ISOLATION OF PANCREATIC AMYLASE AND LIPASE INHIBITORS FROM SOME SELECTED PLANT SEEDS

A PROJECT REPORT

Submitted by

S. ANUGRAKA (0810204003)

G. COORI CHANDINI CHAMAYA (0810204007)

D. SOWMIYA (0810204045)

T.V.S. VAISHNAVI (0810204050)

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

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BONAFIDE CERTIFICATE

Certified that this project report **"ISOLATION OF PANCREATIC AMYLASE AND LIPASE INHIBITORS FROM SOME SELECTED PLANT SEEDS"** is the bonafide work of **"S. ANUGRAKA (0810204003), G. COORI CHANDINI CHAMAYA (0810204007), D. SOWMIYA (0810204045), T.V.S. VAISHNAVI (0810204050)"** who carried out the project work under my supervision.

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We are very grateful to **Dr. S. Ramachandran**, Principal, Kumaraguru College of Technology, Coimbatore-641 049 for providing all the essential facilities in the College.

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ABSTRACT

Diabetes mellitus is a chronic metabolic disorder characterized by increased blood glucose level (Hyperglycaemia). The most prevalent types are Type 1(IDDM) & Type 2 (NIDDM). Six different fruit seeds such as *Mangifera indica* (mango), *Phoenix dactylifera* (dates), *Annona reticulate* (custard apple), *Citrullus lanatus* (watermelon), *Artocarpus heterophyllus* (jack fruit) and *Prunus domestica* (plum) were taken for the study. The aim of the present investigation was focussed on the screening of pancreatic α amylase and lipase polyphenolic inhibitors from the seeds. Among different varieties, *Prunus domestica* (plum) seeds possessed highest pancreatic amylase (98.73%) inhibitory activity and *Phoenix dactylifera* (dates) recorded lowest inhibitory activity(51.2%).Similarly, *Mangifera indica* (mango) seeds possessed highest (45.71%) lipase inhibitory activity and *Phoenix dactylifera* (dates) recorded lowest (0.01%) inhibitory activity. The effect of single factors such as agitation rate, time, temperature and solid-liquid ratio on the extraction of the α-amylase and lipase inhibitors from dates seeds were optimized by RSM(Response Surface Methodology). The results proved that under optimize condition at 70°C, 150rpm, 7.5 minutes, 1:15 material ratio, highest pancreatic amylase inhibitory activity (99.24%) was observed. Similarly, at 80°C, 200 rpm, 10 minutes and 1:20 material ratio, highest lipase inhibitory activity (31.42%) was observed. Simultaneously, extraction (Pressurized Hot Water, temperature 180°C for 5 minutes) of *Prunus domestica* (plum) seed showed potent pancreatic amylase(94.29%) and lipase(22.85%) inhibitory activities. The TLC analysis revealed the presence of flavonoids and phenolic acids .The HPLC/DAD/MS analysis of PTLC (Preparative Thin Layer Chromatography) isolates revealed the compounds like Quercetin, Flavonol, Flavonoids glycoside melonate, Genistein, Chalcone derivative, 3,8'-diprenyl,5,7,4'-trihydroxy flavonone, Isosakurametin and 2 unknown compounds.

KEYWORDS: Diabetes mellitus, Response Surface Methodology, Pressurized Hot water extraction (PHWE), Flavonoids, *Prunus domestica* (plum), *Phoenix dactylifera* (dates) , *Mangifera indica* (mango).

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LIST OF ABBREVIATIONS

OD	Optical Density
µg	Microgram
mg	Milligram
g	Gram
ml	Millilitre
µl	Microlitre
µM	Micromolar
UV	Ultra Violet
$AlCl_3$	Aluminium Chloride
%	Percentage
mins	Minutes
**s ` **	Seconds
nm	Nanometer
cm	Centimeter
W	Watts
LC-MS	Liquid Chromatography-Mass Spectrometry
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
ANOVA	Analysis of Variance

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ANOVA	Analysis of Variance

CHAPTER 1

INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a chronic disease in which there are high levels of glucose in the blood. The prevalence of diabetes is rising all over the world due to population growth, aging, urbanisation and an increase of obesity and physical inactivity. The International Diabetes Federation (IDF) estimates the total number of people in India with diabetes to be around 50.8 million in 2010, rising to 87.0 million by 2030 (Ramachandran *et al*., 2010). It is necessary to develop novel therapeutic agents.It is classified into two types. Body's failure to produce insulin (Type 1 diabetes) referred to as insulin-dependent diabetes mellitus, IDDM or results from insulin resistance (Type 2 diabetes) referred to as non-insulin-dependent diabetes mellitus, NIDDM. (Alemzadeh *et al*., 2011).

1.2 PANCREATIC α-AMYLASE

During digestion process pancreatic amylase hydrolyze starch into maltose that can more easily absorbed by the small intestine. Maltase converts maltose into two glucose molecules which is catabolised to CO_2 and H_2O coupled with ATP production.Pancreatic α-amylases (EC 3.2.1.1) (1,4-α-D-glucan glucanohydrolase; glycogenase) are calcium metalloenzymes that hydrolyze the α(1-4) and (1-6) glycosidic linkage of starch and glycogen, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. In animals, it is the major digestive enzyme, and its optimum pH is 6.7-7.0.(Rydberg *et al*., 1999)

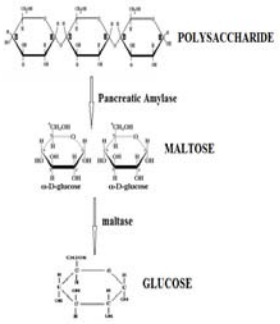


Fig 1.2.1

Hydrolysis of starch by α-Amylase(Brayer, 1995)

1.3 PANCREATIC LIPASE

Lipases (Triacylglycerol acylhydrolase, EC 3.1.1.3) are part of the family of hydrolases that act on carboxylic ester bonds. The physiological role of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol. Lipases can catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. Lipases constitute the most important group of biocatalysts for biotechnological applications (Fariha *et al* ., 2005).

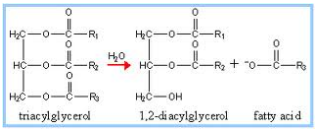


Fig :1.3.1 Hydrolysis of triacylglycerol by Lipase(Young and Hui, 1999)

1.4 MEDICINAL PLANTS

Ayurveda is one of the ancient Indian therapeutic measure for diabetes. traditional medicines is growing rapidly due to the increase side effects by Allopathic mediated drug metabolites. India is the largest producer of medicinal herbs and is called the botanical garden of the world. (Saper, 2004).

Medicinal plants can act as important therapeutic agents applied in the treatment of various ailments like neurological conditions, leprosy, skin diseases, heart disorders, cerebro vascular diseases and hypertension. Brahmi (Bacopa monnieri) is useful for treating the diseases of the brain and to improve memory power. Brahmi-formulations are prescribed in rheumatism, mental disorders, constipation and bronchitis. Amla (*Emblica officinallis*) is used as digestive aid, carminative, laxative, anti-pyretic and tonic. It is prescribed in colics, jaundice, hemorrhage, flatulence and many other disorders. Ashwagandha (*Withania somnifera*) is a nervine tonic, anabolic and aphrodisiac. It is used also to treat general weakness and rheumatism. Aloe Vera contains over 20 minerals, all of which are essential to the human body. Aloe vera is believed to help in sustaining youth, due to its positive effects on the skin. Hence it is called ghee kunvar or ghee kumaari. Its properties include soothing, moisturizing, and healing .Aloe vera gel is used as an ingredient in commercially available lotions, yogurt, beverages, and some desserts.(Yizhong Cai, 2004)

1.4.1 Polyphenols

Polyphenols constitute one of the most common and widespread groups of substances in flowering plants. They are considered as secondary metabolites involved in the chemical defence of they contain at least one aromatic ring with one or more hydroxyl groups in addition to other
substituents. The biological properties of polyphenols include antioxidant, anticancer, antifungal, antibacterial and anti-inflammatory effects (Soares 2007). The water-soluble phenolic compounds possess molecular weight ranging from 500 to 4,000 Da and possess 12 to 16 phenolic hydroxyl groups with 5 to 7 aromatic rings per 1,000 Da.

1.4.2 Flavonoids

Flavonoids are class of secondary metabolites and water soluble polyphenolic compounds containing 15 carbon atoms(fig 1.4.2.1). It can be visualized as two benzene rings which are joined together with a short three carbon chain. The flavonoids consist of 6 major subgroups: chalcone, flavones, flavonol, flavonone, anthocyanins and isoflavonoids. These compounds appear to have the capacity of radical scavenging and metal ion complexation. Green and black tea contains about 25% flavonoids. Other important sources of flavonoids are apple (quercetin), citrus fruits (rutin) (Justesen *et al*., 2001). such compounds display activities like antiviral, antimicrobial,anti-inflammatory chelation of metals, inhibition of enzymes and regulation of gene expression.

mediators.The highest Amylase and Lipase inhibitory activities of flavonoids are related to number of hydroxyl groups present in the molecule. Glycosylation of flavones and flavonols enhanced their affinities for α-amylase as well as lipase.

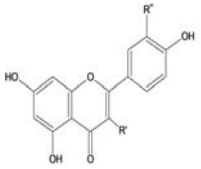


Fig : 1.4.2.1 Basic structure of Flavonoid(Ververidis, 2007)

CHAPTER 2

REVIEW OF LITERATURE

2.1 AMYLASE

In humans, the pancreatic amylase is responsible for cleaving large malto-oligosaccharides to smaller oligosaccharides, which are then substrates for intestinal glucosidases. This digestion process is important for glucose absorption from the intestine to the blood, and in principle, control of human pancreatic amylase activity (HPA) can be used as a means of controlling blood glucose levels. In fact, HPA activity has been correlated to post-prandial blood glucose levels (Shin Numavo *et al*., 2004).

2.1.1. MECHANISM OF ACTION

The enzyme α- amylase consist of three domains: a catalytic domain, a starch binding domain and a linker domain. The starch binding domain of amylase enzyme was located at the N- terminus and attached to catalytic binding domain by a small linker domain. Three carboxyl groups (Asp 206, Glu230 and Asp297) of α- amylases are essential for catalysis and/or postulated to be involved in hydrolysis of starch. Trp indole ring is involved in binding of the glycon part of the cleaved substrate and it controls its release from the active site. Starch binding domains are not found in most of endo acting α- amylases, but they are present in exo acting α- amylases. Trp120 was postulated to be important for stabilization of the transition state during catalysis and glu180 was found to be important for substrate binding with the highest affinity for binding glucose in glucoamylase. The hydrolysed starch granules by the action of α- amylase were relatively uniform size and dependent on the type of the complex (James *et al*., 2009)

2.2 LIPASE

Fat in the diet consists of cholesterol, saturated and unsaturated fats. Apart from food, cholesterol is also present in the gut that comes from the heart are excreted with bile into the small intestine. In the body fat is stored in the form of triglycerides. Pancreatic lipase (PL), lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) are enzymes responsible for the digestion of triglycerides coming from the diet, the plasma lipoproteins and the adipocytes, respectively. Pancreatic lipase which is isolated from the pancreas, catalyzes the hydrolysis of triglycerides in the intestines to become monoglyceride and long-chain fatty acids. Long-chain free fatty acid is percursor of cholesterol. If the pancreatic lipase activity increases, the concentration of cholesterol and lipid in the blood also increase (Diego Moreno *et al*., 2005).

2.2.1. MECHANISM OF ACTION

The catalytic triad of lipases with an α/β-hydrolase fold is composed of three amino acids (Serine, Histidine, Aspartate/Glutamate), which are far apart in the primary sequence but spatially very close in the folded protein. The interaction of the negatively charged residue Asp or Glu allows the His residue to act as a general base which can remove a proton from the hydroxyl group of the active site Ser. The thus generated nucleophilic alkoxide ion on the Ser residue is proposed to attack the carbonyl group of the estrified substrate forming an acyl-enzyme intermediate. Another important component for the catalytic mechanism is the oxyanion-hole which is composed of properly arranged H-bond donors (mostly main-chain NH groups). The oxyanion hole helps to stabilize a reaction intermediate during catalysis when the carbonyl oxygen carries a partial negative charge.

The active serine residue of lipases is embedded in the short consensus sequence GXSXG (with X being any amino acid), a motif also found in esterases, thioesterases and proteases. The active site of lipases in the 'closed' form is shielded from the surface by protective surface loops called the 'lid'. Upon activation, the lid undergoes a conformational rearrangement exposing the active site serine and creating the active, open form of the enzyme. Both, the open and the closed form of lipases have been observed in X-ray structures of lipases.

2.3 AMYLASE INHIBITORS

Inhibitors of amylase have been successfully used in the treatment of diseases such as diabetes or obesity (Shin Numavo *et al*., 2004). α-amylase inhibitors play a major role in managing postprandial hyperglycemia (PPHG) in diabetic patients. They inhibit the action of α-amylase, an enzyme that catalyzes the endohydrolysis of α (1→ 4) glucosidic linkage present in starch and thereby, leading to a reduction in starch hydrolysis. This kind of inhibition shows beneficial effects on glycemic index control in diabetic patients.

The chemical nature of amylase inhibitors may be proteinase or non proteinase (polyphenols). Generally, proteinase inhibitors are widely distributed among cereals and legumes. The favoured hypotheses about physiological roles of proteinase enzyme inhibitors in seeds are that they act as storage or reserve proteins as regulators of endogenous enzyme or as defensive agents against the attacks of animal predators and insect or microbial pests. Low molecular weight plant-derived molecules such as polyphenols, luteolin, myricetin, and quercetin were potent inhibitors against porcine pancreatic α-amylase and the potency of inhibition correlated with the number of hydroxyl groups on the B ring of the flavonoid scaffold. α-amylase and its inhibitors are drug-design targets for the development of compounds to treat diabetes, obesity and hyperlipaemia. These inhibitors show remarkable structural variety leading to different modes of inhibition (Sathishkumar *et al.,* 2011). The known α-amylase inhibitors used in management of diabetes are acarbose and miglitol, but these drugs are known to be associated with gastrointestinal side effects. Therefore, it becomes necessary to identify α- amylase inhibitors from natural sources having fewer side effects (Hema *et al.,* 2011).

2.3.1 MECHANISM OF ACTION

Suppression of α-amylase enzyme in the human digestive system would delay the degradation of starch and oligosaccharides to monosaccharides before they can be adsorbed. This would decrease the absorption of glucose and consequently reduce postprandial blood glucose level (Hema *et al.*, 2011). Figure 2.3.1.1 shows the normal mechanism of action of this class of enzyme involves the formation and hydrolysis of a glycosyl-enzyme intermediate with general acid/base catalytic assistance via transition states with substantial oxocarbenium ion character (Sinnott *et al.,* 1990). A combination of inductive destabilization of these positively charged transition states by the electronegative fluorine at C-2 and loss of crucial hydrogen bonding interactions with the 2-hydroxyl serves to substantially destabilize these two transition states, dramatically slowing both steps. Incorporation of a relatively reactive leaving group such as fluoride or 2,4-dinitrophenolate as aglycone accelerates the first (glycosylation) step sufficiently that the glycosyl-enzyme intermediate is formed, but then hydrolyzes only very slowly, thereby resulting in inactivation.

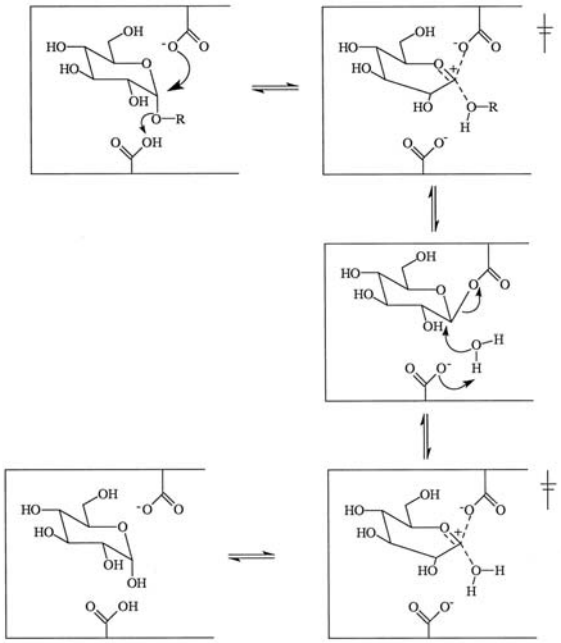


Figure 2.3.1.1:Difluoroglycosides as α-Glycosidase Inhibitors(Curtis Braun *et al*., 1995).

2.4 LIPASE INHIBITOR

The excess cholesterol is one of the factors causing constriction of blood vessels, called atherosclerosis, because calcification and hardening of blood vessel walls. In the brain, atherosclerosis causes a stroke, while in the heart causing coronary heart disease (CHD). So it is necessary to inhibit lipase enzyme. Lipase ihibitors can be obtained from natural products and herbal plants (Diego Moreno *et al*., 2005). The leaves of mango (ML) are also used as an antidiabetic agent in Nigerian folk medicine. Mangiferin present in *Mangifera* extracts in addition to different *Salacia reticulata* polyphenols resulted in enhanced lipolysis and inhibited PL and LPL activities in female Zucker rats (Diego Moreno *et al.,* 2005).

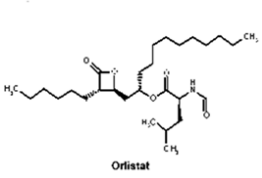


Figure 2.4.1:Sructure of Orlistat(Subandia and Indah Langitasaria, 2010).

Orlistat is one of the blood fat-lowering drugs, and effective in overcoming obesity. Orlistat prevents the absorption of 30% fat. This drug works by inhibiting pancreatic lipase activity. However, the use of the drug give side effects, primarily arise in the gastrointestinal tract such as oily spots in the colon, excessive flatus. (Subandia and Indah Langitasaria, 2010).

2.4.1 MECHANISM OF ACTION

The lipase inhibitor Orlistat belongs to a new class of anti-obesity drugs. It prevents the lipolysis of dietary triglycerides (TG), and thus reduces the subsequent intestinal absorption of fat. Human pancreatic lipase (HPL) has been the main target in the development of Orlistat. The inhibition of human gastric lipase (HGL) by Orlistat has also been investigated *in vitro* but had not yet been studied in the course of test meals. In most clinical studies, the effects of Orlistat have been estimated indirectly from the fecal fat excretion levels, but the inhibition exerted on digestive lipases and the levels of lipolysis were not measured simultaneously *in vivo*.

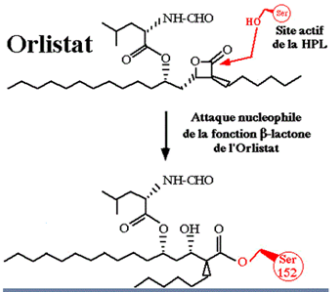


Figure 2.4.1.1: Mechanism of pancreatic lipase inhibition by Orlistat (tetrahydrolipstatin)(Physiol, 2001)

2.5 *Mangifera indica*

Mango (*Mangifera indica* L.) is an economically important tropical fruit. Mango contains various classes of polyphenols, carotenoids, and ascorbic acid, which demonstrate different health-promoting properties, mainly from their antioxidant activities. The antibacterial activity of five flavonoids (epicatechin-3-*O*-glucopyranoside (**1**), 5-hydroxy- 3-pyrano chromene-4 (**2**), 6 taxifolin-7-*O*-D-glucoside (tricuspid) (**3**), quercetin-3-*O*-glucopyranoside (**4**) and epicatechin (**5**)), were isolated from the leaves of mango
(Qudsia *et al*., 2009). Mangiferin from stem bark of *Mangifera indica* (mango) showed anti-diabetic and anti-atherogenic effects. The results showing that *Mangifera indica* might be effective in lowering post prandial hyperglycemia.(Dineshkumar *et al*.,2010)

Figure 2.5.1: Seeds of *Mangifera indica* (Diego Moreno *et al*., 2005)

The mango stem bark is astringent, antihelmintic and used to treat haemoptysis, haemorrhage, diarrhoea, ulcers, diphtheria and rheumatism and also it stops vomiting. Aliphatic constituents such as coumarin, mangiferine, sequioterpinenoids, triterpenoids and phenolics have been reported from the stem barks of different cultivators of *M. indica* and also isolation and characterization of phytoconstituents from the bark was also described (Ansari *et al*.,

2012). A standard aqueous stem bark extract from selected species of *Mangifera indica*, named Vimang, has been used as phytomedicine in Cuba. Studies on the pharmacological properties of mangiferin reveal that this flavonoid compound possesses antitumor, antiviral, antioxidant, antidiabetic, immunomodulatory, and vascular modulatory activity. Neelapu *et al*., 2012 has provided the preliminary data on the antiulcer potential of *M.indica* leaves and support the traditional uses of the plant for the treatment of gastric ulcer.

2.6 *Annona squamosa L*

The claim by some tribal populations in parts of Northern India that the young leaves of the custard apple tree, *Annona squamosa* L. belongs to family Annonaceae has antidiabetic properties (Annie *et al*., 2004).

Figure 2.6.1: Seeds of *Annona squamosa L* (Morton, 1992; Murdiatia *et al.,* 2000).

A compound isolated from Noni roots viz., 1-methoxy-2-formyl-3- hydroxyanthraquinone suppressed the cytopathic effect of HIV infected MT-4 cells, without inhibiting cell growth (Umezawa, 1992). An ethanol extract of the tender Noni leaves induced paralysis and death of the human parasitic nematode worm, A*scaris lumbricoides* within a day (Raj, 1975). Noni has been used in the Philippines and Hawaii as an effective insecticide (Morton, 1992; Murdiatia *et al.,* 2000).

2.7 *Prunus domestica*

Prunus domestica L. originated from the Caucasus region in West Asia. It belongs to the Rosaceae family and is one of the species of plum (Chopra *et al*., 1956) and it is sold as fresh or dried fruit. The dried fruit is also referred to as a dried plum. In general, fresh prunes are freestone cultivars (the pit is easy to remove), whereas most other plums grown for fresh consumption are clingstone (the pit is more difficult to remove). (Stacewicz *et al*., 2001)

Figure 2.7.1: Seeds of *Prunus domestica* (Janic *et al*., 2008)

Prunes and their juice contain mild laxatives including phenolic compounds (mainly as neochlorogenic acids and chlorogenic acids) and sorbitol (Janic *et al*., 2008). HPLC analysis of prune components was carried out according to the method of Donovan *et al*. (1998). From the bark of *Prunus domestica*, the compounds 3-CQA, 5-CQA, caffeic acid, *p*-coumaric acid, and rutin were detected. Antioxidative activities of the chlorogenic acid isomers, such as scavenging activity on superoxide anion radicals and inhibitory effect against oxidation of methyl linoleate, were also evaluated (Nakatani *et al*., 2000). Flavonols like quercetin derivatives were commonly found in plum (Dae *et al*., 2003)

2.8 *Artocarpus heterophyllus*

Artocarpus heterophyllus , commonly known as the jackfruit tree and belonging to the family Moraceae, is an exotic tree originally native to the Western Ghats of India. The fruits are of dietary use and are an important source of carbohydrate, protein, fat, minerals and vitamins. The bark, roots, leaves, and fruit are attributed with diverse medicinal properties and were used in the various traditional and folk systems of medicine to treat a range of ailments. Preclinical studies have shown that jackfruit possesses antioxidant, anti-inflammatory, antibacterial, anticariogenic, antifungal, antineoplastic, hypoglycemic, wound healing effects and α-amylase inhibitory activity (Manjeshwar *et al*., 2011).

Figure 2.8.1: Seeds of *Artocarpus heterophyllus* (Hema *et al*., 2011).

Hema *et al*., 2011 has proposed that methanol extract of the root bark of *A. integrifolia* posses antidiabetic activity and it was examined by α-amylase inhibitory assay.

2.9 *Phoenix dactylifera*

The date palm (*Phoenix dactylifera*) is a palm in the genus *Phoenix*, cultivated for its edible sweet fruit.and it is probably originated from lands around the Persian Gulf. Inhibitors of amylase from *Phoenix dactylifera* were assessed and characterized (Morton,1987)

Figure 2.9.1: Seeds of *Phoenix dactylifera* (Muhammad Ashraf *et al*., 2007)

Aqueous extract of phoenix dactylifera was evaluated for their anti hyperlipidaemic efficacy in High fat diet (HFD) induced albino obese rats. Phytochemical constituents of phoenix dactylifera may be responsible to prevent theobesity Phytochemically the whole plant contains carbohydrates, alkaloids, steroids, flavonoids, vitamins and tannins. The extract of Phoenix dactylifera resulted in significant reduction in LDL cholesterol level and there by it prevents atherosclerosis and other cardiovascular disease (Vembu *et al.,* 2012). Dates at tamar stage contain a wide array of flavones considered to be strong mutagenic and the antimicrobial activity of flavonoid glycosides extracted from the date fruits and its antioxidant property were also evaluated (Samy *et al.,* 2012)

2.10 *Citrullus lanatus*

Watermelon (*Citrullus lanatus*) is one of the fruit that can be used as a medicinal plant. Its endocarp contains a number of beneficial vitamins and nutrients such as vitamin C, vitamin A, vitamin B6, the amino acid citrulline, beta carotene, lycopene, magnesium, protein, dietary fiber, thiamine, riboflavin, and niacin.

Figure 2.10.1 Seeds of *Citrullus lanatus* (Subandia and Indah Langitasaria, 2010).

Watermelon endocarp has the nature Hypolipidemic (lower blood fat). The extract of its endocarp can decrease lipid levels in the blood. The presence of chemical compounds can affect the activity of an enzyme. The content of these compounds in watermelon endocarp extract may be influenced by several factors including: type of watermelon, skin thickness of watermelon, the age of a watermelon (fruit maturity), the weight, diameters fruit and fruit storage time.(Subandia and Indah Langitasaria, 2010).

In spite of several literature documentation on the above mentioned seeds, it was found that the amylase and lipase inhibitory activities were not scientifically explored. In this regard, our laboratory has focussed in the screening and isolation of the above said inhibitors.

CHAPTER 3

MATERIALS AND METHODS

3.1 COLLECTION OF PLANT MATERIAL

Six different fruits which are commonly consumed to be screened for pancreatic amylase and lipase inhibition were selected. Only the seeds of the fruits were used for this study. The fruits used as follows.

- Mango (*Mangifera indica)*
- Jack fruit (*Atrocarpus heterophyllus*)
- Date (*Phoenix dactylifera*)
- Water melon (*Citrullus lanatus*)
- Plum (*Prunus domestica*)
- Custard apple (*Annona squamosa*)

All these fruits were purchased from local market and all the above plants were identified and authenticated for their scientific names by TNAU, Coimbatore.

3.2 PREPARATION OF EXTRACT

The seeds of each fruits were washed with distilled water thrice until the dust and other particles were removed and were air dried for some time. Then they were kept in Hot air oven at 50˚C for overnight. All the samples were then ground into fine powder using mixture grinder. The samples were stored at 4˚C for further use.

3.2.1 Preparation of hot water extract

In a clean dry 250 ml conical flask, 0.5 g of the powdered material was weighed and extracted with 25 ml of distilled water by placing it in water bath at temperature 90˚C for 5 minutes. The suspension was filtered using Whatman No.2 filter paper, the filtrate obtained is precipitated by adding 10% of Ammonium sulphate and centrifuged at 5000 rpm for 10 minutes and the supernatant is used for experimental analysis.

3.2.2 Preparation of Pressurized hot water extract (PHWE)

In a clean dry pressurized vessel 0.5 g of the powdered Date's seed was weighed and extracted with 100 ml distilled water .The vessel is placed in oil bath at 180˚C, 10.027 bar pressure for 5 minutes as shown in figure 3.2.2.1. The suspension was filtered using Whatman N0.2 filter paper. The filtrate obtained is precipitated by adding 10% of Ammonium sulphate and centrifuged at 5000 rpm for 10 minutes and the supernatant part is subjected to assay procedures

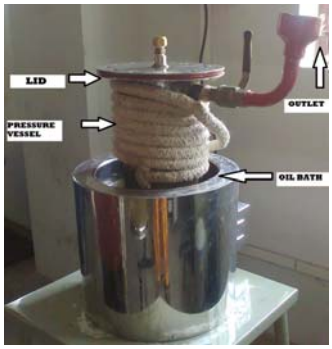


Figure 3.2.2.1: Pressurized Vessel in Oil Bath

3.3 PANCREATIC AMYLASE INHIBITORY ASSAY

Reagents required

- Phosphate buffer (pH 6.9)
- 1% Starch
- Amylase enzyme
- DNS (Dinitro salicylic acid)

Procedure

Series of test tubes were taken as tests with corresponding blanks and 1 control. 1 ml of phosphate buffer was added to the test tubes. 0.1 ml of sample was added to all the test tubes except control. Then 0.5 ml of 1% starch solution was added to all the tubes. 0.1 ml of amylase enzyme was added to all the tubes except blank. Incubate the tubes at 37˚C for 10 minutes. Then 1 ml of DNS reagent was added to all the tubes and kept in boiling water bath for 10 minutes and orange colour obtained was measured spectrophotometrically at 540 nm.

Calculation

Enzyme activity = (concentration of maltose x volume of enzyme x dilution factor) / (molecular weight of maltose x incubation time)

% Inhibition = ((OD of control – OD of test) / (OD of control)) *100

3.4 LIPASE INHIBITORY ASSAY (KIT PROCEDURE, AGAPPE DIAGNOSTICS LTD., INDIA)

The pancreatic lipase activity of the kit purchased and the linearity was checked to ensure that the kit can be further used for the inhibition studies.

Reagent Composition

Lipase R1	**2x10mL**
Goods Buffer (pH 8.0)	40mmol/L
Taurodeoxycholate	3.4mmol/L
Deoxy cholate	6.4mmol/L
Calcium Chloride	12mmol/L
Colipase	1.7mg/L
Lipase R2	**1x5mL**
Tartrate Buffer (pH 4.0)	1.5mmol/L
Taurodeoxycholate	3.4mmol/L
Colour Substrate	0.13mmol/L
Lipase Calibrator	**1x3Ml**
Lipase calibrator	107U/L

.

Procedure

Series of eppendrofs were taken and marked as blank, calibrator and sample. 1 ml of lipase reagent 1(R1) was added to all the tubes and 20 µl of calibrator was added to all the tubes except blank. Then 20 µl of seed extract was added to sample tube and 20 µl of distilled water was added to blank. The contents were mixed carefully and incubated for 5 minutes at 37˚C and then 250 µl of lipase reagent 2 (R2) was added to all the tubes. The contents were mixed once again and incubated for 2 minutes. The absorbance was recorded against reagent blank and the change in absorbance was also measured per minute (ΔOD/min) during 2 minutes.

3.5 OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY

To optimise the extraction conditions for flavonoids and phenolic acids, four variables were selected and they are time, temperature, solid to liquid ratio and agitation rate (rpm)(table 3.5.1). The optimum extraction conditions were determined by Response Surface Methodology. A single factor analysis of variance was adopted to investigate the effect of each factor on the extraction of flavonoids and phenolic acids.

Table 3.5.1 Response Surface Methodology

RUNS	SOL: LIQ	TIME	TEMPERATURE	RPM
1	1:15	7.5	70	150
2	1:20	10	60	200
3	1:10	10	80	100
4	1:20	5	60	200
5	1:5	7.5	70	150
6	1:10	5	80	200
7	1:20	5	60	100
8	1:20	5	80	200
9	1:20	10	60	100
10	1:10	10	80	200
11	1:15	7.5	70	150
12	1:15	7.5	70	150
13	1:10	5	60	100
14	1:20	10	80	200
15	1:25	7.5	70	150
16	1:15	12.5	70	150
17	1:15	2.5	70	150
18	1:15	7.5	70	150
19	1:10	10	60	200
20	1:15	7.5	70	150

21	1:15	7.5	50	150
22	1:10	5	80	100
23	1:10	5	60	200
24	1:20	5	80	100
25	1:10	10	60	100
26	1:10	10	80	100
27	1:5	10	60	200
28	1:15	7.5	90	150
29	1:20	10	80	100
30	1:15	7.5	70	150
31	1:15	7.5	70	250

3.6 THIN LAYER CHROMATOGRAPHY (TLC) AND PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

Reagents Required

- Silica gel
- Ethyl acetate
- Ethanol
- Distilled water
- Chloroform
- Formic acid
- Liquid ammonia
- Ferric chloride

Procedure

Glass plates (20 cm x10 cm) were taken. Silica gel dissolved in water was applied as thin layer to the glass plate with the help of an applicator. The plates were dried for 24 hrs to remove water or moisture and other adsorbed substance from the surface so as to activate the plate. 100µl of the plum seed sample was spotted on each plate using micropipette. Spot was placed 2 cm above the base of the plate and the spotting area should not be immersed in the mobile phase. The development tank has to be saturated for 24 hrs with mobile phase ethyl acetate: ethanol: water in the ratio of 5:1:5 for flavonoids and with chloroform: ehanol: formic acid in the ratio of 85:15:1 for phenolic acids. The glass plates with the spotted area were immersed in the separate tanks and developed. The plates were dried before spraying liquid ammonia and ferric chloride (detecting agent). Moderate amount of the reagent has to be sprayed to the plate so that it always appears dull and flat. The plates were then viewed under while light and short and long UV to detect the phytoconstituents present.

The same procedure was followed for Preparative Thin Layer Chromatography (PTLC) with the glass plates of size 20 cm x 20 cm and the samples were spotted along the length of the plate using the sample template. The development procedure was the same as that of TLC.

The silica gel containing the fluorescent band was scrapped under UV light and the silica gel was centrifuged with phosphate buffer (pH 6.9) to transfer the phytochemicals into the buffer. The supernatant was collected and stored at 4˚C for further use.

3.7 LC-PDA-MS (ESI+) analysis

The liquid chromatography electrospray mass spectropmetry experiment was performed on Varian Inc, (USA) 410 Prostar Binary LC with 500 MS IT PDA Detectors.. The column was C_{18}, 250 x 4.6 mm, i.d. 5µm. The mobile phase A was made up of acetonitrile while B was made of 0.1% formic acid (pH 4.0, adjusted with ammonium hydroxide) aqueous solution. The gradient elution was performed at 1 ml/min with an initial condition of 12% of mobile phase A and 88% of mobile phase B for 10 min. The mobile phase A was increased to 15% at 20 min and linearly increased to 60% at 90 min and then increased to 100% at 95 min. The eluates were monitored by PDA (Multi wavelength) detector at 260 nm. About 20µl of the PTLC *Prunus domestica* (plum) seed isolates were introduced into the ESI source and the mass spectra were scanned in the range 100-1000amu and the maximum ion injection time was set 200nS. Ion spray voltage was set at 5.3 KV and capillary voltage 34 V. The MS scan ran upto 26.67 minutes.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 PANCREATIC AMYLASE AND LIPASE INHIBITION

4.1.1 HOT WATER EXTRACTION

The hot water extract obtained from various plant seeds were screened for the presence of pancreatic amylase and lipase inhibitory activities. The results were depicted in the table 4.1.1

Table 4.1.1: Inhibitory activity(%) of amylase and lipase inhibitors

Seeds	% Inhibition	
	Amylase	Lipase
***Mangifera indica* (MANGO)**	88.59	**45.71**
Annona squamosa (CUSTARD APPLE)	95.55	5.71
***Prunus domestica* (PLUMS)**	**98.09**	2.00
Artocarpus heterophyllus (JACK FRUIT)	96.82	17.14
Phoenix dactylifera (DATES)	51.20	0.1
Citrullus lantus (WATER MELON)	94.92	0.4

The results of the assay implied the hot water extraction of *Prunus domestica* (PLUMS) showed a maximum of 98.09 % pancreatic amylase inhibitory activity and *Mangifera indica* (MANGO) showed a maximum of 45.71 % pancreatic lipase inhibitory activity and extraction of *Phoenix dactylifera* (DATES) shows minimum inhibitory activity on both amylase (51.20%) and lipase (0.1 %). During hot water extraction normally the temperature of water is raised, there is a steady decrease in its permitivity, viscosity and surface tension, but an increase in its diffusivity characteristics. This results in the interaction of solute particles with the water molecules, swells up (hypotonic) and the phytoconstituents may be released (Chin Chye Teo, 2010).

4.2 OPTIMIZATION OF AMYLASE INHIBITORY ACTIVITY OF *Phoenix dactylifera* SEEDS BY RESPONSE SURFACE METHODOLOGY

The optimisation of flavonoids for *Phoenix dactylifera* (DATES) has been done and optimum conditions for amylase inhibition was found to be at Solid : liquid ratio (1:15), Time (7.5 mins), Temperature (70˚C) and RPM (150) and maximum amylase inhibitory activity was found to be 99.24 %. The optimum conditions for lipase inhibition is found to be at Solid : liquid ratio (1:20), Time (10mins), Temperature (80˚C), RPM (200) and maximum lipase inhibitory activity was found to be 31.42 %.

4.2.1 OPTIMISATION 0F *Phoenix dactylifera* (DATES) USING RESPONSE SURFACE METHODOLOGY (RPM 100)

Table 4.2.1: Optimisation at 100 RPM

RUN	RATIO (solid : liquid)	TIME (min)	TEMPERATURE (°C)	%INHIBITION (AMYLASE)	%INHIBITION (LIPASE)
7	20	5	60	96.77	5.71
9	20	10	60	96.39	8.57
13	10	5	60	97.91	2.85
22	10	5	80	97.53	17.14
24	20	5	80	98.48	20.00
25	10	10	60	97.53	11.42
26	10	10	80	95.63	20.00
29	20	10	80	96.38	17.14

4.2.2 OPTIMISATION 0F *Phoenix dactylifera* (DATES) USING RESPONSE SURFACE METHODOLOGY (RPM 200)

Table 4.2.2: Optimisation at 200 RPM

RUN	RATIO (solid:liquid)	TIME(min)	TEMPERATURE (°C)	%INHIBITION (AMYLASE)	%INHIBITION (LIPASE)
2	20	10	60	93.92	5.71
4	20	5	60	88.21	17.14
6	10	5	80	86.31	0.01
8	20	5	80	93.92	5.71
10	10	10	80	86.31	17.14
14	**20**	**10**	**80**	93.54	**31.42**
19	10	10	60	86.31	0.02
23	10	5	60	93.54	8.57

4.2.3 OPTIMISATION 0F *Phoenix dactylifera* (DATES) USING RESPONSE SURFACE METHODOLOGY (RPM 150)

Table 4.2.3: Optimisation at 150 RPM

RUN	RATIO (solid:liquid)	TIME (mins)	TEMPERATURE (˚C)	%INHIBITION (AMYLASE)	%INHIBITION (LIPASE)
1	15	7.5	70	94.49	8.57
5	5	7.5	70	94.30	8.57
11	15	7.5	70	93.16	11.42
12	15	7.5	70	95.63	17.14
15	25	7.5	70	91.44	17.14
16	15	12.5	70	89.92	0.02
17	15	2.5	70	88.40	0.01
18	**15**	**7.5**	**70**	**99.24**	0.02
20	**15**	**7.5**	**70**	**99.24**	25.71
21	15	7.5	50	79.66	0.01
28	15	7.5	90	85.74	0.03
30	15	7.5	70	85.17	5.71

Tests were performed to select the relevant factors (temperature, time and ratio) and the experimental ranges for these independent variables. In general, efficiency of the extraction of a compound is influenced by these multiple parameters (Myers and Montgomery, 2002). Experimental data were fitted to a second-order polynomial model and regression coefficients obtained using MINITAB 15 TRIAL VERSION. The generalized second-order polynomial model used in the response surface analysis was as follows:

4.2.4 CONTOUR PLOT AND SURFACE PLOT FOR AMYLASE AND LIPASE INHIBITION FROM OPTIMISATION OF *Phoenix dactylifera* (DATES)

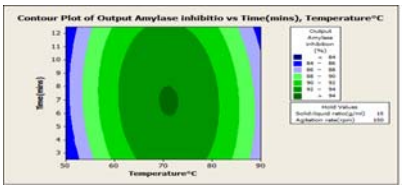


Fig.4.2.4.1:Contour Plot of Output Amylase Inhibition vs Time(mins),Temperature(°C)

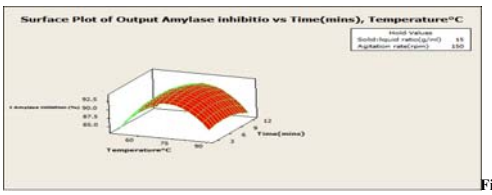


Fig.4.2.4.2 Surface Plot of Output Amylase Inhibition vs Time(mins),Temp.(°C)

The regression equation is
Output Amylase inhibition (%) = 95.0 + 0.0403 Temperature˚C - 0.060 Time(mins)
+ 0.090 Solid:liquid ratio(g/ml)
- 0.0421 Agitation rate(rpm)

Where 95.0, 0.0403, 0.060, 0.090, and 0.0421 are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and temperature, time, agitation rate and ratio are the independent variables. Minitab software was employed to generate response surfaces and contour plots while holding a variable constant in the second-order polynomial mode. Temperature and solid:liquid ratio are found to be directly proportional while time and agitation rate is indirectly proportional to the amylase inhibition (%).

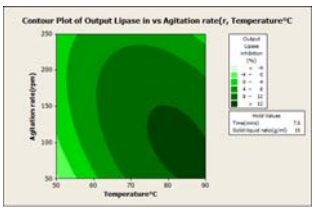


Fig4.2.4.3: Contour Plot of Output Amylase Inhibition vs Agitation rate(rpm), Temperature(°C)

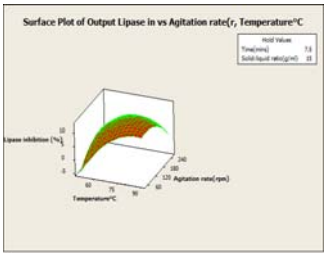


Fig 4.2.4.4: Surface Plot Output Amylase Inhibition vs Agitation rate(rpm), Temperature(°C)

The regression equation is
Output Lipase inhibition (%) = - 15.1 + 0.222 Temperature˚C + 0.480 Time(mins)
+ 0.468 Solid:liquid ratio(g/ml)
- 0.0089 Agitation rate(rpm)

Where 15.1, 0.222, 0.480, 0.468 and 0.0089 are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and temperature, time ,agitation rate and ratio are the independent variables. Temperature, time and solid:liquid ratio are found to be directly proportional while agitation rate is indirectly proportional to the amylase inhibition (%).

EFFECT OF TEMPERATURE

The effect of temperature on the aqueous extraction level was investigated over the range of 50 to 90°C. Regression analysis demonstrated the quadratic and linear models for amylase was found to be (r= 5.626) and (r= 4.598) while lipase have(r=3.616) and (r=1.783). According to the results, there was no significant difference (P< 0.05) observed between 50 and 90°C. Therefore, a fixed maximum temperature (axial value) of 70°C was adopted. Increasing temperature cause opening of cell matrix, and flavonoids availability increases. Under High temperature, solvent viscosity decreases and diffusivity increases, so efficiency of extraction increases.

Table4.2.4.1:One-way ANOVA: Output Amylase inhibition (%) versus Temperature˚C

```
Source          DF     SS    MS     F      P
Temperature˚C    4  232.8  58.2  3.01  0.036
Error           26  502.6  19.3
Total           30  735.4

S = 4.397   R-Sq = 31.65%   R-Sq(adj) = 21.14%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean  StDev  ---------+---------+---------+---------+
50      1  79.660      *  (-----------*-----------)
60      8  93.822  4.371                  (----*----)
70     13  92.362  4.197                 (---*---)
80      8  93.513  4.743                  (----*---)
90      1  85.740      *          (-----------*-----------)
                          ---------+---------+---------+---------+
                                77.0      84.0      91.0      98.0

Pooled StDev = 4.397


Grouping Information Using Tukey Method

Temperature˚C   N    Mean  Grouping
60              8  93.822  A
80              8  93.513  A
70             13  92.362  A B
90              1  85.740  A B
50              1  79.660    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Temperature˚C

Individual confidence level = 99.30%


Temperature˚C = 50 subtracted from:

Temperature˚C    Lower  Center   Upper
60               0.511  14.162  27.814
70              -0.655  12.702  26.059
80               0.201  13.853  27.504
90             -12.122   6.080  24.282

Temperature˚C  ----+---------+---------+---------+-----
60                        (--------*---------)
70                        (-------*--------)
80                        (--------*--------)
90                 (-----------*----------)
```

```
              ----+---------+---------+---------+-----
                -15         0        15        30


Temperature˚C = 60 subtracted from:

Temperature˚C    Lower  Center  Upper  ----+---------+---------+---------+-----
70              -7.244  -1.460  4.324           (---*---)
80              -6.746  -0.310  6.126            (---*---)
90             -21.734  -8.082  5.569  (--------*--------)
                                       ----+---------+---------+---------+-----
                                         -15         0        15        30


Temperature˚C = 70 subtracted from:

Temperature˚C    Lower  Center  Upper  ----+---------+---------+---------+-----
80              -4.634   1.150  6.934            (---*---)
90             -19.979  -6.622  6.735   (--------*--------)
                                       ----+---------+---------+---------+-----
                                         -15         0        15        30


Temperature˚C = 80 subtracted from:

Temperature˚C    Lower  Center  Upper  ----+---------+---------+---------+-----
90             -21.424  -7.773  5.879  (--------*--------)
                                       ----+---------+---------+---------+-----


                                         -15         0        15        30
```

EFFECT OF SOLID:LIQUID RATIO

The impact of the solid-to-liquid ratio on the extraction was tested at four various ratios (1:5, 1:10, 1:15, 1:20, 1:25; w:v). Also, the results of the one-way Analysis of Variance revealed that there were significant differences among the ratios studied. The optimun material ratio was found to be 1:15 and according to the obtained results, it was demonstrated that whichever the ratio chosen above 1:20, the quantity of phenolic compounds extracted will remain the same. Generally in conventional extraction techniques a higher volume of solvent will increase the recovery . Successful extraction of biologically active principles from plant samples is mainly based upon type of solvent used in the extraction procedure. Properties of a good solvent includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate (Das et al., 2010). the yield of flavonoids increased with the increase of solvent to material ratio.

Table4.2.4.2:One-way ANOVA: Output Amylase inhibition (%) versus Solid:liquid ratio(g/ml)

```
Source                    DF     SS    MS     F      P
Solid:liquid ratio(g/ml)   4   80.7  20.2  0.80  0.535
Error                     26  654.7  25.2
Total                     30  735.4

S = 5.018   R-Sq = 10.97%   R-Sq(adj) = 0.00%
```

```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level   N   Mean  StDev  -----+---------+---------+---------+----
 5      1  94.30      *       (----------------*----------------)
10      8  92.63   5.42            (-----*-----)
15     13  90.80   5.63          (---*----)
20      8  94.70   3.14                (-----*-----)
25      1  91.44      *  (----------------*----------------)
                          -----+---------+---------+---------+----
                            84.0      90.0      96.0     102.0

Pooled StDev = 5.02


Grouping Information Using Tukey Method

Solid:liquid
ratio(g/ml)    N    Mean  Grouping
20             8  94.701  A
 5             1  94.300  A
10             8  92.634  A
25             1  91.440  A
15            13  90.798  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Solid:liquid ratio(g/ml)

Individual confidence level = 99.30%


Solid:liquid ratio(g/ml) =  5 subtracted from:

Solid:liquid
ratio(g/ml)     Lower  Center   Upper
10            -17.247  -1.666  13.914
15            -18.746  -3.502  11.742
20            -15.179   0.401  15.982
25            -23.634  -2.860  17.914

Solid:liquid
ratio(g/ml)     +---------+---------+---------+---------
10                    (-----------*-----------)
15                 (------------*------------)
20                   (-----------*-----------)
25              (-----------------*----------------)
                +---------+---------+---------+---------
               -24       -12         0        12


Solid:liquid ratio(g/ml) = 10 subtracted from:

Solid:liquid
ratio(g/ml)     Lower  Center   Upper
15             -8.437  -1.836   4.765
20             -5.277   2.068   9.412
25            -16.774  -1.194  14.387

Solid:liquid
ratio(g/ml)     +---------+---------+---------+---------
15                   (----*-----)
20                     (-----*-----)
25                 (------------*------------)
                +---------+---------+---------+---------
               -24       -12         0        12
```

```
S = 5.080   R-Sq = 8.77%   R-Sq(adj) = 0.00%


                                Individual 95% CIs For Mean Based on Pooled StDev
Level   N   Mean  StDev     +---------+---------+---------+---------
 2.5    1  88.40      *     (----------------*----------------)
 5.0    8  94.08   4.60                 (-----*-----)
 7.5   13  91.37   5.64              (----*----)
10.0    8  93.25   4.48                 (-----*-----)
12.5    1  89.92      *       (----------------*----------------)
                           +---------+---------+---------+---------
                          78.0      84.0      90.0      96.0

Pooled StDev = 5.08


Grouping Information Using Tukey Method

Time(mins)   N    Mean  Grouping
 5.0         8  94.084  A
10.0         8  93.251  A
 7.5        13  91.368  A
12.5         1  89.920  A
 2.5         1  88.400  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Time(mins)

Individual confidence level = 99.30%


Time(mins) =  2.5 subtracted from:

Time(mins)    Lower  Center   Upper  --------+---------+---------+---------+-
 5.0        -10.089   5.684  21.457           (------------*------------)
 7.5        -12.464   2.968  18.401         (-----------*-----------)
10.0        -10.922   4.851  20.624           (------------*------------)
12.5        -19.510   1.520  22.550    (-------------------*-------------------)
                                       --------+---------+---------+---------+-
                                             -12         0        12        24


Time(mins) =  5.0 subtracted from:

Time(mins)    Lower  Center   Upper  --------+---------+---------+---------+-
 7.5         -9.398  -2.715   3.967           (-----*----)
10.0         -8.268  -0.832   6.603            (-----*------)
12.5        -19.937  -4.164  11.609   (------------*------------)
                                       --------+---------+---------+---------+-
                                             -12         0        12        24


Time(mins) =  7.5 subtracted from:

Time(mins)    Lower  Center   Upper  --------+---------+---------+---------+-
10.0         -4.800   1.883   8.565           (-----*----)
12.5        -16.881  -1.448  13.984     (------------*------------)
                                       --------+---------+---------+---------+-
                                             -12         0        12        24


Time(mins) = 10.0 subtracted from:

Time(mins)    Lower  Center   Upper  --------+---------+---------+---------+-
12.5        -19.104  -3.331  12.442    (------------*------------)
                                       --------+---------+---------+---------+-
```

EFFECT OF TIME

The optimum time for the maximum amylase inihibitory activity was found to be 7.5 minutes. The range of time was determined based on the practical and economical aspects. It was probably because longer time will increase cost. However, even at longer time, there was not much difference in extraction of phenolic compounds when compared to shorter time. Time does have a significant effect on extraction of phenolic compound. It was obvious that a shorter time will extract the same amount of phenolic extracts as longer time while saving cost and is more practical. Excessive extraction time is not useful to extract more phenolic antioxidants
(Silva et al. 2007). Polymers and wall-bound phenolics retained in cells that was extracted out as well as the polymerization reaction that occurs and new components produced probably a reason to the increase of total phenolic contents at a longer extraction time (Spigno, 2007). The short extraction time was aimed to decrease ploy phenolics degradation and long ones to maximize extraction, but the concentration of tannins in the extracts tend to fall rather than rise after few hours (Mane, 2007). The above results also shows the significant increase in inhibition due to increase in temperature.

Table4.2.4.3:One-way ANOVA: Output Amylase inhibition (%) versus Time(mins)

```
Source      DF     SS    MS     F      P
Time(mins)   4   64.5  16.1  0.62  0.649
Error       26  670.9  25.8
Total       30  735.4
```

-12 0 12 24

4.3 IDENTIFICATION AND ISOLATION OF FLAVONOIDS BY THIN LAYER CHROMATOGRAPHY(TLC) AND PREPARATIVE THIN LAYER CHROMATOGRAPHY(PTLC)

TLC is a simple technique used for identifying various compounds. The R_f values of standards such as quercetin and rutin were found to be 0.856 and 0.871, respectively. The plum seed extract has revealed an R_f of about 0.678 while the seed extract of dates has revealed the R_f value as 0.878 proving the possibilites of the presence of flavonols and their glycosides. The UV detection of flavonoids in both the extracts has also proved the presence of phenolic acids.

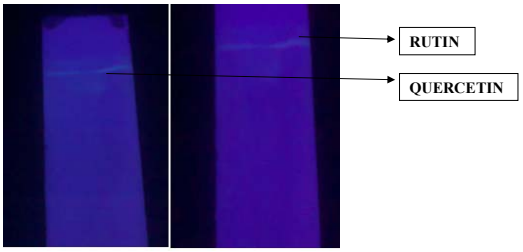


Figure 4.3.1.1: TLC analysis of standards visualised under far UV light

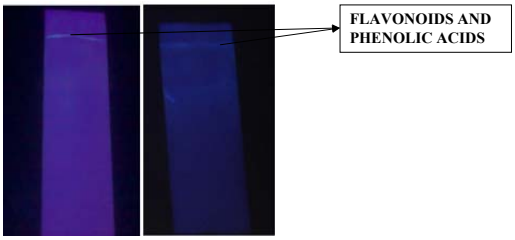


Figure 4.3.1.2: Identification of flavonoids and phenolic acids of *Prunus domestica* and *Phoenix dactylifera* by TLC under UV light after spraying ammonium hydroxide

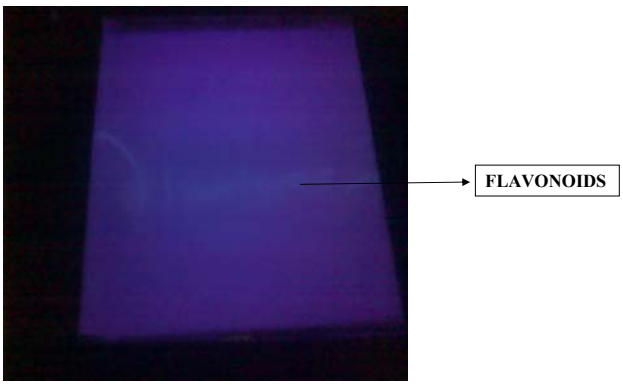


Figure 4.3.2.1: Isolation of flavonoids by PTLC technique

The colour from the above plates shows the presence of flavonoids. Flavonoids are a major class of oxygen-containing heterocyclic natural products that are widespread in green plants. Isolated flavonoids have been shown to possess a host of important biological activities, including antifungal and antibacterial activities (Qudsia Kanwal, 2009). One of the major problems with the preparative separation of flavonoids is their sparing solubility in solvents employed in chromatography. Moreover, the flavonoids become less soluble as their purification proceeds. There is no single isolation strategy for the separation of flavonoids and one or many steps may be necessary for their isolation. The choice of method depends on the polarity of the compounds and the quantity of sample available (Hasler, 1992).

4.4 PRESSURISED HOT WATER EXTRACTION (PHWE)

PHWE is done at Temperature of 150˚C for 5 minutes where Supercritical state is obtained. Here the amylase inhibition was found to be 94.29% and Lipase inhibition is found to be 22.85%. Supercritical state is achieved when the temperature and the pressure of a substance is raised over its critical value. Supercritical water extraction was found to recover all the antraquinones present in the roots even at 170 °C. Increase in water temperature increases the solubility of the organic compound. (Shotipruk *et al*., 2004) Super critical fluid plays a major role in PHWE.

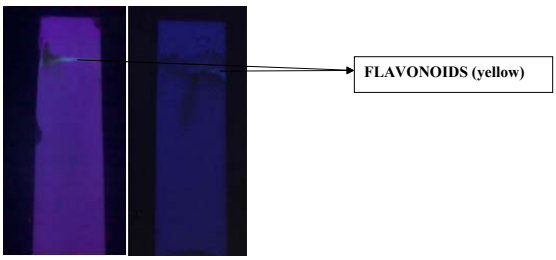


Figure 4.3.1.3: Identification of flavonoids and phenolic acids of *Phoenix dactylifera* by TLC under UV light after spraying Aluminium Chloride

Thin-layer chromatography (TLC), polyamide chromatography, and paper electrophoresis were the major separation techniques for phenolics. Of these methods, TLC is still the workhorse of flavonoid analysis. It is used as a rapid, simple, and versatile method for polyphenolics in plant extracts and in fractionation work. (Yang, 2001).

A classical stationary phase of silica gel is widely used to separate many apolar flavonoids such as flavonols and isoflavonoids. Various mobile phases like ethanol/water (55:45, v/v), petroleum ether/ethyl acetate (70:30), petroleum ether/acetone/formic acid (35:10:5), chloroform/ethyl acetate (60:40), toluene/chloroform/acetone (40:25:35), *n*-hexane/ethyl acetate/acetic acid (31:14:5) or (60:40:3) and chloroform/methanol/formic acid (44.1:3:2.35) has been reported for effective identification and separation of polyphenolics (G´omez-Caravaca, 2005). Similarly, in most of the TLC polyphenol compounds analysis 366 nm was adopted as a common wavelength (Vembu, 2012).

4.3.2 PREPARATIVE THIN LAYER CHROMATOGRAPHY (PTLC)

Preparative TLC has been cosidered as the most basic equipment for the best separation and purification of polyphenols like flavonoids and phenolic acids.It is also used for monitoring the reactions in a large scale manner and is a method that requires the least financial outlay. It is normally employed for milligram quantities of sample. Preparative TLC in conjunction with open-column chromatography used for purifying natural products, although centrifugal TLC have found application in the separation of flavonoids (Andrew marston, 2006). Single strong spot observed in the PTLC of plum seed extract was sucessfully isolated and subjected for LC-MS analysis.

Table 4.4.1 % Inhibition of Amylase and lipase inhibitors.

INHIBITORS IN PHWE	%INHIBITION
Amylase	94.29
Lipase	22.85

The supercritical fluid has characteristics of both gases and liquids. Compared with liquid solvents, supercritical fluids have several major advantages:

(1) The dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or/and temperature;

(2) The supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer (Wang and Weller, 2006). The solubility of a target compound in a supercritical fluid is a major factor in determining its extraction efficiency. The temperature and density of the fluid control the solubility.

Plant particle size is an also important for a good SFE process. Large particles may result in a long extraction process because the process may be controlled by internal diffusion. However, fine powder can speed up the extraction but may also cause difficulty in maintaining a proper flow rate (Chemat *et al*., 2004).

The extraction time has been proven to be another parameter that determines extract composition. Lower molecular weight and less polar compounds are more readily extracted during supercritical fluid extraction since the extraction mechanism is usually controlled by internal diffusion (Cherchi *et al*., 2001)

4.5 LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

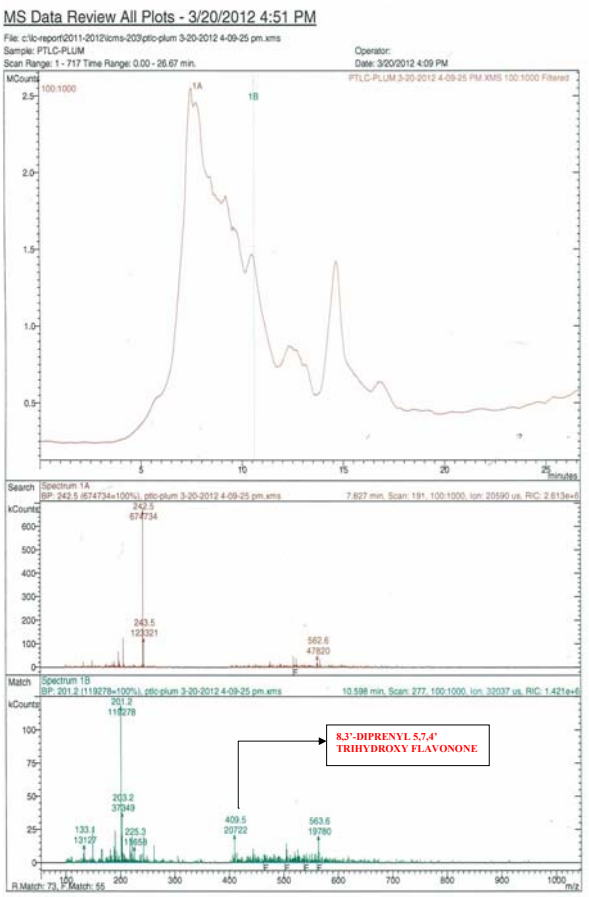
Phenolic acids and flavonoids seem to be universally distributed in the plant kingdom, essential for the growth and reproduction of plants, and are produced as a response to defence against pathogens. The importance of antioxidant activities of phenolic and flavonoid compounds and their possible usage 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.

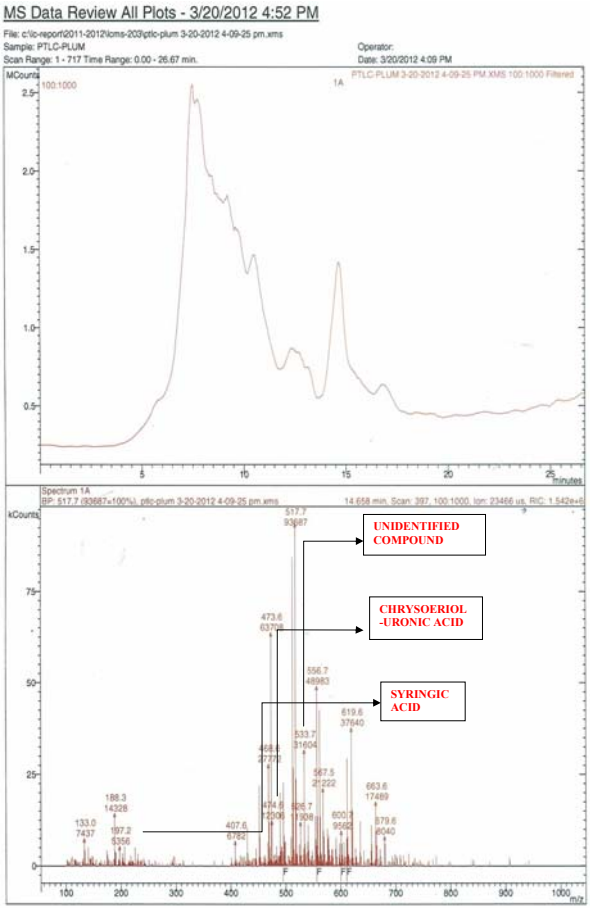
Due to the abundance of different classes of flavonoids and phenolic acids and their diverse chemical properties, a variety of separation and identification methods have been developed using Thin Later Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and Gas Liquid Chromatography (GLC).

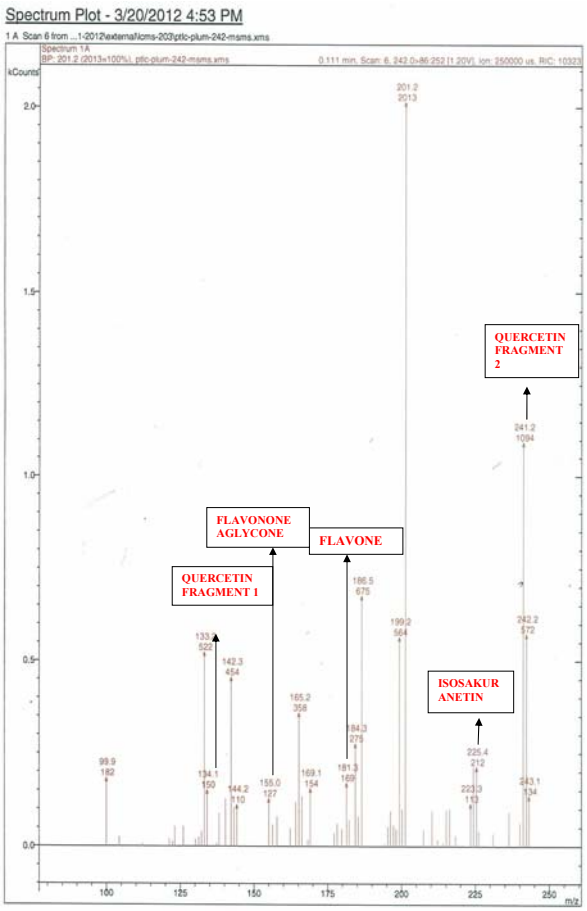
The PTLC isolate of aqueous plum seed extract was sent to IIT BOMBAY to perform LCMS with ESI for first peak. The instrument had the following specifications:

1. Direct Infusion Mass with ESI and APCI Negative and Positive mode ionization, mass ranging from 100 to 1000 amu.
2. LCMS ION TRAP
3. HPLC with PDA Detector
4. HPLC PDA Detector- Mass Spectometer.
5. The molecular masses of phenolic acids were assigned by electrospray ionization mass.

The MS data is as follows.







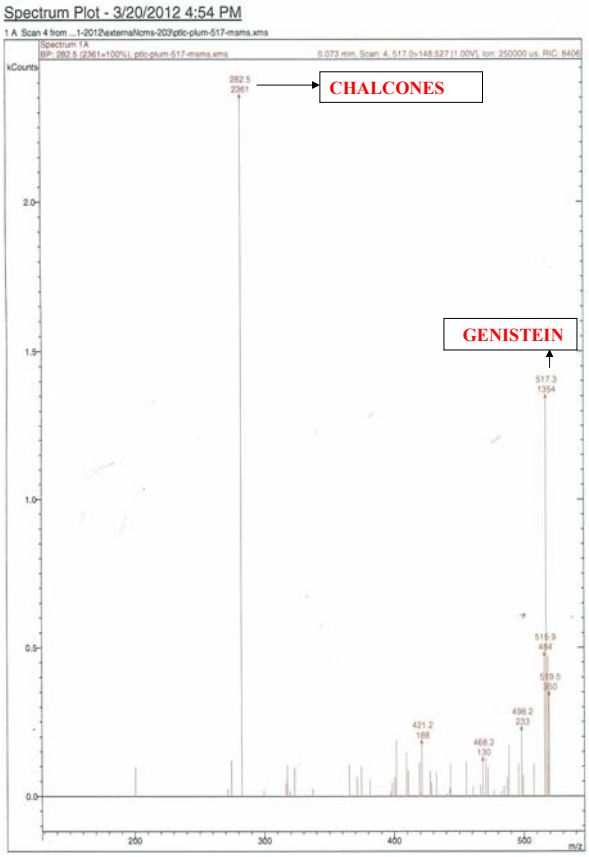


Fig.4.5.1 Chromatogram and MS Data.

Fragmentation behaviour of phenolic acids and flavonoids 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 and UV data together with HPLC of flavonoids and phenolic acids allowed structural characterization of compounds present in the plum seed extract. The compounds present are

Table 4.5.1 : Validation of LC-MS report

S.NO	COMPOUND	MOLECULAR WEIGHT	MS^1 PARENT ION	MS^2 BASE PEAK M/Z
1.	Quercetin fragment 1	134	137	153
2.	Quercetin fragment 2	241.2	241	201
3.	Flavonol	125	121	
4.	Flavone	181.3	179	
5.	Flavonone aglycone	155	151	107
6.	Isosakurametin	225.4	226	
7.	Genistein	517	519	
8.	Chalcone	282.5	280	
9.	8,3'-diprenyl 5,7,4' trihydroxy flavonone	409	409.5	
10.	Syringic acid	197.2	197	
11.	Chrysoeriol- uronic acid	474.6	475	298.9
12.	Undentified compound	533.7		

According to Kayano *et al.,* (2011) the MS/ MS spectrum of standard quercetin revealed two distinct peaks at *m/z* 137 and 153 and base peaks with *m/z* 241 and 201. A similar MS/ MS spectrum has been recorded in the present studies at *m/z* 134.1 and 155 and base peaks at *m/z* 241.2 and 201.2 has proved the presence of **Quercetin** in the sample.

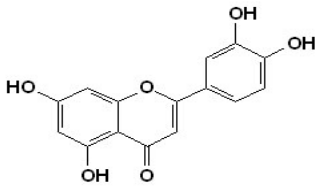


Figure 4.5.2: Structure of Quercetin

Previous studies on the fragmentation MS/ MS spectra of Syringic acid has revealed the base peaks at *m/z* 197. A similar documentation was also made in the present investigation as m/z 197.2 and this revealed the presence of **Syringic acid** in the sample. (Hung *et al*., 2011)

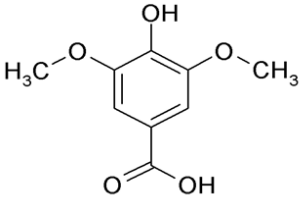


Figure 4.5.3: Structure of Syringic acid.

Similarly, an exhaustive investigation on the fragmentation of **flavonol, flavone** and **flavonone aglycones** have been performed by Fabre *et al.,* (2001). According to their studies, a consecutive lossess of CO and CO_2 have been observed in quercetin fragmentation that yielded a resonance-stabilized ion. Another different fragmentation mode was also observed i.e., loss of B ring (*m/z* 121) with subsequent retrocyclisation yielded a $^{1,2}A^-$ ion (*m/z* 179) and subsequent loss of –CO (*m/z* 151) and CO_2 (*m/z* 107). The present investigation was well agreed with the above mentioned results with a loss of B ring (*m/z* 125) and subsequent retrocyclisation with the formation of $^{1,2}A^-$ ion (*m/z* 181.3). The loss of –CO has yielded (*m/z* 155) which proved the presence of quercetin in the sample.

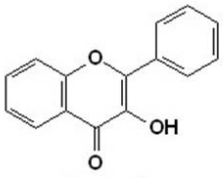


Figure 4.5.4: Structure of Flavonol

Previous studies on the fragmentation MS/ MS spectra of Isosakurametin (flavonone) has revealed the base peaks at *m/z* 226 with a loss of CO2 and CH3. A similar documentation was also made in the present investigation i.e., a base peak at *m/z* 225.4 has been recorded which proved the presence of **Isosakurametin** (Nakatani *et al*., 2000)

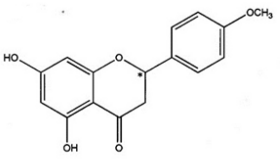


Figure 4.5.5: Structure of Isosakurametin.

According to Mebrahtu *et al*., 2004 the MS/MS spectrum of genistein glucosyl melanoate revealed 2 distinct peaks (m/z 519 and 291). A similar MS/MS spectrum has been recorded in the present study at (m/z 517) has proved the presence of **Genistein** in the sample. Previous studies on the fragmentation of MS/MS Ion peaks at (*m/z* 251, 285 and 271) are the characteristic positive ions of the three isoflavone aglycone moieties, daidzein, glycitein and genistein. (Yu wang *et al*., 2008)

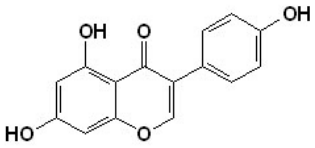


Figure 4.5.6: Structure of Genistein

According to Ana *et al*., 2009 the MS/MS spectrum of Chrysoeriol uronic acid revealed 2 distint peaks were obtained as m/z 475 and 298.9. A similar MS/MS spectrum has been recorded in the present study at m/z 474.6 has proved the presence of Chrysoeriol uronic acid in the sample.

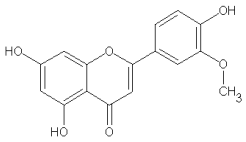


Figure 4.5.7: structure of Chrysoeriol

According to Chedwick *et al* 2004 , the EIMS spectrum of compound **1** revealed [m+] ion peak at m/z 280.163, indicating the molecular formula C18H16O3 (cal. m/z 280). Further peaks were obtained at m/z 279.1588 due to proton migration. Compound **1** was identified **as 6, 4'** dihydroxy 3*l* propen chalcones. A similar documentation was also made in present investigation at base peak m/z 282.5 has been recorded which proves the presence of **chalcone derivative**.

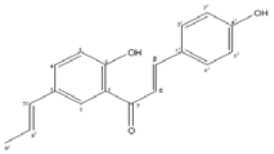


Figure 4.5.8: Structure of Chalcone

The MS spectrum recorded at *m/z* 409.5 was found to be **8, 3'-diprenyl 5, 7, 4' trihydroxy flavonone** and this has been previously documented as *m/z* 409 from the root of *F. Strobilifera* (Madan *et al.,* 2008)

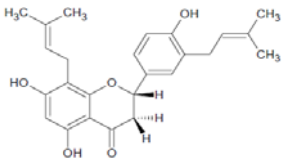


Figure 4.5.9: Structure of 8, 3'-diprenyl 5, 7, 4' trihydroxy flavonone

CHAPTER 5

CONCLUSION

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose level. The regulation of plasma glucose level and glucose metabolism is one of the most important homeostatic mechanisms operated in an *in vivo* system. This can be achieved by the inhibition of pancreatic α-amylase and lipase. Many plant sources have been exploited to identify the phytochemicals that may efficiently inhibit pancreatic amylase and lipase. In this study, six different fruit seeds were selected and screened for the maximum pancreatic amylase and lipase inhibition. Of the six fruit seeds, hot water extraction of plum was found to exhibit the maximum pancreatic amylase inhibitory activity (98.73%) while dates seeds showed the lowest activity (51.2%). Similarly, the highest lipase inhibition was recorded in mango seeds (45.71%) and lowest in dates seeds (0.01%). Response surface methodology (RSM) meditaed statistical optimization has proved that at 70°C, 150rpm, 7.5 minutes and 1:15 material ratio was the optimal conditions for highest pancreatic amylase inhibitory activity (99.24%). Similarly, 80°C, 200 rpm, 10 minutes and 1:20 material ratio was observed as the optimal conditions for highest lipase inhibitory activity (31.42%). Simultaneous pressurized hot water extraction (PHWE) has showed that at 180°C with 5 minutes time duration the *Prunus domestica* (plum) seed has showed potent pancreatic amylase (94.29%) and lipase (22.85%) inhibitory activities, respectively. The HPLC/ DAD /MS analysis of PTLC (Preparative Thin Layer Chromatography) isolates revealed the compounds like Quercetin, Genistein, Chalcone derivative, 3,8'-diprenyl,5,7,4'-trihydroxy flavonone, Isosakurametin and 2 unknown compounds. It was concluded that the selected seeds possessed high pancreatic amylase inhibitory activity than lipase activity.

CHAPTER 6

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