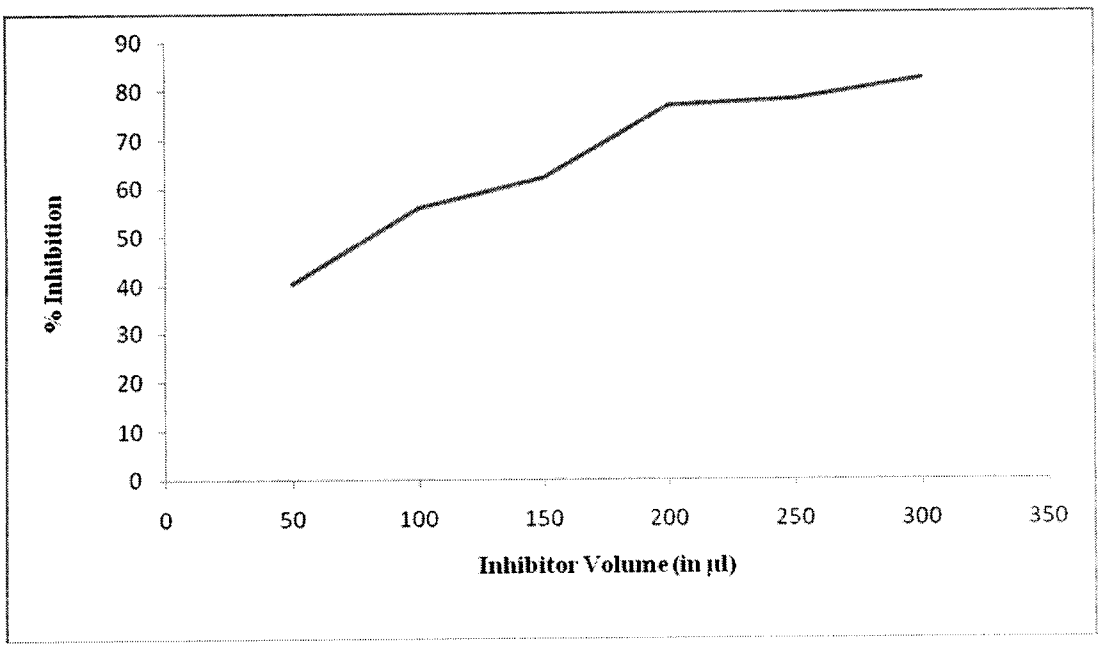
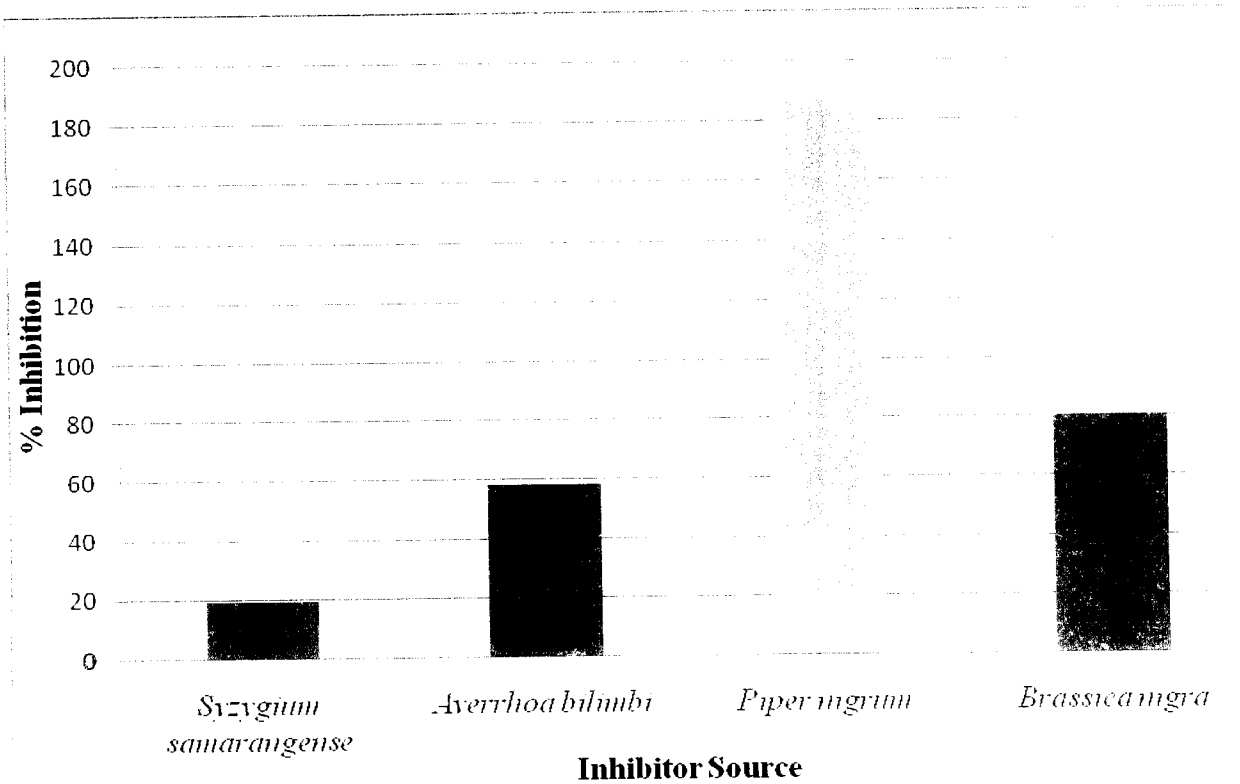


Table 4.6 IC_{50} values of α-Glucosidase inhibitors from various plant extracts

Inhibitor Source	IC_{50} values (in µl)
Syzygium samarangense (Leaf)	19.3 (2.41 mg/ml)
Averrhoa bilimbi (Leaf)	58.2 (7.27mg/ml)
Piper nigrum (seeds)	190 (23.75 mg/ml)
Brassica nigra (seeds)	80 (10mg/ml)

Fig 4.6 Comparison of IC_{50} values of various plant extracts



Guava leaf extract, a plant well known for alpha-glucosidase inhibition has been reported to have an IC_{50} value of 2.1 mg/mL for maltase by Yoriko Deguchi et al. (2010). The IC_{50} values for the plants investigated in this study are tabulated and *Syzgium samarangenese* was found to have an IC_{50} value comparable to the one reported for guava (2.41 mg/ml), which leads to the inference that it is a promising plant in terms of alpha-glucosidase inhibitory activity. Additionally, other members of the Syzgium family have been reported to have alpha amylase activity as well, making this plant a likely candidate as a versatile plant source that may potentially inhibit not only alpha-glucosidase but also alpha-amylase thus acting as an effective herbal preparation for a natural therapy for hyperglycemia.

4.4 AMMONIUM SULPHATE PRECIPITATION

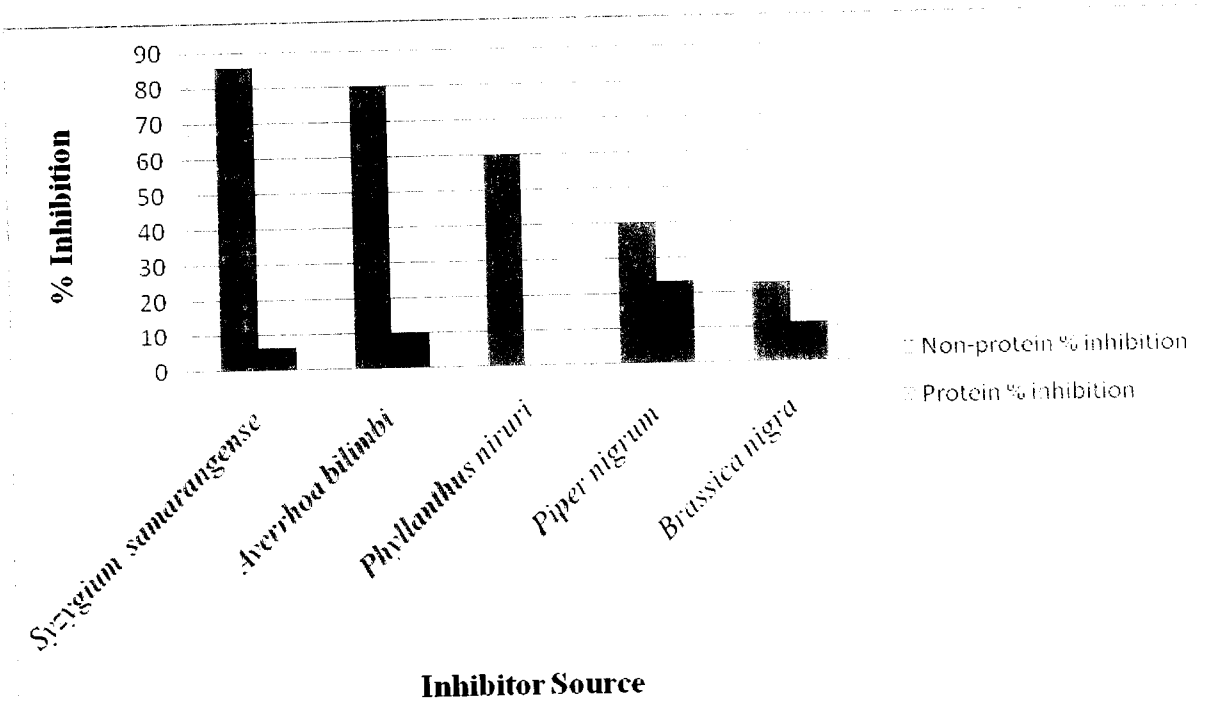
Aqueous extract of the plants which showed significant inhibition against α-Glucosidase were subjected to ammonium sulphate precipitation to check the nature of the inhibitor (i.e.) whether the proteinaceous or non-proteinaceous part is responsible for inhibition. The results of Ammonium sulphate precipitation are presented in Table 4.7 and Fig 4.7

It was inferred from the results that the supernatant which contains the low molecular weight non-proteinaceous part had % inhibition similar to the crude extract in the case of *Syzygium samarangense* and *Averrhoa bilimbi*. Inhibition studies carried out prove that the low molecular weight fraction has greater inhibitory action on alpha glucosidase as against very poor inhibition by the high molecular weight protein fraction. Hence it indicates that non-proteinaceous content in the supernatant might contain polyphenols which are responsible for inhibition.

Table 4.7 % Inhibition of protein and non protein fractions after Ammonium sulphate precipitation

PLANT NAME	NON PROTEIN % INHIBITION	PROTEIN % INHIBITION
Syzygium samarangense (Leaf)	85.65	6.23
Averrhoa bilimbi (Leaf)	80.23	10.25
Phyllanthus niruri (Leaf)	59.78	No inhibition
Piper nigrum (seeds)	40.25	23.54
Brassica nigra (seeds)	22.65	11.23

Fig 4.7 Comparison of % inhibition of protein and non- protein fractions of various plant extracts after Ammonium sulphate precipitation

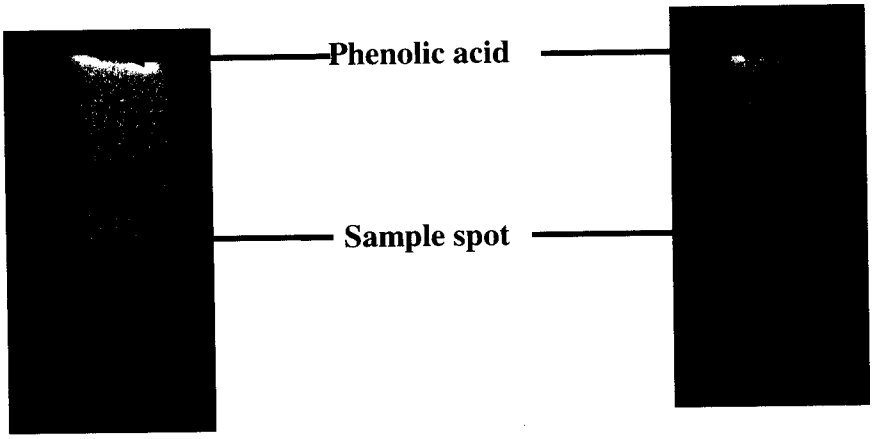


4.5 SEPARATION OF COMPOUNDS BY TLC

The aqueous extract of Syzygium samarangense and Averrhoa bilimbi was taken and Thin Layer Chromatography (TLC) was performed with various solvents. The extract was directly used as a source of inhibitor for α- Glucosidase. TLC plates were sprayed with 0.1% ethanolic solution of Aluminium chloride reagent. In case of Micro TLC *Syzygium samarangense* and *Averrhoa bilimbi* with Chloroform: Acetic acid (9:1) as mobile phase solvent showed flourescence in longwave UV light indicating the presence of phenolic acids (shown in Fig 4.8). TLC was then performed with various inhibitor volumes and it was found that the plates with 50µl inhibitor volume gave better fluorescence (as shown in Fig 4.9)

Macro TLC was then performed with different solvents and it was found that *Syzygium samarangense*, with Chloroform: Acetic acid (9:1) as mobile phase solvent showed flourescence in longwave UV light indicating the presence of phenolic acids (Fig 4.10) whereas *Averrhoa bilimbi* with Ethyl acetate: Ethanol: Water (5:1:5) as mobile phase solvent showed flourescence in longwave UV light indicating the presence of flavonoids (shown in Fig 4.11).

Fig 4.8 Separation of compounds by micro Thin Layer Chromatography

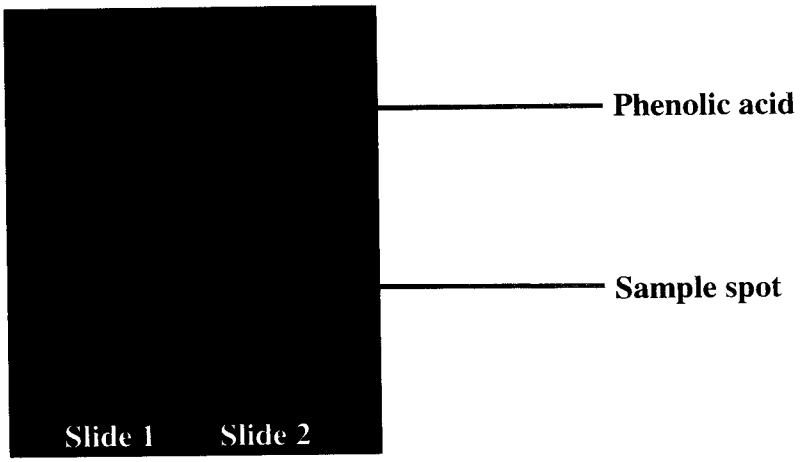


Syzygium samarangense *Averrhoa bilimbi*

Volume of sample spotted : 75µl

Mobile phase: Chloroform: acetic acid:: 9:1

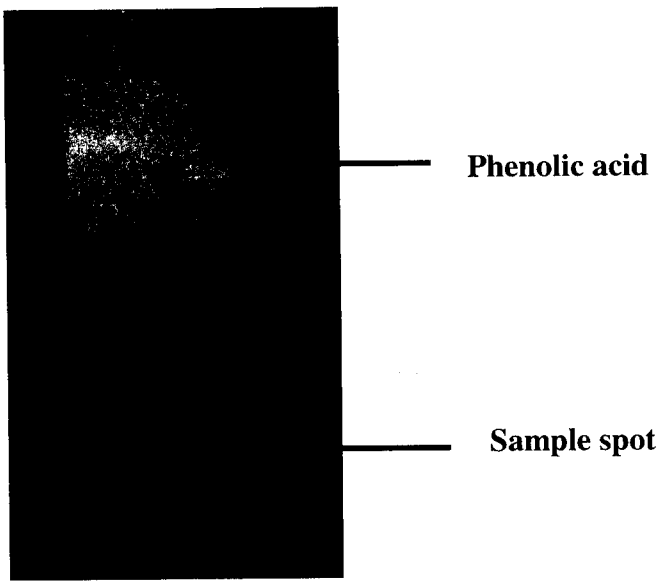
Fig 4.9 Comparison of different volumes of inhibitors - *Syzygium samarangense*



Volume of sample: Slide1: 50µl, **Slide 2:** 75µl

Mobile phase: Chloroform: acetic acid:: 9:1

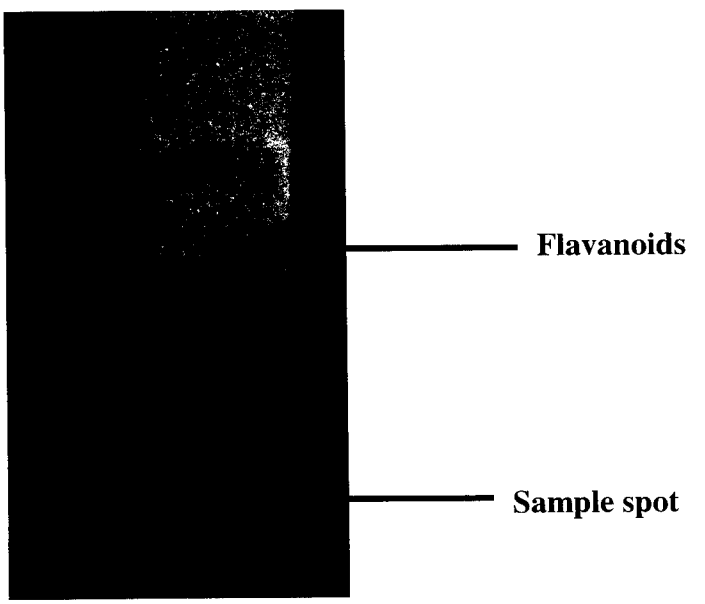
Fig 4.10 Separation of compounds by macro TLC - *Syzygium samarangense*



Volume of sample: 200 µl

Mobile phase used: Chloroform: acetic acid:: 9:1

Fig 4.11 Separation of compounds by macro TLC - *Averrhoa bilimbi*



Volume of sample: 200 µl

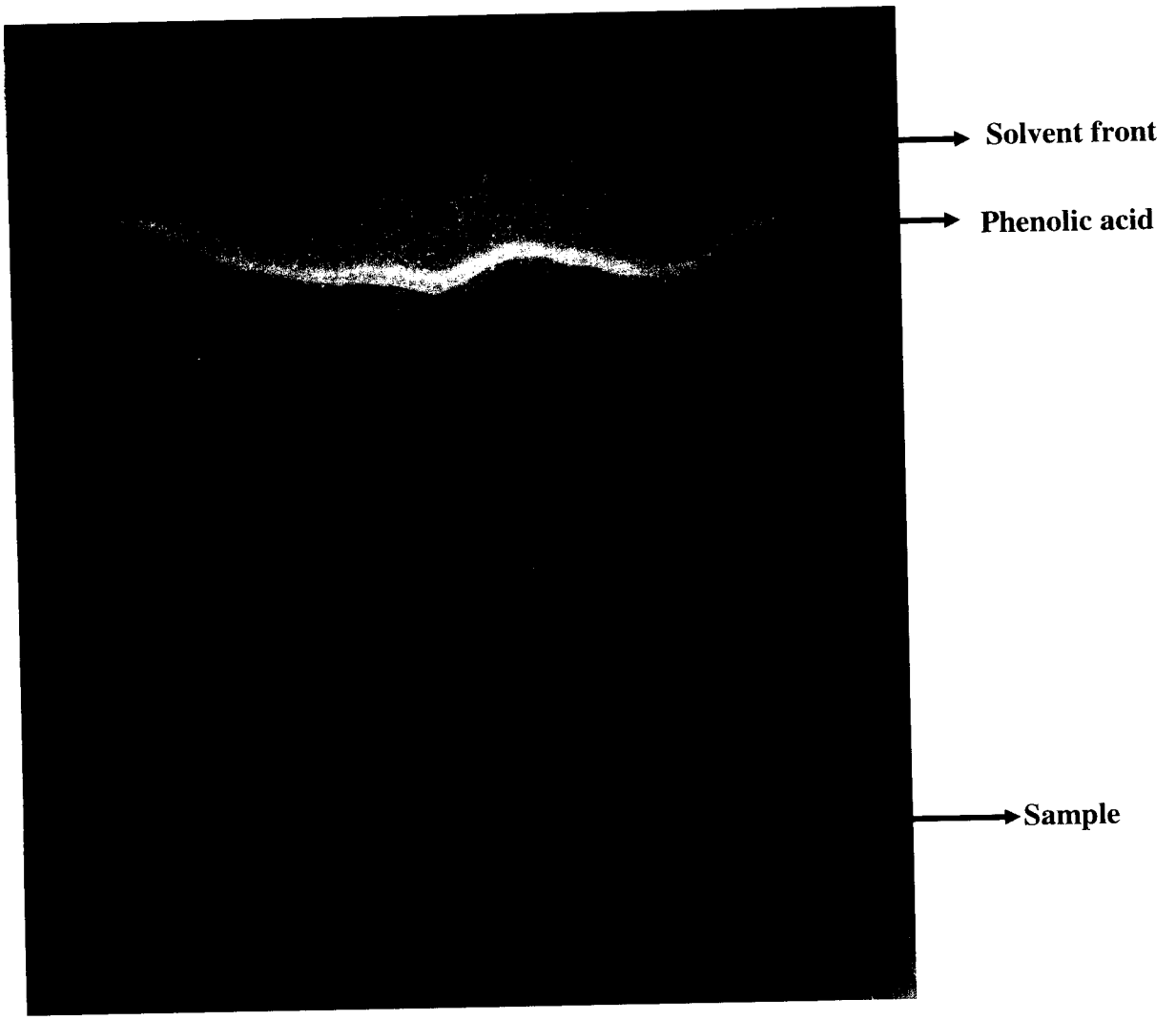
Psidium guajava (guava plant) has been reported to have alpha-glucosidase inhibition activity largely due to its flavanoid content, specifically quercetin, quercetin-3-O-α-L-arabinofuranoside, quercetin-3-O-β-arabinopyranoside, quercetin-3-O-β-D-glucoside as per the report by A M Metwally et al. (2010). Guava is rich in tannins, phenols, triterpenes, flavonoids, saponins, carotenoids and lectins. Much of guava's therapeutic activity is attributed to quercetin and its derivatives. (Zhongjun M. A, et al. 2004) TLC using hot water extract from ***Averrhoa bilimbi*** showed significant flavonoid and anthocyanin spots. ***Syzygium samarangense*** on the other hand showed a rich phenolic acid content.

4.6 PREPARATIVE TLC

Preparative TLC was done to obtain the purified compounds in adequate concentration. It was found that the plate with *Syzygium samarangense* gave a clear bluish green band which indicates that it is rich in phenolic acids (Fig 4.13). The plate with *Averrhoa bilimbi* showed three bands. The topmost band was green in colour indicating the presence of anthocyanins, the middle band in dark orange indicating the presence of phenolic acids followed by a clear band at the bottom indicating the presence of flavonoids (Fig 4.14)

The portion of the silica gel which showed fluorescence was scrapped out and extracted in equal volume of distilled water and was centrifuged at 10,000 rpm for 10 mins. The supernatant was used as a source for inhibitor assay.

Fig 4.12 Separation of compounds by Preparative TLC - *Syzygium samarangense*

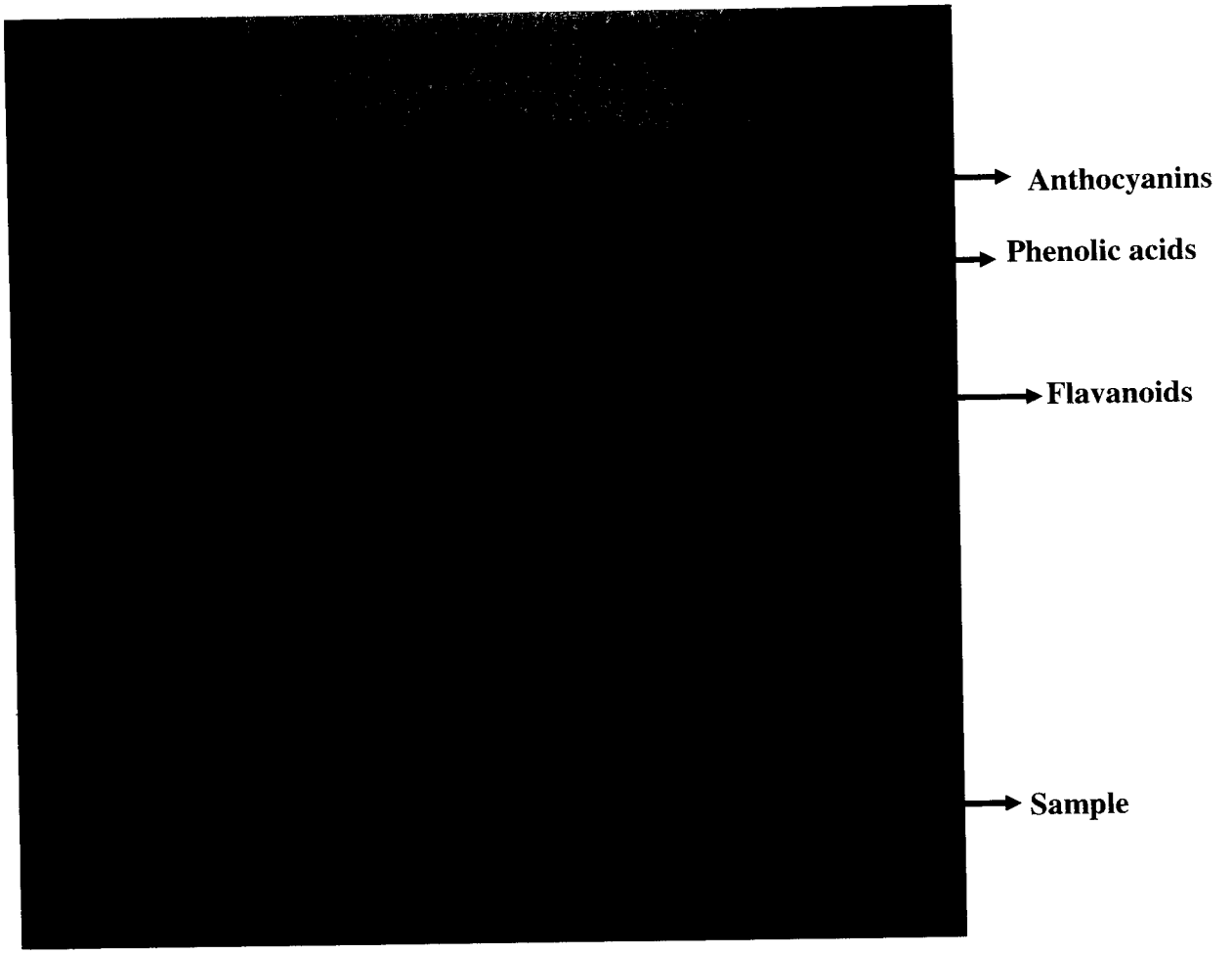


Sample: Crude hot water extract of dried, powdered leaves of *Syzygium samarangense*

Volume of sample loaded: 2250µl (150µl per spot)

Mobile phase: Chloroform: acetic acid:: 9:1

Fig 4.13 Separation of compounds by Preparative TLC - *Averrhoa bilimbi*



Sample: Crude hot water extract of dried, powdered leaves of *Averrhoa bilimbi*

Volume of sample loaded: 2250µl (150µl per spot)

Mobile phase: Ethyl acetate: ethanol: water : 5:1:5

4.6.1. INHIBITORY STUDY USING PURIFIED SAMPLE FROM PTLC

The supernatant obtained by centrifuging the sample scrapped out from the preparative TLC plate mixed with equal volume of distilled water is used as a source for inhibitory assay. The results of the assay (shown in Table 4.8 and Fig 4.15) confirmed that phenolic acids in *Syzygium samarangense* are responsible for **93%** inhibition against α-Glucosidase. In *Averrhoa bilimbi* **flavanoids** recorded **70.9%** inhibition while **anthocyanins** showed **60%** inhibition against α- Glucosidase.

Purified phenolic acid band when assayed for alpha-glucosidase inhibitory activity showed percentage inhibition comparable with that of the crude sample (93%). Thus, it can be reasonably assumed that the phenolic acid fraction was largely responsible for the high levels of inhibition exhibited by the crude sample. Rich phenolic compound repertoire may have beneficial effect on glucose homeostasis. Whilst the results from dietary human interventions are still scarce pertaining to phenolic acids, there is a wealthy of data published with different diabetic animal models. (Bondia-Pons, I et al. 2009).

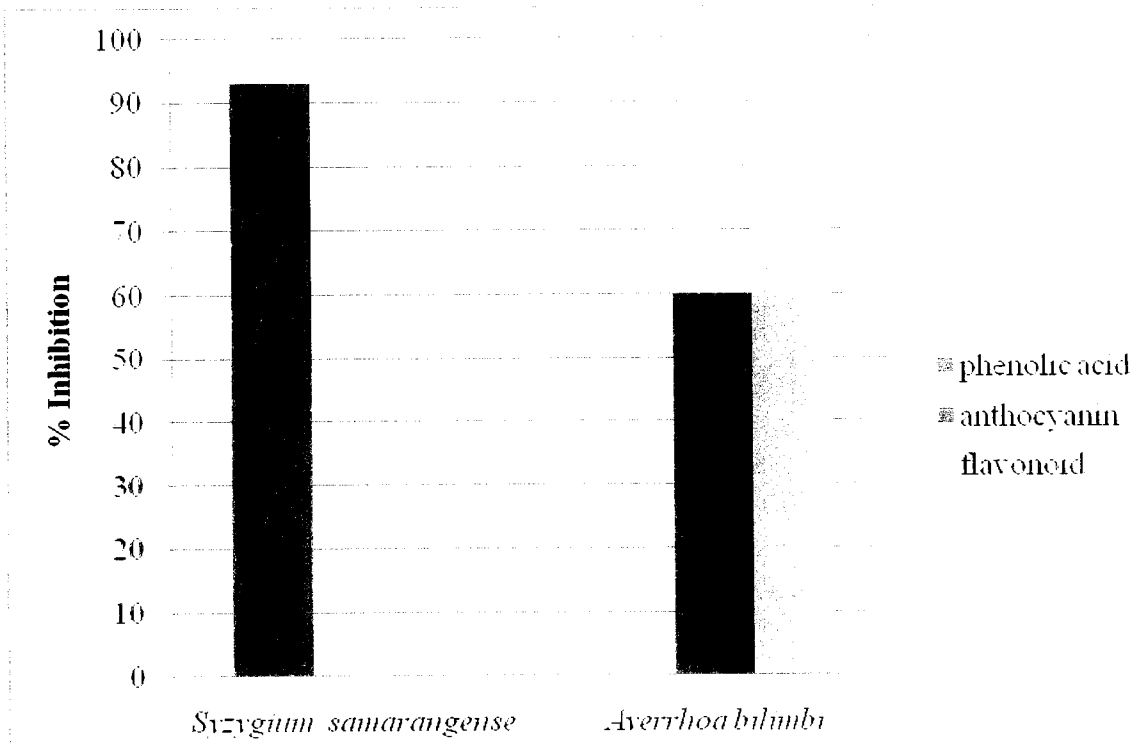
Phenolic compounds such as quercetin, rutin, caffeic acid, gallic acid and chlorogenic acid in *Syzgium cumini* L are very important for anti oxidant and anti-diabetic activities and are responsible for a host of health benefits. (Zhi Ping Ruan et al. 2008).

The Na+-dependent Sodium-Glucose Linked Transporter (SGLT1) -mediated glucose transport was inhibited by chlorogenic, ferulic, caffeic and tannic acids ,quercetin monoglucosides, tea catechins and naringenin. The glucose transport by GLUT2 was inhibited by quercetin, myricetin, apigenin and tea catechins. (Welsch et al. 1989). Thus, polyphenols are attributed to control hyperglycemia via a variety of mechanisms, making them effective and versatile natural source to control hyperglycemia.

Table 4.8 % Inhibition by the purified fractions obtained from Preparative TLC

Plant Name	Active Compound	% Inhibition
Syzygium samarangense	Phenolic Acid	93
Averrhoa bilimbi	Flavanoids	70.9
	Anthocyanins	60

Fig 4.14 Comparison % inhibition showed by the various active compounds



4.7. STORAGE STABILITY

The storage stability of the most effective α-Glucosidase inhibitor (i.e) *Syzygium samarangense* was studied by performing the inhibitory assay at various temperatures for different time periods. The dried powdered leaves of ***Syzygium*** *samarangense* were incubated at 4^0 C (in cold room) , room temperature (i.e) 27^0 C and 37^0 C in an incubator and the α-Glucosidase inhibitory assay was performed after 0, 2, 7, 14 and 28 days. The results are tabulated in Table 4.9

Table 4.9 Storage stability assay for *Syzygium samarangense* showing % inhibition at various temperatures and different time periods

	% Inhibition		
Time (in days)	4^0C	27^0C	37^0C
0	97.25	97.65	96.12
2	95.23	95.40	91.12
7	94.87	94.31	90.11
14	93.45	94.12	89.92
28	93.33	93.42	89.66

The results of the storage stability test shows that the inhibitor is stable and gives maximum inhibition of 97.65% at room temperature (27^0C) followed by 97.25 at 4^0 C on the 0^{th} day. It is also found that α-Glucosidase inhibitory activity decreases as time increases.

4.8 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The PTLC isolate of aqueous *Syzgium samarangenes* extract was sent to IIT BOMBAY to perform FTIR.

The instrument had the following specifications:

4.8.1 Instrument Details:

Make : Nicolet Instruments Corporation, USA

Model : MAGNA 550

Specification : Range - 4000 cm-1 to 50 cm-1

Fig 4.15 Fourier Transform Infrared (FT-IR) Data Plot of PTLC isolate of aqueous *Syzgium samarangenes* extract

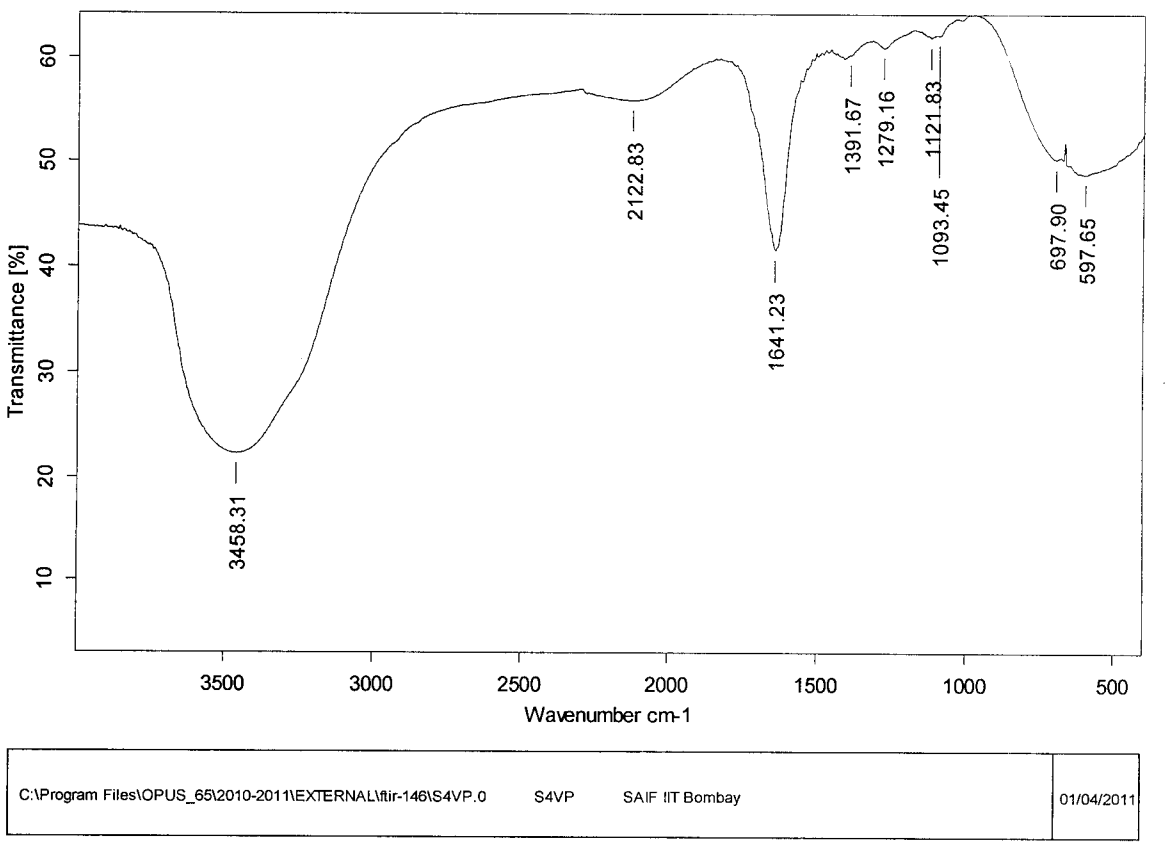


Table 4.10 Functional groups corresponding to the peak transmittance

PEAK (%TRANSMITTANCE)	FUNCTIONAL GROUP
3458.31	Alcohol
1641.23	Non acid carbonyl

4.9 LIQUID CHROMATOGRAPHY – MASS SPECTROMETER (LC- MS)

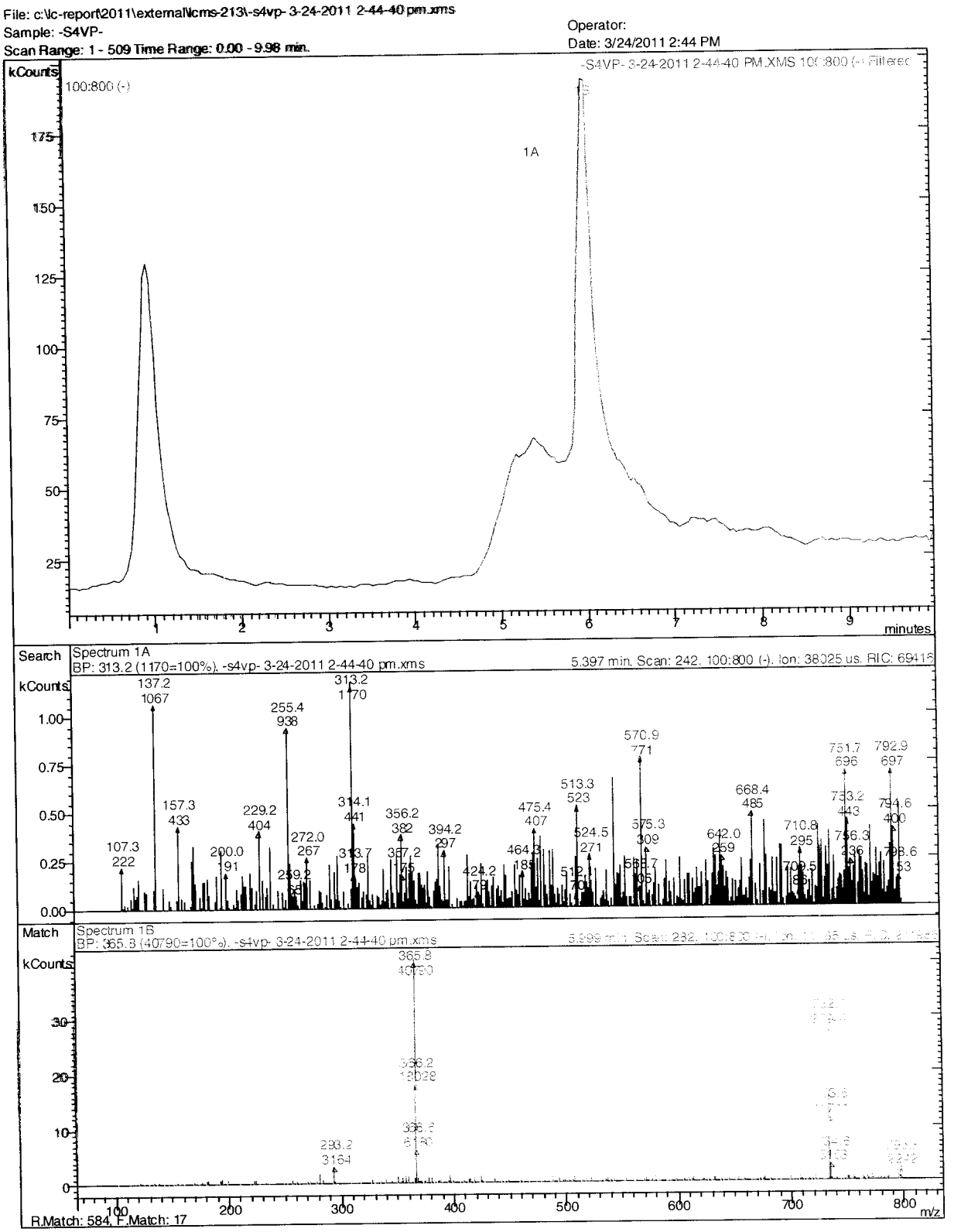
Phenolic acids seem to be universally distributed in the plant kingdom, essential for the growth and reproduction of plants, and are produced as a response to defense against pathogens. The importance of antioxidant activities of phenolic compounds and their possible usage in processed foods as a natural antioxidant has received attention in recent years. These compounds are diverse in structure but are characterized by hydroxylated aromatic rings (e.g., flavan-3-ols) and polymerized into larger molecules.Due to the abundance of different classes of phenolic acids and their diverse chemical properties, a variety of separation and identification methods have been developed using thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas liquid chromatography (GLC).

The PTLC isolate of *Syzgium Samaragenes* extract was sent to IIT BOMBAY to perform LCMS with ESI for every peak. The instrument had the following specifications:

1. Direct Infusion Mass with ESI & APCI Negative & Positive mode ionization, mass ranging from 50 to 2000 m/e
2. LCMS / MS & MS^n ION TRAP
3. HPLC with PDA Detector
4. HPLC PDA Detector – Mass spectrometer

The molecular masses of phenolic acids were assigned by electrospray ionization mass spectrometry.

Fig 4.16 Mass Spectrometry Data Plot of PTLC isolate of *Syzgium Samaragenes* extract



Fragmentation behaviour of phenolic acids was investigated using ion trap mass spectrometry in negative mode. The fragmentation rule in mass spectrum offers the ability to identify the related unknown compounds. The MS, MS^n and UV data together with HPLC retention time (RT) of phenolic acids allowed structural characterization of the compounds present in the aqueous cinnamon extract. The compounds present are listed in Table 4.11

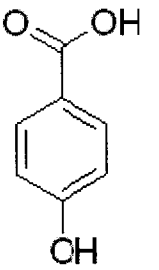
Table 4.11 Validation of LC- MS report

S.No	Compound	Retention time (min)	Molecular weight	MS^1 Parent ion	MS^2 Base peak m/z	MS^2 secondary peaks m/z (intensity)
1	*Ferrulyl quinic acid*	5.999	365.8	365.8	-	-
2	Gallic alcohol	5.397	157.3	157.3	-	-
3	4-Hydroxybenzoic acid	5.397	137.2	137.2	-	-
4	Salicyl alcohol derivative	0.159	185	185	-	-
5	Pinobanksin-3-O-acetate	5.397	313	313	-	-
6	Gallolyted procyanidin	5.999	732.6	732.6	271	255
7	Unidentified compound	0.111	693.6	693.6	-	-
8	Unidentified compound	5.379	255.5	255.6	-	-

4.9.1 Structural Details Of compounds Identified:

1. 4-Hydroxybenzoic Acid

Fig 4.19 Structure of 4-Hydroxybenzoic Acid



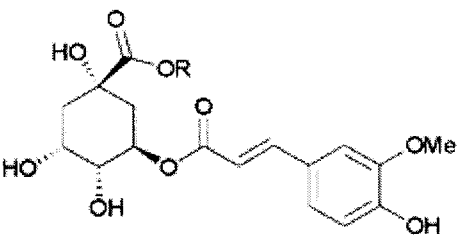
Molecular Formula : $C_7H_8O_3$

Molar mass : 138.12074g/mol

From a comparison with the findings of Isao Murakami et al (2007), it is inferred that the peak corresponding to peak value 137.2 is indicative of 4-hydroxy benzoic acid. The paper cited above shows identical peak value using SSI (sonic spray ionisation).

2. 5-Ferulolylquinic Acid

Fig 4.20 Structure of 5-Ferulolylquinic Acid



R = H 5-O-Feruloylquinic acid

Polyphenol class : Phenolic acids

Polyphenol sub-class : Hydroxycinnamic acids

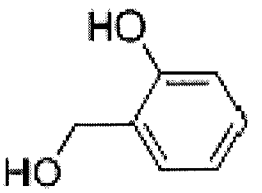
Molecular weight : 368.0

Chemical Formula : $C_{17}H_{20}O_9$

Feruloyl quinic acid was identified by comparison of the MS/MS data to that described in the literature (Deborah H. Markowicz Bastos et al, 2007). The identification of the corresponding compound was based on the search of the molecule together with the interpretation of its MSn fragmentations. At the retention time of 5.397 min, the peak corresponding to 237 was observed which had its MS2

3. **Salicyl Alcohol Derivative**

Fig 4.21 Structure of Salicyl Alcohol



Chemical Formula : $C_7H_8O_2$

Molecular weight : 124.1372

Nominal mass : 124 Da

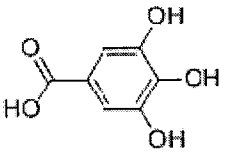
Average mass : 124.1372 Da

Monoisotopic mass : 124.052429 Da

It was concluded that the peak value of 185 corresponds to a derivative of salicyl alcohol, which is based on an LC-MS peak that is indicative of deprotonated salicyl alcohol from the findings of Bala´zs Blazics et al. (2010)

4. **Gallic Acid**

Fig 4.22 Structure of Gallic acid



Chemical Name : Gallic acid

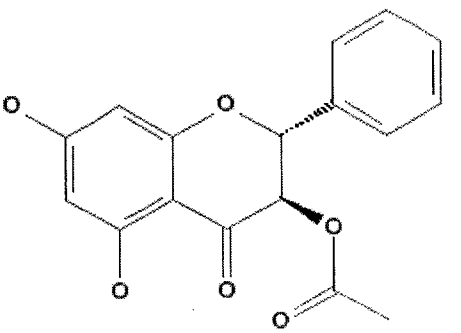
Molecular Formula : $C_7H_6O_5$

Formula Weight : 170.12

Presence of gallic acid derivative was inferred with regard to the peak value 157.3 upon comparison with the LC-MS data reported by Rui Song et al. (2010) Whose m/z value has been shown to be close to169.07 with fragments of peak value 125.01.

5. Pinobanksin-3-O-acetate

Fig 4.23 Structure of Pinobanksin-3-O-acetate



Molecular Formula : $C_{15}H_{12}O_5$

Formula Weight : 272.25

The peak value 313 showed high abundance and was concluded to indicate the phenolic acid Pinobanksin-3-O-acetate upon comparison of the main fragments that were tabulated to be 271 and 253 in the LC-MS findings of S.I. Falcão et al. (2010)

CONCLUSION AND FUTURE PRESPECTIVES

CHAPTER 5

CONCLUSION

Nine plants which were known to have anti-hyperglycaemic activity, but not known to have alpha glucosidase inhibition mechanism were chosen to screen for alpha glucosidase inhibition. Out of these, four best inhibitors were tested for alpha glucosidase inhibition at different concentrations. IC_{50} values for each were calculated. *Syzgium samarangenes* and *Aveorrhea bilimbi* showed the best inhibition and was thus chosen for further studies. These inhibitors were then purified using Thin Layer Chromatography and the results showed presence of anthocyanins ans flavanoids in *Averrohea bilimbi* extract and phenolic acids in *Syzgium samaragenes*. Inhibitory assays with the plant extracts showed higher inhibition with the phenolic acid extract of *Syzgium samarangenes*. The FTIR and LC-MS of Syzgium samarangenes showed the presence of few compounds as inhibitors. The results showed that the extract contains 4-Hydroxybenzoic acid, Sailcyl alcohol derivative, Gallic alcohol, Pinobanksin-3-O-acetate, 5-Ferulolylquinic Acid. Four compounds are yet to be identified. 4-hydoxybenzoic acid is a known alpha glucosidase inhibitor,(Young-In Kwon et al, 2010). Gallic acid is also a known inhibitor (Kati Hanhineva, 2010).So its derivative gallic alcohol is also inferred to b a potential inhibitor in combination with other molecules. 5-ferrulolylquinic acid is known to be an amylase inhibitor (Yusaku Narita and Kuniyo Inouye, 2011) and its inhibition of alpha glucosidase independently or along with other compounds make it an effective molecule in controlling hyperglyceimic condition. Alpha glucosidase inhibition by Pinobanksin-3-O-acetate and salicyl alcohol derivatives has so far not been reported.

FUTURE PLANS

Isolation of individual components identified from detailed LC-MS analysis and study of its efficacy of alpha-glucosidase inhibition followed by comparative *in-vivo* studies of the crude plant extract and isolated compounds, at different concentrations. This can further help in optimising the dosage of the plant extract for case specific alpha glucosidase inhibition thus controlling hyperglycaemia. The storage stability and effective route of administration can also be considered for investigation.

REFERENCES

REFERENCES

1. Adlercreutz , H., Mazur W., (1997) 'Phyto-oestrogens and Western diseases', Ann Med Vol.No.29, pp.95-120.
2. Ahmad Gholamhosinian and Hossein Fallah, (2008) 'Inhibitory effects of some Iranian plant extracts against α-Glucosidase'
3. Arts, I.C.W., Hollman, P.C.H., (2005) 'Polyphenols and disease risk in epidemiologic studies' Am J Clin Nutr, Vol.No.81, pp.317-325.
4. Bala´zs, Blazics, Ildiko´ and Papp, A (2010) 'LC–MS Qualitative Analysis and Simultaneous Determination of Six Filipendula Salicylates with Two Standards' Chromatographia pp 61–67
5. Beckman, C.H (2000) 'Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants' Physiol. Mol. Plant Pathol Vol.No.57 pp.101-110.
6. Biljana Kaurinovic, Mira Popovic and Sanja Vlaisavljevic, (2010) 'Molecules In Vitro and in Vivo Effects of Laurus nobilis L. Leaf Extracts'
7. Chen, W.P., Chi, T.C., Chuang, L.M. and Su, M.J. (2007) 'Resveratrol enhances insulin secretion by blocking K(ATP) and K(V) channels of beta cells' Eur J Pharmacol Vol.No.568 pp.269-277.
8. Chiba, S., Kimura, A. and Matsui, H. ((1983) Agri,Biol.Chem, Vol.No.44, pp.1741-1746.
9. Clifford, M.N (2000) 'Chlorogenic acids and other cinnamates' Nature, 'Occurence, dietary burden, absorption and metabolism' J Sci Food Agric Vol.No.80 pp.1033-43.
10. Cooke, D.W. and Plotnick L (2008) 'Type 1 diabetes mellitus in pediatrics', Pediatr Rev 29 (11) pp.374–384.
11. Cooke, D.W., Plotnick, L. (2008) 'Type 1 diabetes mellitus in pediatrics', Pediatr Rev Vol.No.29 pp.374–384.
12. Coutinho, P.M. and Reilley, P.J. (1994) Protein Engg Vol.No.7 pp.749-760.

13. Crespy, V., Morand, C., Besson, C., Manach, C., Demigne, C. and Remesy, C. (2002) 'Quercetin, but not its glycosides, is absorbed from the rat stomach' J Agric Food Chem Vol.No.50 pp.618-21
14. D'Archivio, M., Filesi, C., Benedetto, R.D., Gargiulo, R., Giovannini, C.and Masella, R. (2000) 'Polyphenols, dietary sources and bioavailability', Ann Ist Super Sanità.
15. Dangles, O., Dufour, C., Manach, C., Morand , C. and Remesy, C. (2001) 'Binding of flavonoids to plasma proteins', Methods Enzymol Vol.No.335 pp.319-333.
16. Day, A.J. and Williamson, G. (2001) 'Biomarkers for exposure to dietary flavonoids: a review of the current evidence for identification of quercetin glycosides in plasma', Br J Nutr Vol.No.86 pp.105-110.
17. Dembinska-Kiec, A., Mykkänen, O., Kiec-Wilk, B. and Mykkänen, H. (2008) 'Antioxidant phytochemicals against type 2 diabetes',Br J Nutr Vol.No.99 pp.109-1.
18. Dufour, C., Loonis, M. and Dangles, O. (2007) 'Inhibition of the peroxidation of linoleic acid by the flavonoid quercetin within their complex with human serum albumin', Free Radic Biol Med Vol.No.43 pp.241-252.
19. Dufour, C., Loonis, M. and Dangles, O. (2007) 'Inhibition of the peroxidation of linoleic acid by the flavonoid quercetin within their complex with human serum albumin', Free Radic Biol Med Vol.No.43 pp.241-52.
20. Duthie, G.G., Pedersen, M.W., Gardner, P.T., Morrice, P.C., Jenkinson, A.M., McPhail, D.B. and Steele, G.M. (1998) 'The effect of whisky and wine consumption on total phenol content and antioxidant capacity of plasma from healthy volunteers' Eur J Clin Nutr Vol.No.52 pp.733-736.
21. Falany, C.N., (1997) 'Enzymology of human cytosolic sulfotransferases', Faseb J Vol.No.11 pp.206-216.
22. Gee, J.M., DuPont, M.S., Rhodes, M.J. and Johnson, I.T. (1998) 'Quercetin glucosides interact with the intestinal glucose transport pathway', Free Radic Biol Med Vol.No.25 pp.19-25.
23. Graf, B.A., Milbury, P.E. and Blumberg, J.B. (2005) 'Flavonols, flavonones, flavanones and human health: Epidemological evidence', J Med Food Vol.No.8 pp.281-290.
24. Greean, F., Edward, Y., Hauri, H.P., Povey, S., Ho, N.W., Pinto, M. and Swallow, D. (1987) 'Gene' , Vol.No.57, pp.101-110.

25. Halliwell, B., Zhao, K. and Whiteman, M. (2000) 'The gastrointestinal tract: a major site of antioxidant action', Free Radic Res Vol.No.33 pp.819-830.
26. Halliwell, B., Zhao, K. and Whiteman, M. (2000) 'The gastrointestinal tract: a major site of antioxidant action', Free Radic Res Vol.No.33 pp.819-830.
27. Harikumar, K.B. and Aggarwal, B.B. (2008) 'Resveratrol: a multi-targeted agent for age-associated chronic diseases', Cell Cycle Vol.No.7 pp.1020-1035.
28. Hermans, M.M.P., Kroos, M.A., van Beeumen, J., Oostra, B.A. and Reuser, A.J.J. (1991) J. BioL Chem., Vol.No.266 pp 13507-13512.
29. Hiromi, K., Nitta, Y., Numatta, C. and Ono, S. (1973) 'Biochem,Biophysics Acta', Vol.No.302 pp.362- 375.
30. Hoefsloot, L.H., Hoogevee-westerveld, M., Kroos, M.A., Van Beeumen, J., Reuser, A.J.and Ostra, B.A. (1990), Biochem.J. Vol.No.272 pp.493-497.
31. Hollman, P.C., Tijburg, L.B. and Yang, C.S. (1997) 'Bioavailability of flavonoids from tea', Crit Rev Food Sci Nutr Vol.No.37 pp.719-738.
32. Isao Murakami, Takumi Nakamura, Yukiko Ishibashi, Ryo Shibuya, Eri Ayano, Yuko –Morita-Murase, Yoshiko Nagata and Hideko Kanazawa, (2006) Dept Of Pharmaceutical Chemistry, Kyortsu University Of Pharmacy-Tokyo, pp105-8512.
33. Iwanami, S., Matsui, H., Kimura, A., Ito, H., Mori, H., Honma, M. and Chiba, S. (1995) Biosci. Biotech. Biochem., Vol.No.59 pp.459-463
34. James, A.A., Blackmer, K. and Racioppi, J.V. (1989) 'Gene', pp.73-83.
35. Karthic, K., Kirthiram, K.S., Palvanan, T., Sadasivam, S. and Thayumanavan, B. (2008) 'Identification of α amylase inhibitors from Syzygium cumini Linn seeds', Indian Journal of Experimental Biology Vol.46 pp. 677-680.
36. Kimura, A., Takata, M., Fukushi, Y., Mori, H., Matsui, H., and Chiba, S., (1992) Biosci. Biotech. Biochem, Vol.No.61, pp.1901-1098.
37. Kimura, A., Takatta, M., Sakai, O., Matsui, H., Takai, N., Takayanagi, T., Nishimura, I., Uozumi, T. and Chiba, S. (1992) Biosci, Biotech, Biochem, Vol.No.56, pp.1368-1370.
38. Kondratyuk, T.P. and Pezzuto, J.M., (2004) 'Natural Product Polyphenols of Relevance to Human Health', Pharm Biol Vol.No.42 pp.46-63.
39. Kuhnau, J. (1976) 'The flavonoids - A class of semi-essential food components: their role in human nutrition' World Rev Nutr Diet Vol.No.24 pp.117-191.

50. Pandey, K.B. and Rizvi, S.I. (2009) 'Protective effect of resveratrol on markers of oxidative stress in human erythrocytes subjected to in vitro oxidative insult', Phytother Res.
51. Pandey, K.B., Mishra, N. and Rizvi, S.I. (2009) 'Protective role of myricetin on markers of oxidative stress in human erythrocytes subjected to oxidative stress', Nat Prod Commun Vol.No.4 pp.221-226
52. Parr, A.J., Bolwell, G.P., (2000) 'Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile', J Agric Food Chem Vol.No.80 pp.985-1012.
53. Polyxeni Alexiou and Vassilis, J. and Demopoulos (2010) 'Plants in traditional and modern medicine' Chemistry and Activity pp.69-175.
54. Price, K.R., Bacon, J.R. and Rhodes, M.J.C. (1997) 'Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*)', J Agric Food Chem Vol.No.45 pp.938-942.
55. Quaroni and Semenza, G. (1976) J. BioL Chem., Vol.No.251, pp.3250-3253.
56. Renaud, S. and de Lorgeril, M. (1992) 'Wine, alcohol, platelets, and the French paradox for coronary heart disease', Lancet Vol.No.339 pp.1523-1526.
57. Rendell (2004) 'Advances in diabetes for the millennium: drug therapy of type 2 diabetes' MedGenMed: Medscape general medicine 6 (3 Suppl): 9. PMC 1474831.PMID 15647714.) .
58. Richard Cantrill, (2008) 'Chemical and Technical Assessment of Paprika Extract', pp.6-8.
59. Rizvi, S. I. and Zaid, M. A. (2001) 'Insulin like effect of epicatechin on membrane acetylcholinesterase activity in type 2 diabetes mellitus', Clin Exp Pharmacol Physiol Vol.No.28 pp.776-784.
60. Rizvi, S.I. and Zaid, M.A (2001) 'Intracellular reduced glutathione content in normal and type 2 diabetic erythrocytes: effect of Insulin and (-)epicatechin', J Physiol Pharmacol Vol.No.52 pp.483-488.
61. Rizvi, S.I. and Zaid, M.A. (2005) 'Impairment of sodium pump and Na/H exchanger in erythrocytes from non-insulin dependent diabetes mellitus patients: effect of tea catechins', Clin Chim Acta Vol.No.354 pp.59-67.
62. Rui Song, Lei Xu, Zunjian Zhang and Yuan Tian, (2010) 'Determination of Gallic Acid in Rat Plasma by LC-MS-MS' ChromatographiaVol.71 pp.1107–1111

63. Scalbert, A., Manach, C., Morand, C. and Remesy, C. (2005) 'Dietary polyphenols and the prevention of diseases', Crit Rev Food Sci Nutr Vol.No.45 pp.287-306
64. Setchell, K.D., Faughnan, M.S., Avades, T., Zimmer- Nechemias, L., Brown, N.M., et al. (2003) 'Comparing the pharmacokinetics of daidzein and genistein with the use of 13C-labeled tracers in premenopausal women', Am J Clin Nutr Vol.No.77 pp.411-9.
65. Shahidi, F. and Naczk, M. (1995) 'Food phenolics, sources, chemistry, effects, applications', Lancaster, PA: Technomic Publishing Co Inc.
66. Simon, B.F., Perez-Ilzarbe, J., Hernandez, T., Gomez- Cordoves, C. and Estrella, I. (1992) 'Importance of phenolic compounds for the characterization of fruit juices', J Agric Food Sci Vol.No.40 pp 1531-1535.
67. Snyder, M. and Davidson, N. (1983) J.Mol.Biol., Vol.No.166 pp101-118.
68. Soraia, I., Falcão and Miguel Vilas-Boas (2010) 'Phenolic characterization of Northeast Portuguese propolis: usual and unusual compounds' Anal Bioanal Chem Vol.396 pp.887–897.
69. Sosulski, F.W., Krygier, K. and Hogge, L. (1982) 'Importance of phenolic compounds for the characterization of fruit juices', J Agric Food Chem Vol.No.30 pp.337-340.
70. Spencer, J.P., Abd El Mohsen, M.M., Minihane, A.M. and Mathers, J.C. (2008) 'Biomarkers of the intake of dietary polyphenols: strengths, limitations and application in nutrition research', Br J Nutr Vol.No.99 pp.12-22.
71. Spencer, J.P., Chowrimootoo, G., Choudhury, R., Debnam, E.S., Srai, S.K., Rice-Evans, C. (1999) 'The small intestine can both absorb and glucuronidate luminal flavonoids', FEBS Lett Vol.No.458 pp.224-230.
72. Stahl, E. (1988) 'Thin layer chromatography', 2nd edition, pp.210-243
73. Thai, J. (2010) Pharmaceutical sciences, 2nd edition, pp 317-325.
74. Tibbot, B.K. and R.W.Skadsen (1996), Plant Mol Biol Vol.No.30, pp.229-241.
75. Toda, M., Kawabata, J. and Kasai, T. (2000) 'α-Glucosidase inhibitors from clove (*Syzgium aromaticum*)', Biosci. Biotechnol. Biochem., Vol.64 pp.294-298.
76. Wink, M. (1997) 'Compartmentation of secondary metabolites and xenobiotics in plant vacuoles', Adv Bot Res Vol.No.25 pp.141-169.
77. Yoriko Deguchi and Kouji Miyazaki (2010) Nutrition & Metabolism , Vol.77 pp 9-13
78. Young, J.F., Nielsen ,S.E., Haraldsdóttir, J., Daneshvar, B., Lauridsen, S.T., Knuthsen, P., Crozier, A., Sandström, B. and Dragsted, L.O. (1999) 'Effect of fruit

juice intake on urinary quercetin excretion and biomarkers of antioxidative status', Am J Clin Nutr Vol.No.69 pp.87-94.

79. Young-In Kwon, M.S., Dhiraj Vattem and Kalidas Shetty (2010) 'Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension', Asia Pac J Clin Nutr Vol.107 pp.108- 117
80. Yusaku Narita and Kuniyo Inouye (2010) ' Inhibitory effects of chlorogenic acids from green coffee beans and cinnamate derivatives on the activity of porcine pancreas α-amylase isoenzyme', Journal of Food Chemistry Vol. 127 pp. 1532-1539.