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SCREENING OF α-AMYLASE INHIBITOR AND ANTIOXIDANT ACTIVITY IN LEAFY VEGETABLES

A PROJECT REPORT

Submitted by

V. NANDHINI

NIJI ROY THOMAS

V. NITHYA

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Coimbatore-641006

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BONAFIDE CERTIFICATE

Certified that this project report titled **"SCREENING OF α-AMYLASE INHIBITOR AND ANTIOXIDANT ACTIVITY IN LEAFY VEGETABLES"** is the bonafide work of **"Ms.Nandhini.V, Ms.Niji Roy Thomas, Ms.Nithya.V"** who carried out the project work under my supervision.

SIGNATURE 27/4/09

Dr. P. Rajasekaran
Professor and Head

Department of Biotechnology
Kumaraguru College of Technology
Coimbatore- 641 006

SIGNATURE

Dr. B. Thayumanavan
Supervisor
Professor
Department of Biotechnology
Kumaraguru College of Technology
Coimbatore- 641 006

CERTIFICATION OF EVALUATION

COLLEGE : Kumaraguru College of Technology

BRANCH : Biotechnology

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NAME OF THE STUDENTS	TITLE OF THE PROJECT	NAME OF THE SUPERVISOR WITH DESIGNATION
V. NANDHINI NIJI ROY THOMAS V. NITHYA	SCREENING OF α-AMYLASE INHIBITOR AND ANTIOXIDANT ACTIVITY IN LEAFY VEGETABLES	Dr B. THAYUMANAVAN Professor

The report of the project work submitted by the above students in partial fulfilment for the award of the degree of Bachelor of technology in Biotechnology of Anna University was evaluated and confirmed.

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V. NANDHINI

NIJI ROY THOMAS

V. NITHYA

ABSTRACT

1. ABSTRACT

Diabetes is a metabolic disorder where in human body does not produce or properly uses insulin, a hormone that is required to convert sugar, starches into energy. Major complications of diabetes is post-prandial hyperglycemia(PPHG). α-Amylase inhibitor is used in the treatment of diabetic patients. Pancreatic α-amylase hydrolyses starch to maltose and oligosaccharides in the small intestine. Inhibition of this enzyme reduces the rate of digestion of starch and results in a decrease in the post prandial blood glucose levels in the diabetic patients. Free radicals are produced in the body either naturally or on the exposure of radiation, cigarette smoke, etc. and can be implicated in many diseases like diabetes, arthritis, cancer, Parkinson's disease, Alzheimer's disease, aging and other age related- diseases. In most cases dietary supplement of antioxidants is recommended. Plants are a very good source of α-amylase inhibitor and antioxidants and other nutrients. This project aims to screen twelve such leafy vegetables for the presence of α-amylase inhibitor and antioxidants. The plants chosen for the study were ***Sesbania grandiflora, Marsilea quadrifolia, Eclipta prostrata, Althernanthera sessilis, Cardiospermum halicacabum, Centella asiatica, Solanum nigrum, Hibiscus cannabinus, Moringa oleifera, Basella alba, Amaranthus blitum, Portulaca oleracea.*** The plant constituents were extracted using water. These extracts were subjected to quantitative assay for α-amylase inhibitor assay and *in vitro* free radical scavenging assays such as DPPH scavenging assay and FRAP assay. Estimation of total phenols was also carried out. The results were analyzed and all the plants were observed to possess significant α-amylase inhibiting and antioxidant potential. *Portulaca oleracea* and *Cardiospermum halicacabum* possessed relatively high α-amylase inhibitor and antioxidant activities.

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LIST OF ABBREVIATION

µg	Microgram
µl	Microliter
DPPH	1, 1-diphenyl -2-picryl hydrazyl
FRAP	Ferric ion Reducing Antioxidant power
$FeCl_3$	Ferric Chloride
GDM	Gestational Diabetes Mellitus
g	Gram
IDDM	Insulin Dependent Diabetes Mellitus
mg	Milligram
min	Minute
ml	Millilitre
M	Molarity
NIDDM	Non Insulin Dependent Diabetes Mellitus
PPHG	Post-Prandial hyperglycemia
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
NaOH	Sodium hydroxide
TCA	Trichloro Acetic acid

INTRODUCTION

1. INTRODUCTION

1.1 Diabetes mellitus

Diabetes is defined as a state in which homeostasis of carbohydrate and lipid metabolism is improperly regulated by the pancreatic hormone, insulin resulting in an increased blood glucose level. Diabetic disease is increasing rapidly and consumes vast amounts of resources in all countries (Maher *et al.*, 2006). In India, the incidence of diabetes will increase by 195% in 2025 and the sufferers will be young age individuals (Analava, 2007). The chronic hyperglycaemia of diabetes is associated with the long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Maher *et al.*, 2006).

Diabetes are classified into three type: Type I Diabetes Type II Diabetes and Gestational diabetes

Type I diabetes is also known as **Insulin Dependent Diabetes Mellitus** (IDDM). It is an auto- immune disease. The body's immune system attacks the cells in the pancreas that produce insulin. A predisposition to develop type 1 diabetes may run in families, but genetic causes (a postitive family history) is much more common for type 2 diabetes. Environmental factors, including common unavoidable viral infections may also contribute. Type 1 diabetes is most common in people of non-Hispanic, Northern European descent (especially Finland and Sardinia), followed by African Americans, and Hispanic Americans. It is relatively rare in those of Asian descent. Type 1 diabetes is slightly more common in men than in women.

Type II diabetes is also known as **Non Insulin Dependent Diabetes Mellitus** (NIDDM). It has strong genetic links, meaning that type 2 diabetes tends to run in families. Several genes have been identified and more are under

study which may relate to the causes of type 2 diabetes. Risk factors for developing type 2 diabetes include the following:

- High blood pressure
- High blood triglyceride (fat) levels
- Gestational diabetes or giving birth to a baby weighing more than 9 pounds
- High-fat diet
- High alcohol intake
- Sedentary lifestyle
- Obesity or being overweight
- *Ethnicity,* particularly when a close relative had type 2 diabetes or gestational diabetes: certain groups, such as African Americans, Native Americans, Hispanic Americans, and Japanese Americans, have a greater risk of developing type 2 diabetes than non-Hispanic whites.
- *Aging*: Increasing age is a significant risk factor for type 2 diabetes. Risk begins to rise significantly at about age 45 years, and rises considerably after age 65 years.

Gestational diabetes (or **gestational diabetes mellitus,GDM**) is a condition in which women without previously diagnosed diabetes exhibit high blood glucose levels during pregnancy. It is formally defined as "any degree of glucose intolerance with onset or first recognition during pregnancy".

Gestational diabetes generally have few symptoms and it is most commonly diagnosed by screening during pregnancy (Buchanan *et al.*, 2003). Diagnostic test detect inappropriate high levels of glucose in the blood samples. Gestational diabetes affect 3-10% of the pregnancies, depending on the population studied. No specific cause has been identified, but it is believed that

the hormones produced during pregnancy increase women's resistance to insulin, resulting in impaired glucose tolerance.

Babies born to mothers with gestational diabetes are at increased risk of problems typically such as being large for gestational age (which may lead to delivery complications), low blood sugar, and jaundice. Gestational diabetes is a treatable condition and women who have adequate control of glucose levels can effectively decrease these risks (Holt *et al.*, 2009).

The drugs used to treat diabetes are:

- Sulfonylureas- improve insulin production.
- Metformin (Biguanide class)- regulates inappropriate release of glucose by liver and attenuate insulin resistance to some extent.
- Thiazolidinediones(glitazones)- attenuates insulin resistance.
- α-amylase inhibitor inhibits starch digestion in intestine.
- α-glucosidase inhibitor inhibits digestion of maltose and related oligosaccharides.

But α-amylase and α-glucosidase inhibitors are of microbial origin (acarbose) which are associated with gastro-intestinal side effects such as abdominal pain, flatulence and diarrhoea in the patients (Mioko *et al.*, 2001). Therefore it is necessary to identify the amylase inhibitors from plant sources having lesser side effects.

1.2 Antioxidant

Free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals. Free radicals are atomic or molecular species with unpaired electrons. These unpaired electrons are highly reactive. They are very unstable and react quickly with other compounds, trying to capture the needed

electron to gain stability. The attacked molecules now become free radicals, thus initiating the chain reaction (Mark, 1998). Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions.

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from oxygen. The formation of oxygen radicals could be the reason for the damaging effects of O_2. A class of enzymes called superoxide dismutases (SOD) is responsible for the catalytic removal of superoxide free radical, (Lee *et al.*, 2001). An average person has around 10,000–20,000 free radicals attacking each body cell every day. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide are often generated as by-products of metabolic processes. *In vivo*, they may also cause great damage to cell membranes and DNA.

Antioxidants are capable of stabilizing, or deactivating free radicals before they attack the cells (Mark, 1998).Antioxidants are defined as the substance that when present in low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substance (Halliwell and Gutteridge, 1989). Synergism, antagonism, co-antioxidants and oxidation retarders are the other useful concepts related to antioxidants. Synergism can be defined as the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than if they were alone. Antagonism can be defined likewise by substituting "more" with "less", whereas co antioxidants may be defined by substituting "more" with "same". The compounds that reduce the rate of oxidation without showing a distinct lag phase of oxidation are retarders of oxidation.

Antioxidants are divided into two classes: preventive antioxidants and chain breaking antioxidants. Preventive antioxidants inhibit oxidation by reducing the rate of chain initiation. Preventive antioxidants convert the

hydroperoxides to molecular products that are not potential sources of free radicals (Burton *et al.*, 1985). Most biological preventive antioxidants are also peroxide decomposers. Antioxidants can also be manufactured synthetically. These belong to the class of synthetic antioxidants. The main disadvantage with these antioxidants is their side effect when taken *in vivo* (Chen *et al.*, 1992). Most of the natural antioxidants are found to have higher antioxidant activity when compared with that of the synthetic ones. Previous epidemiological studies have consistently shown that consumption of fruits and vegetables has been associated with reduced risk of chronic diseases, such as cardiovascular diseases and cancers (Sargeant *et al.*, 2008) and neurodegenerative disorders, including Parkinson's and Alzheimer's diseases (Gella and Durany, 2009)as well as inflammation and problems caused by cell and cutaneous aging. Diabetic disease is increasing rapidly and consumes vast resources in all countries. The oxidative products, mainly the superoxide anion radical(O_2^-), from diabetic monocytes during oxidative stress lead researchers to give more attention to the protective functions of naturally occurring antioxidants and α-amylase inhibitors in the cells of organism containing them(Maher *et al.*,2006)

Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (Hollman, 2001). Common antioxidants include Vitamin A, Vitamin C, Vitamin E, and certain compounds called carotenoids (like lutein and beta-carotene) (Hayek, 2000). These plant-based dietary antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (Holt *et al*, 2009).

Green leafy vegetables occupy an important place among the food crops as these provide adequate amounts of many vitamins and minerals for humans.

They are rich source of carotene, ascorbic acid, riboflavin, folic acid and minerals like calcium, iron and phosphorous. In nature, there are many underutilized greens of promising nutritive value, which can nourish the ever-increasing human population (Gupta and Prakash, 2009). Many of them are resilient, adaptive and tolerant to adverse climatic conditions.

The nutritional value of green leafy vegetables and the lack of awareness of the antioxidant potential of some unexploited green leafy varieties has provoked us to screen the antioxidant activity and α-amylase inhibitor of twelve under-exploited green leafy vegetables such as ***Sesbania grandiflora, Marsilea quadrifolia, Eclipta prostrata, Althernanthera sessilis, Cardiospermum halicacabum, Hibiscus cannabinus, Centella asiatica, Solanum nigrum, Moringa oleifera, Basella alba, Amaranthus blitum and Portulaca oleracea.***

The main objectives of our study are:

- To screen and compare the *in vitro* antioxidant activities of the twelve plant varieties by different free radical scavenging assays.
- To determine the inhibition of α-amylase activity and
- To estimate total phenol content in the selected plant varieties and to correlate with amylase inhibitory and antioxidant activites.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with the long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Maher *et al.*, 2006). Diabetes is a progressive disease and is one of the major killers in recent times. World Health Organisation (WHO) suggests that world-wide the global population is in the midst of a diabetes epidemic with people in South East Asia and Western Pacific being mostly at risk. The most prevalent form of diabetes is non-insulin dependent diabetes mellitus (NIDDM/ type II). The treatment of type II diabetes is complicated by several factors inherent to the disease and elevated post prandial hyperglycemia (PPHG) is one of the risk.

PPHG is elevated by the action of α-amylase and α-glucosidase, a class of enzymes that helps in the breakdown of complex carbohydrates into simple sugars, such as maltose and glucose (Menakshi *et al.*, 2008). Pancreatic α-amylase hydrolyses starch to maltose and oligo-saccharides in the small intestine, whereas, membrane bound intestinal α-glucosidase hydrolyses di- and oligosaccharides to glucose.

The possible causative factors of diabetes are as follows:

- Diet
- Body mass index
- Effects of migration
- Birth weight and childhood developments
- Effects of life style
- Economic impact of disease

- Other factors(Analava,2007)

2.2 Amylase

α-Amylase is an enzyme that breaks starch into sugar. Amylase is present in human saliva, where it begins the chemical process of digestion. Foods that contain much starch but little sugar are rice and potato. The pancreas also makes amylase to break down dietary starch into di- and trisaccharides which are converted by other enzymes to glucose to supply the body with energy. Plants and some bacteria also produce amylase. All amylases are glycoside hydrolases and act on α-1, 4-glycosidic bonds.

2.3 Classification

Amylase is generally classified into three types namely:

- α-amylase
- β-amylase
- γ-amylase

α-Amylase

α-Amylase (EC 3.2.1.1) (alternate names: 1,4- α-D-glucan glucanohydrolases glycogenase) are calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and 'limit dextrin' from amylopectin. Because it can act anywhere on the substrate randomly, α-amylase tends to be faster-acting than β-amylase (Heidari *et al.*, 2005). In animals, it is a major digestive enzyme and its optimum pH is 6.7 to 7.0.

Salivary amylase (ptyalin)

Amylase is found in saliva and breaks starch down into maltose and dextrin. This form of amylase is also called ptyalin. It will break large, insoluble starch molecules into soluble starches (amylodextrin, erythrodextrin, achrodextrin) producing successively to smaller starches, and ultimately maltose. Ptyalin acts on linear α(1,4) glycosidic linkages, but compound hydrolysis require an enzyme which acts on branched products. Salivary amylase is inactivated in the stomach by acid pH (Nagaraj and Pattabiraman, 1985). In gastric juice adjusted to pH 3.3, ptyalin was totally inactivated in 20 minutes at 37°C. In contrast, 50% of amylase activity remained after 150 minutes of exposure to gastric juice at pH 4.3 (Rosenblum *et al.*, 1988). Both starch, the substrate of ptyalin, and the product (short chains of glucose) are able to partially protect it against inactivation by gastric acid.

Genetic variation in human ptyalin (salivary amylase)

The salivary amylase gene has undergone duplication during evolution, and DNA hybridization studies indicate that many individuals have multiple tandem repeats of the gene. The number of gene copies correlates with the levels of salivary amylase, as measured by protein blot assays using antibodies to human amylase. Gene copy number is associated with apparent evolutionary exposure to high starch diets. Increased copy number of the salivary amylase gene may have enhanced survival coincident to a shift to a starchy diet during human evolution .

Pancreatic amylase

Pancreatic α-amylase randomly cleaves the α(1-4) glycosidic linkages of amylase to yield limit dextrin, maltose and maltotriose. It adopts a double displacement mechanism with retention of anomeric configuration. α- amylase hydrolyses starch to maltose and oligosaccharides in the small intestine, whear

as, membrane bound intestinal α- glucosidase hydrolyses di- and oligosaccharides to glucose. (Karthic et al.,2008). Inhibition of these enzymes reduces the rate of digestion of starch and result in a decrease in the post prandial blood glucose levels in diabetic patients.

2.4 Plants

Ayurveda and other traditional approaches as described anti-diabetic potentials in more than 800 plants in the sub-continent. Vegetables are among the numerous plant adjuncts tried for the treatment of diabetes. A few vegetables that are commonly consumed in india have being claimed to possess anti-diabetic potency. Majority of these studies have documented the beneficial effect of fruit of bitter gourd and leaf of ivy gourd when administered orally as a single dose and limited number of studies on other vegetables such as cabbage, leafy vegetables, beans and tubers have shown the beneficial hypoglycaemic influence in both experimental and humans.(Patel and Srinivasan, 1997).

As per Bazzano *et al.*,(2008), maltivatiate-adjusted diabetes hazard and consumption of fruits n leafy vegetables associated with a lower hazard of diabetes and appears to have a protective effect against coronary heart disease.

It is estimated that more than 200 species of plants exhibit hypoglycaemic properties. Some of the most extensively studied plants are *Syzygium cumini* , wheat and roselle tea(*Hibiscus sabdariffa*) of *Syzygium cumini* Chloroform extract has effective inhibition IC(50)=5.06μg/ml on porcrine α- amylase and *B.spectabilis* aqueous extract showed inhibition(IC(50)=11.16 μg/ml) with murine pancreatic glucosidase. The effect could be possibly because of the high- phenolic contents present in the crude extracts (Meenakshi et al.,2008).

Inhibitor activity of common buckwheat *(Fagopyrum esculentum)* and tartary buckwheat *(Fagopyrum tartaricum)* showed that tartary buckwheat flour had an average higher inhibitory activity (44.8±3.2 *DIg)* than commom

buckwheat flour (37.8±2.4 *DIg*). No significant regional differences were detected among the same species in the level of inhibitory activity. It was also demonstrated that the inhibitor was unthermostable. When it was heated at 80°C, 90°C and 100°C for different time respective, a fat lot or no inhibitory activity was displayed, during buckwheat seeds germination, the inhibitory activity was gradually lost. The study suggested that α-amylase-inhibitor in buckwheat flour might be not act as an anti-nutritional factor.

Six ethno-botanically known plants having antidiabetic property namely, *Azadirachta indica Adr. Juss.; Murraya koenigii* (L.) *Sprengel; Ocimum tenuflorum* (L.) (syn: Sanctum); *Syzygium cumini* (L.) *Skeels* (syn: Eugenia jambolana); *Linum usitatissimum* (L.) and *Bougainvillea spectabilis* were tested for their ability to inhibit glucosidase activity. The chloroform, methanol and aqueous extracts were prepared sequentially from either leaves or seeds of these plants. It was observed that the chloroform extract of *O. tenuflorum*; *B. spectabilis*; *M. koenigii* and *S. cumini* have significant alpha-amylase inhibitory property. Plants extracts were further tested against murine pancreatic, liver and small intestinal crude enzyme preparations for glucosidase inhibitory activity. The three extracts of *O. tenuflorum* and chloroform extract of *M. koenigii* showed good inhibition of murine pancreatic and intestinal glucosidases as compared with acarbose, a known glucosidase inhibitor (Bhat *et al.*,2008).

The research work carried out on enzyme inhibitors, antioxidants and related studies in the plants selected for the study are reviewed and presented below.

2.4.1 *Centella asiatica:*

A study was carried out to investigate the effects of *Centella asiatica* leaf on lipid metabolism of oxidatively stressed rats. The rats were fed 0.1% hydrogen peroxide (H_2O_2) with either 0.3% (w/w) *C. asiatica* extract, 5% *C. asiatica* powder (w/w), or 0.3% (w/w) alpha-tocopherol for 25 wk. Results of

the study showed that *C. asiatica* powder significantly ($P < 0.05$) lowered serum low-density lipoprotein compared to that of control rats (rats fed H_2O_2 only). At the end of the study *C. asiatica*-fed rats were also found to have significantly ($P < 0.05$) higher high-density lipoprotein and lower triglyceride level compared to rats fed only normal diet. However, cholesterol level of rats fed both *C. asiatica* extract and powder was found to be significantly ($P < 0.05$) higher compared to that of control rats. It was interesting to note that consumption of *C. asiatica* significantly decreased body and liver weights of the rats. Histological examinations revealed no obvious changes in all rats studied. Quantitative analysis of *C. asiatica* leaf (Fig.2.4.1) revealed high concentration of total phenolic compounds, in particular, catechin, quercetin, and rutin.(Hussin et al., 2009).

2.4.2 *Basella alba*

Recent evidence suggests that the vitamin A equivalency of beta-carotene from plant sources is lower than previously estimated. *Basella alba* (Fig.2.4.2) is rich in retinol. A diet providing approximately 200 microg RE/d. Mean changes in vitamin A stores in the vegetable and beta-carotene groups were compared with the mean change in the retinyl palmitate group to estimate the relative equivalency of these vitamin A sources (Haskell.,2004).

2.4.3 *Eclipta prostrata*

The plant *Eclipta prostrate* (Fig.2.4.3) is used in the traditional medical practices of India to treat hepatic diseases and hyperlipidemia. The total alcoholic extract of the plant when tested for antihyperlipidemic potential, exhibited a dose-dependent activity in albino rats when compared to standard drugs. The activity was assessed by studying the lipid profiles of serum, liver and heart of the control and drug-treated animals. The results lend support to the traditional use of *Eclipta prostrata* in the treatment of hyperlipidemia. *E.*

prostrata juice inhibited cancer invasion and migration, without affecting cell adhesion. Cell migration was inhibited in a variety of cancer cell types and in endothelial cells, with IC50 values of 31-70 μg/ml, much lower than the IC50 values for cytotoxicity of 203-1,217 μg /ml for cancer cells and >4,000 microg/ml for endothelial cells. Fifty percent inhibition of angiogenesis by *E. prostrata* juice was observed at 200 μg /egg. *E. prostrata* juice inhibited cancer and endothelial cell migration *in vitro* and also showed *in vivo* anti-angiogenic activity (Lirdprapamongkol *et al.*, 2008).

2.4.4 *Solanum nigrum*

Solanum nigrum Linne (SNL) (Fig.2.4.4) has been used in traditional Chinese medicine for centuries because of its diuretic and antipyretic effects. The crude polysaccharides isolated from *Solanum nigrum Linne* (SNL-P) has effect on tumor growth. SNL-P had a significant growth inhibition effect on cervical cancer (U14) of tumor-bearing mice (Li *et al.*, 2007).

Of the 30 plants tested, 13 showed antifungal activity (40%) against one ore more human pathogenic fungi. The strongest inhibition was exhibited by *Azima tetracantha* (fruits), *Sansevieria ehrenbergii* (fruits) and *Solanum incanum* (fruits).Ten methanol extracts, especially those of Acacia asak barks and *Solanum nigrum* fruits, showed effective free radical scavenging activities in the DPPH assay (Mohamed *et al.*,2007).

2.4.5 *Sesbania grandiflora*

The plant *Sesbania grandiflora* (Fig.2.4.5) is claimed to be useful for various ailments, and one such use is for the treatment of renal calculi. The leaf juice of *S. grandiflora* was evaluated for median lethal dose, gross behavioral changes, antiurolithiatic and antioxidant activities. The *in vivo* antioxidant parameters lipid peroxidation, glutathione reductase and catalase were monitored. The plant juice was also evaluated for scavenging of nitric oxide and

2-diphenyl-2-picryl hydrazyl free radicals. Two alpha-glucosidase inhibitors were isolated from the flowers of *Sesbania grandiflora* and named SGF60 and SGF90. The procedure involved extraction with phosphate buffer, precipitation with ammonium sulfate, ion-exchange chromatography on DEAE-cellulose and gel filtration on Superdex-200. These proteins were identified by using tandem mass spectrometry. The result showed that the plant has antidiabetic property.

2.4.6 *Alternanthera sessilis*

The leaves of *Alternanthera sessilis (Linn.)* (Fig.2.4.6) were exhaustively extracted by Soxhlet apparatus with different solvents like, petroleum ether (40-60°C), chloroform, acetone, methanol and distilled water in ascending order of the polarity. All the five extracts were subjected to antimicrobial screening by using the cup plate and turbidimetric methods. The chloroform extract among all five extracts showed maximum zone of inhibition and significant MIC values in above two methods respectively. Hence chloroform extract show maximum for the screening of wound healing activity (Sunil *et al.*, 2008).

2.4.7 *Amaranthus blitum*

The major α-amylase inhibitor (AAI) present in the seeds of *Amaranthus hypocondriacus* (Fig.2.4.7), a variety of the purified AAI strongly inhibits the a-amylase activity of insect larvae *(Zibolium castaneum* and *Prostephanus truncatus)* and does not inhibit proteases and mammalian α-amylases.

Fig 2.4.1 ***Centella asiatica***

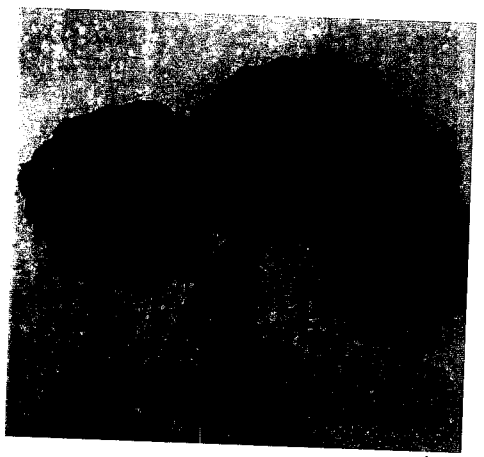


Fig 2.4.2 ***Basella alba***

Fig 2.4.3 ***Eclipta prostrata***

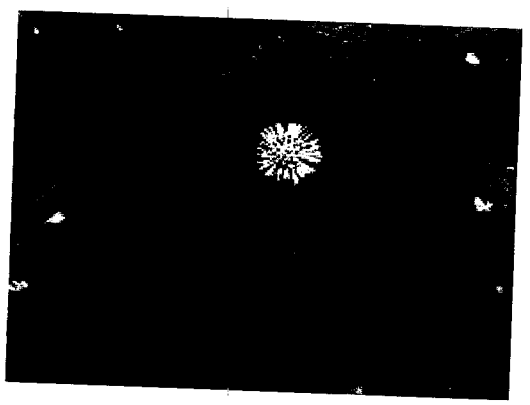


Fig 2.4.4 ***Solanum nigrum***

Fig 2.4.5 ***Sesbania grandiflora***

Fig 2.4.6 Alternanthera sessilis

Fig 2.4.7 *Amaranthus blitum*

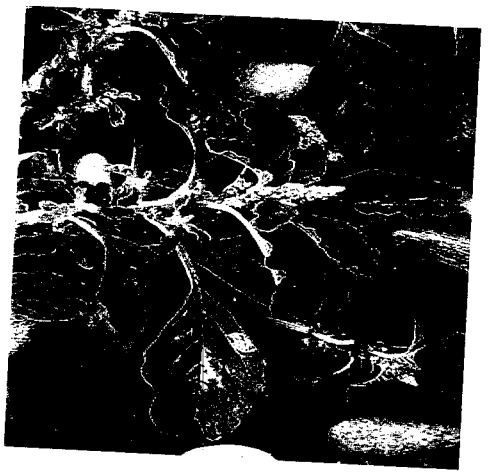


Fig 2.4.8 *Cardiospermum halicacabum*

Fig 2.4.9 *Moringa oleifera*

Fig 2.4.10 *Portulaca oleracea*

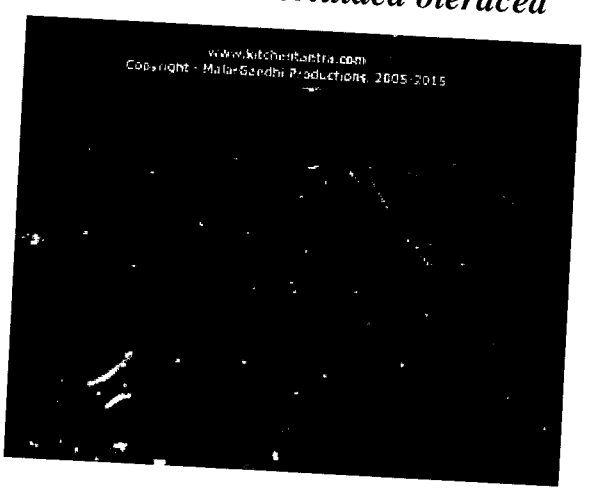
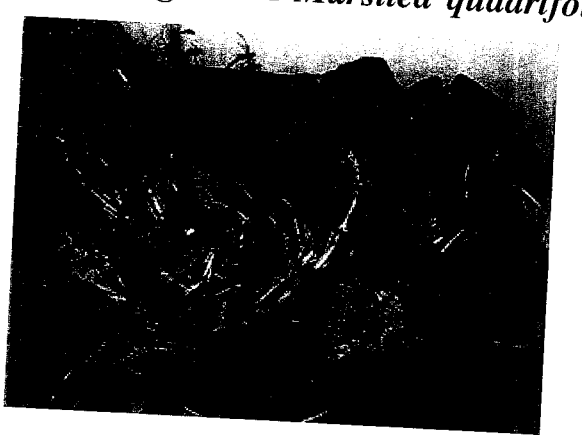


Fig 2.4.11 *Hibiscus cannabinus*

Fig 2.4.12 *Marsilea quadrifolia*



2.4.8 *Cardiospermum halicacabum*

Ethanol extract of *Cardiospermum halicacabum* Linn. (Fig.2.4.8) (Sapindaceae), in a concentration dependant manner (200–600 mg/kg) inhibited gastric ulcers induced by oral administration of absolute ethanol. Further, the extract administration to rats resulted in an increase in levels of gastric glutathione and a decrease in alkaline phosphatase activity. The extract also exhibited potent *in vitro* hydroxyl radical scavenging and inhibition of lipid peroxidation activities. The extract was found to be devoid of any conspicuous acute and short-term toxicity in rats (Sheeba and Asha., 2006).

2.4.9 *Moringa oleifera*

The medicinal plants are efficient for the treatment of diabetes, 41 plants were used by the patients and the two most frequently cited were *Moringa oleifera* Lam (65.90%) and *Sclerocarya birrea* (A. Rich) Hochst (43.20%) (Diève *et al.*,2008). *M.oleifera* also prevented the deleterious histopathological and ultrastructural perturbations caused by isoproterenol (ISP)-induced model of myocardial infarction. Based on the results, *M.oleifera* (Fig.2.4.9) extract possesses significant cardioprotective effect, which may be attributed to its antioxidant, antiperoxidative, and myocardial preservative properties (Nandave et *al.*, 2009)

2.4.10 *Portulaca oleracea*

Antioxidant activities of three phenolic alkaloids, i.e., oleracein A (OA), oleracein B (OB) and oleracein E (OE), isolated from *Portulaca oleracea* (Fig.2.4.10) were determined, based on scavenging activity against 1,1-diphenyl- 2-picryl-hydrazyl (DPPH) radical and inhibitory effect on hydrogen peroxide-induced lipid peroxidation in rat brain homogenates(Yang *et al.*, 2009). The DPPH radical scavenging activities of these phenolic alkaloids were lower than caffeic acid but higher than ascorbic acid and alpha-tocopherol,

being in the following order: OB > OA > OE. OE was most potent in preventing formation of malondialdehyde (MDA) with an EC (50) value of 73.13 microM, close to that of caffeic acid (72.09 microM). It was demonstrated that phenolic alkaloids served as a new class of antioxidant agents in this plant.

2.4.11 *Hibiscus cannabinus*

As per Neeru and Sharma (2008), *Hibiscus*. species (Malvaceae) (Fig.2.4.11) have been used as a folk remedy for the treatment of skin diseases, as an antifertility agent, antiseptic, and carminative. Some compounds isolated from the species, such as flavonoids, phenolic acids, and polysaccharides, are considered responsible for these activities.

2.4.12 *Marsilea quadrifolia*

A juice made from the leaves of *Marsilea quadrifolia* (Fig.2.4.12) is diuretic and febrifuge. It is also used to treat snake bite and applied to abscesses etc. The plant is anti-inflammatory, diuretic, depurative, febrifuge and refrigerant.

2.5 Plants and Antioxidant activity

Antioxidants also referred to as chelators which bind metal ions such as copper and iron that catalyze lipid peroxidation.

Research on natural antioxidants has become increasingly active in various fields (*Moon and Shibamotol*, 2009). Accordingly, numerous articles on natural antioxidants, including polyphenols, flavonoids, vitamins, and volatile chemicals, have been published. Assays developed to evaluate the antioxidant activity of plants and food constituents vary. Therefore, to investigate the antioxidant activity of chemical(s), choosing an adequate assay based on the chemical(s) of interest is critical. There are two general types of assays widely used for different antioxidant studies. One is an assay associated with lipid

peroxidations, including the thiobarbituric acid assay (TBA), malonaldehyde/high-performance liquid chromatography (MA/HPLC) assay, malonaldehyde/gas chromatography (MA/GC) assay, beta-carotene bleaching assay, and conjugated diene assay. Other assays are associated with electron or radical scavenging, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing/antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay.

To identify the potential of green leafy vegetables (GLV) as antioxidants, methanolic extracts of *Amaranthus* sp., *Centella asiatica, Murraya koenigii* and *Trigonella foenum graecum* were studied for their antioxidant activity in different systems at multiple concentrations. Total antioxidant activity assessed by phosphomolybdenum method, free radical scavenging activity by DPPH, reducing power and ferrous ion chelating activity were determined. The GLV were analyzed for ascorbic acid, total and beta-carotene and total polyphenol contents. The ascorbic acid, total carotene, beta-carotene and total phenolic content (tannic acid equivalents) of the GLV ranged between 15.18-101.36, 34.78-64.51, 4.23-8.84 and 150.0-387.50 mg/100 g GLV, respectively. The extracts were found to have significantly different levels of antioxidant activities in the systems tested. The total antioxidant activity was highest in *Murraya koenigii* (2,691.78 μmol of ascorbic acid/g sample) and least in *Centella asiatica* (623.78 μmol of ascorbic acid/g sample). The extract concentration causing 50% inhibition of DPPH (IC50) was determined (*M. koenigii*<*C.asiatica*<*Amaranthus* sp. <*T. graecum*). The maximum DPPH scavenging activity and reducing power was exhibited by Murraya koenigii. (Guptha and Prakash, 2009) Multiple regression analysis showed that the relationship of total antioxidant activity, free radical scavenging activity, and

reducing power with polyphenol and total and beta-carotene was highly significant.

2.6 Free Radicals

Table 2.6.1 Examples of free radicals (Mark ,1998)

	DESCRIPTION
Superoxide radical ($^{\cdot}O_2^-$)	One-electron reduction state of O_2 . Rather unreactive, but can release Fe^{2+} from iron-sulfur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor of metal catalyzed $^{\cdot}OH$ formation.
Hydrogen peroxide (H_2O_2)	Two-electron reduction state of O_2. Formed by dismutation of $^{\cdot}O_2^-$ or by the direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
Hydroxyl radical ($^{\cdot}OH$)	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive and will attack most cellular components.
Organic hydroperoxide (ROOH)	Formed by radical reactions with cellular components such as lipids and nucleobases.
Alkoxy ($RO^{\cdot}$) and Peroxy ($ROO^{\cdot}$) radicals	Oxygen centered organic radicals. Lipid forms precipitate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
Hypochlorous acid (HOCl)	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize

	protein constituents, including thiol groups, amino groups and methionine.
Peroxynitrite ($OONO^-$)	Formed in a rapid reaction between $\cdot O_2^-$ and $NO\cdot$. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo cleavage to form hydroxyl radical and nitrogen dioxide.

2.6.1 Biological significance of free radicals

Indirect evidence suggests that free radicals and excited-state species play a key role in both normal biological functions and in the pathogenesis of certain human diseases. For example, generation of activated species by inflammatory cells is a major microbicidal mechanism and may also mediate important components of the inflammatory response. They also have important roles in redox signaling. The free radicals may also be involved in the prevention of aging by the induction of a process known as mitohormesis. They are also involved in the induction of host defense genes and mobilization of ion transport systems. Their roles in signaling are crucial. In particular, platelets involved in wound repair and blood homeostasis release ROS to recruit additional platelets to the sites of injury. Thus reactive oxygen species play an indispensable role in the normal functioning of biological system.

In recent years, the use of some synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects. This concern has resulted in an increased interest in the investigation of the effectiveness of naturally occurring compounds with antioxidant properties. A number of plant and medicinal mushrooms constituents have been recognized to have positive effects when tested against the oxygen reactive compounds. Foods rich in antioxidants have been shown to play an essential role in the prevention of

cardiovascular diseases; cancers, neurodegenerative diseases, the most well known of which are Parkinson's and Alzheimer's diseases, inflammation and problem caused by cell and cutaneous aging (Shahidi and Wanasundara, 1992).

2.6.2 Types of antioxidants

Antioxidants are of different types such as natural or enzymatic antioxidants, non-enzymatic antioxidants, scavenging or chain breaking antioxidants, pharmacologic antioxidants and others.

Enzymatic antioxidants

The enzymes responsible for the defense against the free radical damage include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. SOD is present in two places naturally in the cell. SOD that is present in the mitochondria contains manganese (MnSOD).

Natural non-enzymatic antioxidants

In addition, antioxidants play an important role in defense mechanisms. These are often the three vitamins, Vitamin A, Vitamin C and Vitamin E, and certain molecules like carotenoids (lutein and beta-carotene) and phenolics.

Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation. (Sies,1997).

Reactive Oxygen Species	**Neutralizing Antioxidants**
Hydroxyl radical	Vitamin C, Glutathione, Flavanoids, Lipoic acid.

Superoxide radical	Vitamin C, Glutathione, flavanoids, SOD.
Hydrogen peroxide	Vitamin E, Glutathione, beta carotene, Vitamin C, CoQ10, Flavanoids, Lipoic acid.
Lipid peroxides	Beta caotene, Vitamin E, Ubiquinone, flavanoids, glutathione peroxidase.

Table 2.6.2 Antioxidant Protection System

Endogenous antioxidants	Bilirubin, thiols, Enzymes
Dietary antioxidants	Vitamin C,E, carotenoids, poly phenols
Metal binding proteins	Albumin(Cu), ferritin(Fe), myoglobin(Fe)

(Mark, 1998)

2.6.3Antioxidants from dietary sources

2.6.3.1 Importance of Antioxidant rich dietary sources

The body naturally creates some antioxidants, but it relies heavily on a proper diet to get the rest of its natural antioxidants. Spinach as well as blueberries, apples and several other plant foods are rich in antioxidants. Flaxseed contains lignans, which may have antioxidant effects. Flaxseeds are said to fight cancer due to the fact that the omega-3 fatty acids found within it act as antioxidants in the body. Strawberries are high in antioxidants. The antioxidants in dark chocolate have shown to make a difference in cardiovascular health. Garlic also serves as an antioxidant and some studies even indicate that it can help to protect against cancer. Thus fruits and

vegetables contain essential antioxidants for the body. The effect of *Aloe vera* is interesting because it makes Vitamin C, Vitamin E and other antioxidants work well.

2.6.3.2 Chemical components

The plants have been found to possess the following chemical constituents some of which have an important role in the antioxidant activity of the plant (Castillo *et al.*, 2009)

- Vallarine
- Asiaticoside
- Pectic acids
- Steroids
- Hersaponin
- Bacogenin
- Monnierin
- Triterpene
- Tannin
- Phenolics.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The plants used for the study are tabulated in Table.3.1.

Table 3.1 Plants used for the study

BOTANICAL NAME	VERNACULAR NAME
Sesbania grandiflora	Agathi keerai
Marsilea quadrifolia	Arai keerai
Eclipta prostrate	Karuslanganni keerai
Althernanthera sessilis	Ponanganni
Cardiospermum halicacabum	Modakathan
Centella asiatica	Vallarai keerai
Hibiscus cannabinus	Pulicha keerai
Solanum nigrum	Manathakali
Moringa oleifera	Murungai keerai
Basella alba	Pasalai keerai
Amaranthus blitum	Thandu keerai
Portulaca oleracea	Parupu keerai

The leaves of the above mentioned plants were collected from various places in Coimbatore (Tamil Nadu, India) during the months of February-March 2009. The plant specimens were authenticated at the Botanical Survey of India, Coimbatore.

3.1 Preparation of plant extracts

1. Using pestle and mortar 5g of leaf was ground with 50 ml water.
2. The extract was then centrifuged at 10000 RPM for 15 minutes at 4°C.
3. The pellet was discarded and the supernatant was taken and stored at 4°C for further investigation.

3.2 Preparation of enzyme solution

The porcine pancreatic α-amylase (Sigma A-3176) was used as the source of enzyme. The enzyme stock solution of 1mg/ml was prepared initially using 2M phosphate buffer, pH-6.9. The working solution was prepared by mixing 3ml of the enzyme stock (1mg/ml) with 2mL of the phosphate buffer pH-6.9.

3.3 Assay of α-amylase inhibitor activity (DNS method) (Bernfield , 1955)

3.3.1 Principle

The method of Bernfield (1955) was adopted with some modifications. This method is used to test the presence of free carbonyl group (C=O) which was present in the reducing sugars. This involves the oxidation of the aldehyde functional group; for e.g., aldehyde group in glucose or maltose. Simultaneously, 3, 5-dinitrosalicylic acid (DNS) was reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions:

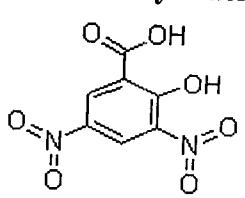
Oxidation

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

←

Reduction

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



Because dissolved oxygen can interfere with glucose oxidation, sulfite, which itself was not necessary for the color reaction, was added in the reagent to

absorb the dissolved oxygen. The above reaction scheme shows that one mole of sugar will react with one mole of 3, 5-dinitrosalicylic acid. However, it was suspected that there were many side reactions, and the actual stoichiometry of the reaction was more complicated than that previously described. The type of side reaction depends on the exact nature of the reducing sugars. Different reducing sugars generally yield different color intensities; thus, it was necessary to calibrate for each sugar. In addition to the oxidation of the carbonyl groups in the sugar, other side reaction such as the decomposition of sugar also competes for the availability of 3, 5-dinitrosalicylic acid. As a consequence, carboxymethyl cellulose can affect the calibration curve by enhancing the intensity of the developed color. One can determine the background absorption on the original cellulose substrate solution by adding cellulase, immediately stopping the reaction, and measuring the absorbance, i.e. following exactly the same procedures for the actual samples. When the effects of extraneous compounds were not known, one can effectively include a so-called internal standard by first fully developing the color for the unknown sample; then, a known amount of sugar was added to this sample. The increase in the absorbance upon the second color development was equivalent to the incremental amount of sugar added.

3.3.2 Materials

- Micro centrifuge tubes
- Pipettes
- Beckhman Coulter Spectrophotometer
- Water bath

3.3.3 Reagents

- **Starch solution, 1%** (Freshly prepared)

• **Dinitrosalicylic acid reagent (DNS reagent):** 1 g of dinitrosalicylic acid was dissolved in 100 ml of 1% NaOH. Then 30 g crystalline phenol and sodium sulphite was added.

3.3.4 Procedure (Nagaraj and Pattabiraman, 1985)

(i) Two test tubes were taken; one was labeled as control (C) and the other as Test (T).

(ii) Reaction with enzyme (αA) and without enzyme (E) along with inhibitor in all test tubes were taken in varying dozes.

(iii) To C, T and αA test tubes 100 µl of the working enzyme was added.

(iv) To the entire test tubes 100 µl phosphate buffer (pH 6.9) was added.

(v) To this 100 µl inhibitor extract (from different leaves) was added to all the tubes.

(vi) Distilled water is added to all the test tubes to make up the volume up to 700 µl.

(vii) The tubes were then incubated at room temperature (RT) for 20 min.

(viii)Freshly prepared substrate (starch 1%) of 200 µl was added to all the tubes.

(ix) The tubes were again incubated at RT for 15 min.

(x) One ml of DNS reagent was added to all the tubes to arrest the reaction.

(xi) The tubes were then kept in boiling water bath for 5 min.

(xii) The tubes were then cooled and the absorbance measured at 540 nm.

3.4 Ferric ion reducing antioxidant power (FRAP) assay (Bharathi kumar *et al.*,2008)

3.4.1 Principle

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this method the Fe^{3+} is reduced to Fe^{2+} in the presence of antioxidants (Reductants) in the extracts. The blue

colour formed is colorimetrically measured at 700 nm. The increase in the absorbance is directly proportional to the concentration of total antioxidants present in the sample.

3.4.2 Reagents

a) Sample preparation

Five gram of dried leaves (fresh leaves were air dried in the incubator at 37°C for two days) was weighed and mixed with 50 ml of water in a conical flask. It was then incubated at room temperature in orbital shaker over night. The contents were filtered with Whitman filter paper or centrifuged and the filtrate was collected. The solvent in the filtrate was evaporated and 50mg of the dried powder was dissolved in 50ml of distilled water. From this, a series of dilution (1:20 [100µg], 1:10 [200 µg] to 1:4 [500 µg]) is prepared for experimental analysis.

b) 0.2M Phosphate buffer (pH 6.6)

26.5ml of 0.2M Na_2HPO_4 was mixed with 73.5ml of 0.2M NaH_2PO_4 (0.2M Na_2HPO_4 = 35.6g/L, 0.2M NaH_2PO_4 = 31.2g/L)

c) Potassium ferricyanide, 1%

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

d) TCA, 10%

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

e) $FeCl_3$, 0.1%

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

3.4.3 Procedure

(i) Two ml (concentration varying from 100 to 500μg) of the extract was pipetted out into a series of tubes.

(ii) To this 2.5 ml of phosphate buffer (pH, 6.6) and 2.5 ml of 1% potassium ferricyanide was added to all the tubes.

(iii) To a "Blank" tube, 4ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were added.

(iv) All the tubes were incubated at 50^0C for 20 minutes.

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

(vi) The tubes were centrifuged at 650xg for 10 minutes and 4ml of supernatant was pipetted out.

(vii) To this 0.5ml of 0.1% $FeCl_3$ was added.

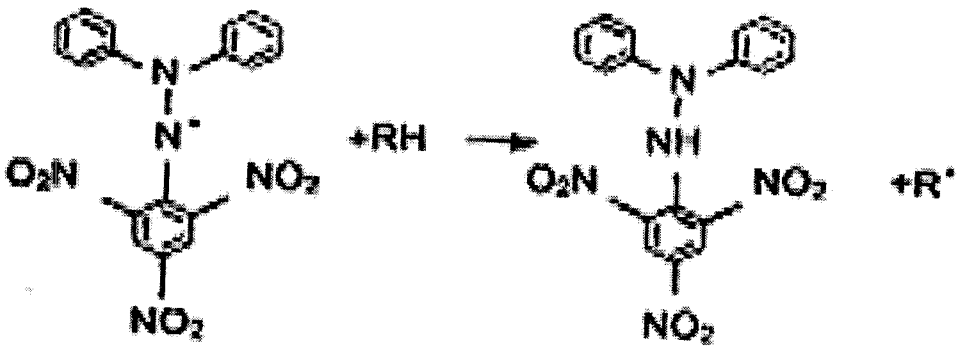
(viii) The blue colour formed was colorimetrically read at 700nm and an increase in the absorbance reading showed an increased antioxidant activity in the leaf extract.

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

3.5.1 Principle

In this 1, 1-diphenyl -2-picryl hydroxyl (DPPH) assay system, due to its odd electron, the stable DPPH free radical produces a color with strong absorbance at 517 nm. When DPPH is placed in an assay system containing free

radical scavengers such as flavonoids, the color vanishes.



The change in absorbance produced in this reaction is a measurement of antioxidant scavenging capacity of test samples. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep violet to light yellow) were measured at 517 nm with a UV/visible spectrophotometer.

% Antioxidant capacity = [(Control – Test) / Control] x 100

3.5.2 Reagents

a) DPPH Reagent

Four mg DPPH (2, 2 - diphenyl-1-picrylhydrazyl) was mixed in 100 ml methanol to get the active reagent. This must be freshly prepared before the assay.

3.5.3 Procedure (Conforti *et al.*, 2008)

(i) Two ml (concentration varying from 100 to 500µg) of the extract was pipetted out into a series of test tubes.

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

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

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

3.6 Estimation of total phenol content

3.6.1 Principle:

The Folin's phenol reagent is a mixture of phosphomolybdate and phosphotungstate is used for the colorimetric assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of the substrate being tested needed to inhibit the oxidation of the reagent.

3.6.2 Materials

a) Stock preparation:

Catechol of 50mg was taken as a standard and made up to 50 ml with distilled water.

b) Working solution:

From the stock, 1ml was taken and made up to 10 ml with distilled water.

c) Folin's reagent

In a clean dry flask, 10 ml of 2N Folin's reagent was made up to 20 ml with distilled water.

d) Sodium carbonate solution, 10%

In a clean dry 100 ml flask, 10g of sodium carbonate was made up to the mark with distilled water.

3.6.3 Procedure (Singleton and Rossi, 1965; Maher *et al.*, 2006)

(i) Working solution was added to 5 different test tubes (concentration varying from 100 to 500 µl) and made up to 3 ml with distilled water.

(ii) Distilled water serves as a blank.

(iii) Half (0.5) ml Folin's reagent was added to all the test tubes and incubated at room temperature for 3 min.

(iv) Two ml of sodium carbonate solution was added and boiled for 1 min.

(v) The blue colour formed was colorimetrically read at 650 nm.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The salient observations made and the results obtained from the experiments conducted related to the screening of twelve green leafy vegetables for porcine pancreatic α-amylase inhibitory activity and antioxidants status in these plants are presented in this chapter. The results of the present investigation are also discussed on the basis of available information in the literature.

4.1 Inhibition of porcine pancreatic α-amylase

The crude aqueous extracts from twelve leafy vegetables were assayed for inhibitory activity against porcine pancreatic α-amylase. The results are presented in tables 4.1 to 4.3 and figure 4.1

Among the twelve leafy vegetables analysed, *Cardiospermum halicacabum* and *Portulaca oleracea* showed strong inhibition of α-amylase. In all the extracts analysed, a dose- dependent increase in the inhibitory activity was also observed (Figure 4.2 – 4.3)

Sigma plot obtained from the dose-dependent inhibition values are used to calculate IC_{50} values for all extracts and the values are shown in figure 4.3. The plant extracts which showed strong inhibition are given below in order: *C. halicacabum*> *P. oleracea*> *H. cannabinus*> *E. prostrata*> *B. alba*> *M.quadrifolia*> *M. oleifera*> *A. blitum*> *S.grandiflora*. Least inhibition was obtained in *C. asiatica*.

α-Amylase inhibitors play a major role in managing PPHG in diabetic patients. These α-amylase inhibitors inhibit the action of α-amylase leading to a reduction in starch hydrolysis which shows beneficial effects on glycemic index control in diabetic patients (Menakshi *et al.*, 2008). Lower IC_{50} value indicates that the plant possess high α-amylase inhibiting property. In the above observations, *Cardiospermum halicacabum* was found to have the least IC_{50} value of 26.87 µl with inhibition of 40-85% at 20-100 µl which indicates that the plant is more potent in inhibiting the activity of α-amylase.

Table 4.1.1: Percentage inhibition of α-amylase activity (100 µl-500 µl)

PLANTS	INHIBITION OF α-AMYLASE (%)					
	100 µl	200 µl	300 µl	400 µl	500 µl	IC_{50}(µl/g)
A.blitum	15.06	37.09	50.69	57.4	76.1	297.0
M.oleifera	18.42	28.7	56.2	67.3	87.6	277.0
M.quadrifolia	18.7	37.2	53.6	62.4	74.5	274.2
S.nigrum	7.5	14.08	51.1	96.0	96.3	294.6
B.alba	12.0	20.2	64.0	64.6	73.4	264.1
E.prostrata	30.7	48.3	52.5	57.8	60.6	233.6
H.cannabinus	48.23	58.9	60.6	64.7	78.2	117.0
S.grandiflora	22.6	31.4	36.1	51.3	96.5	388.5

Table 4.1.2: Percentage inhibition of α-amylase activity (20 µl-100 µl)

PLANTS	INHIBITION OF α-AMYLASE (%)					
	20 µl	40 µl	60 µl	80 µl	100 µl	IC_{50}(µl/g)
P.oleracea	32.8	67.7	73.29	79.30	80.34	29.9
C.halicacabum	44.7	58.5	61.8	73.08	81.85	26.8

Table 4.1.3: Percentage inhibition of α-amylase activity (100 µl-1000 µl)

PLANTS	INHIBITION OF α-AMYLASE (%)										
	100 µl	200 µl	300 µl	400 µl	500 µl	600 µl	700 µl	800 µl	900 µl	1000 µl	IC_{50}(µl/g)
C.asiatica	7.2	9.4	10.7	21.6	22.0	29.3	31.7	40.3	43.2	56.4	959.8
A.sessilis	5.7	5.73	18.4	19.1	29.0	62	71.6	77.2	90.1	92.1	563.5

Fig: 4.1.1: Comparison of IC_{50} values of the plant extracts

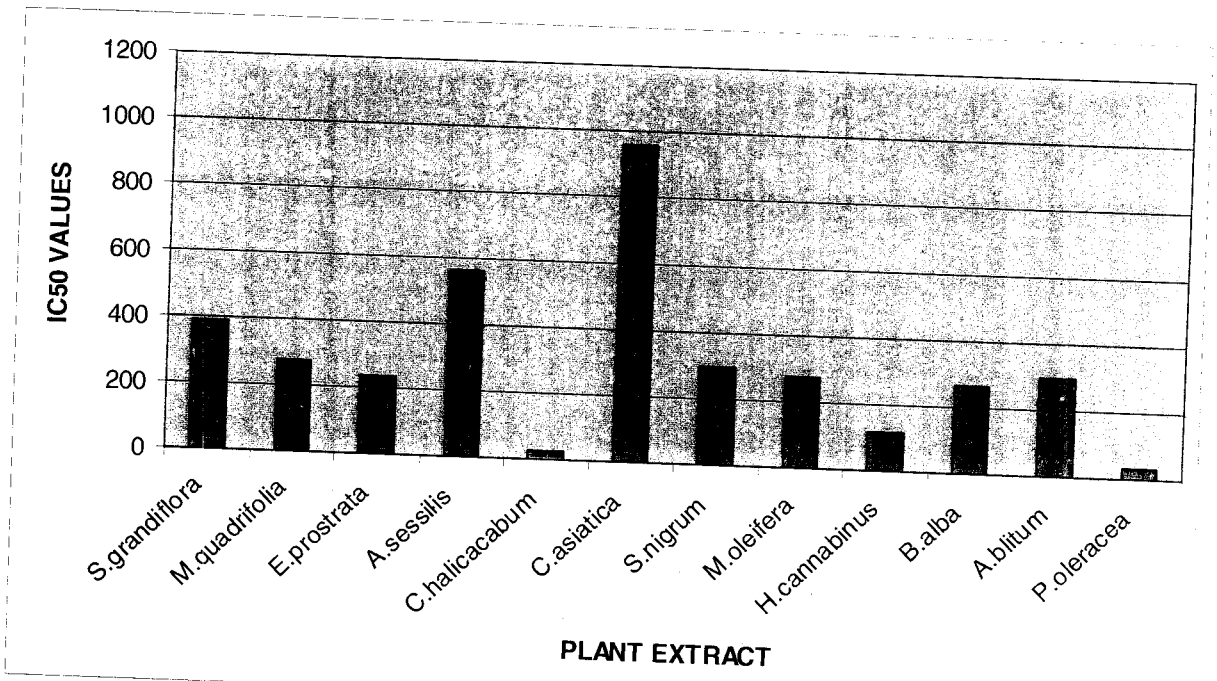
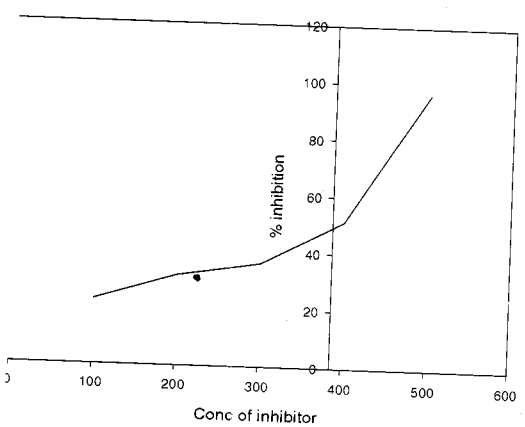
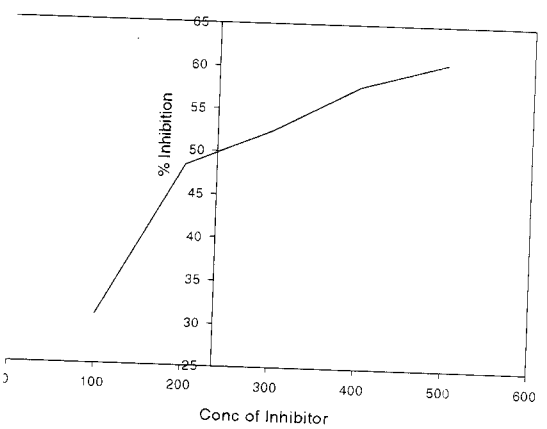


Fig: 4.1.2 Sigma plot for IC_{50} values

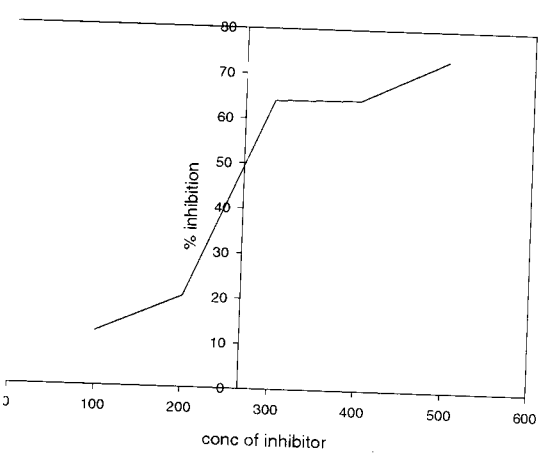
S.grandiflora



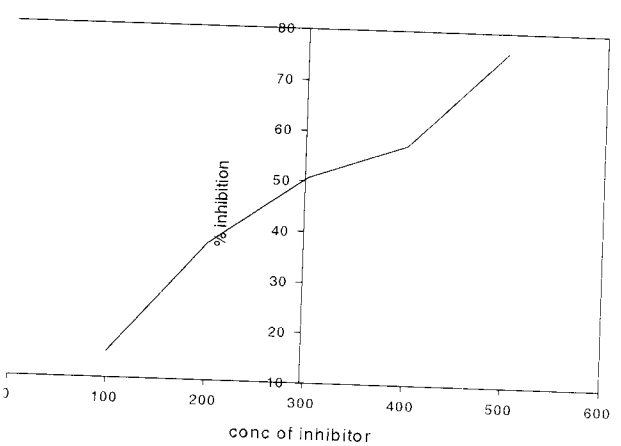
E.prostrata



B.alba

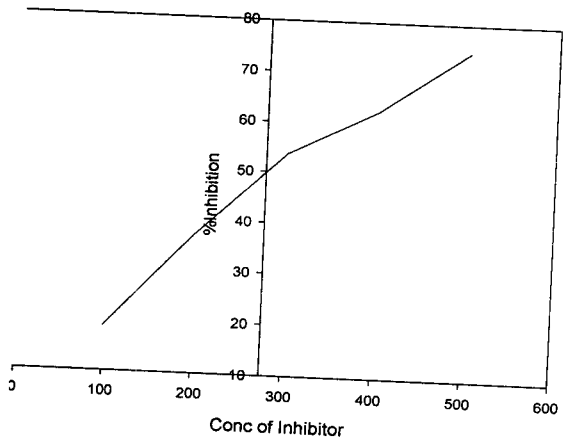


A.blitum

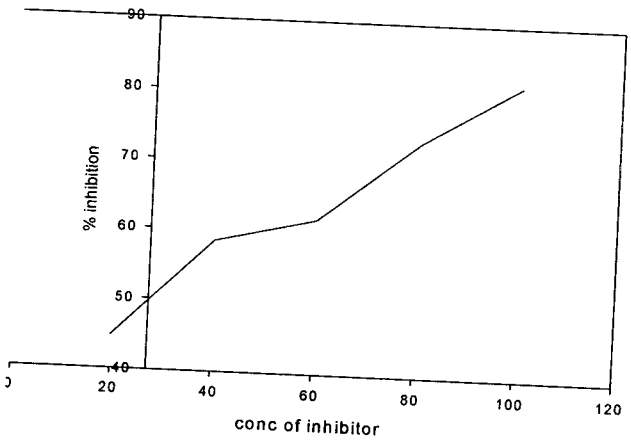


Sigma plot for IC_{50} values continued

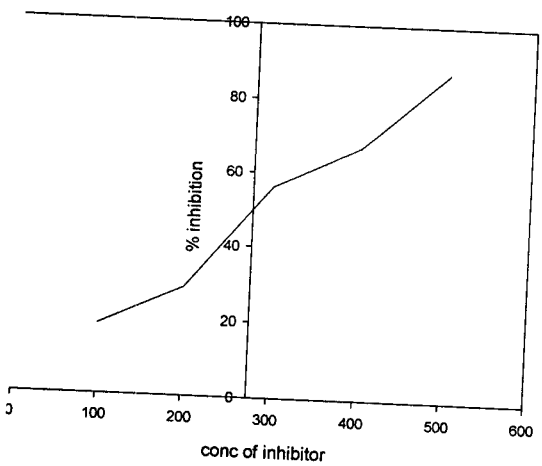
M.quadrifolia



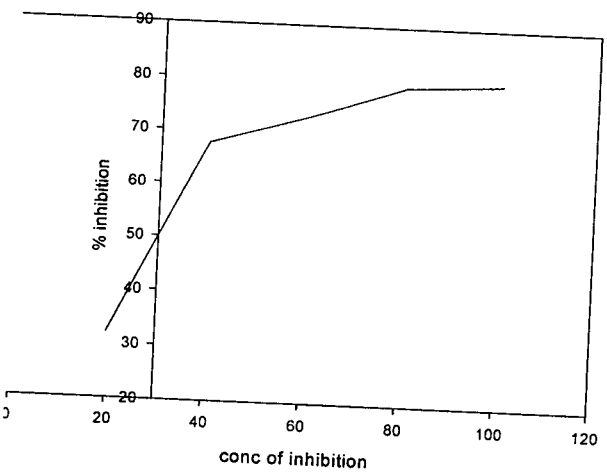
C.halicacabum



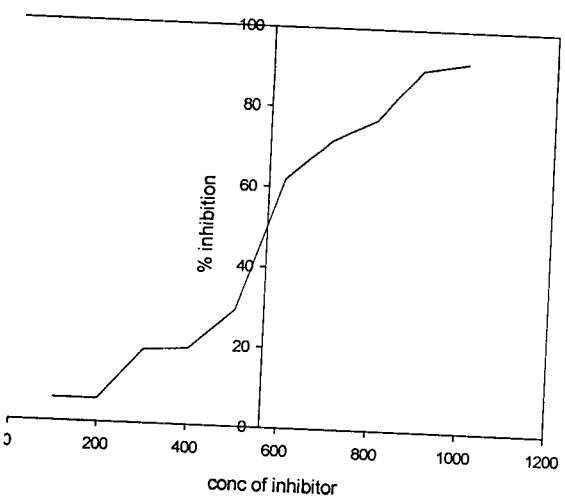
M.oleifera



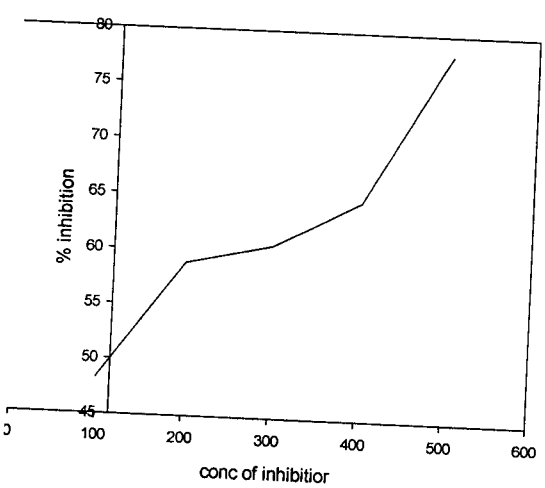
P.oleracea



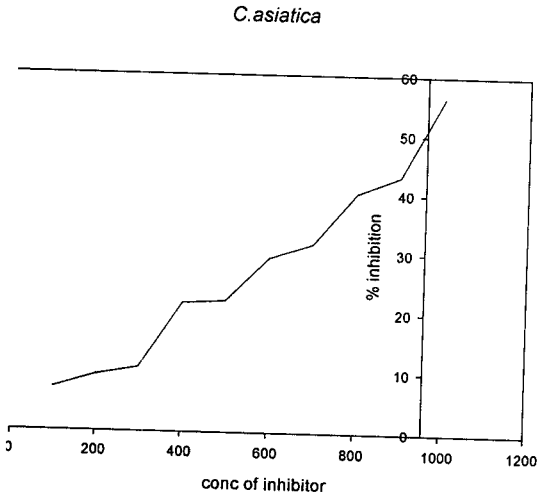
A.sessilis

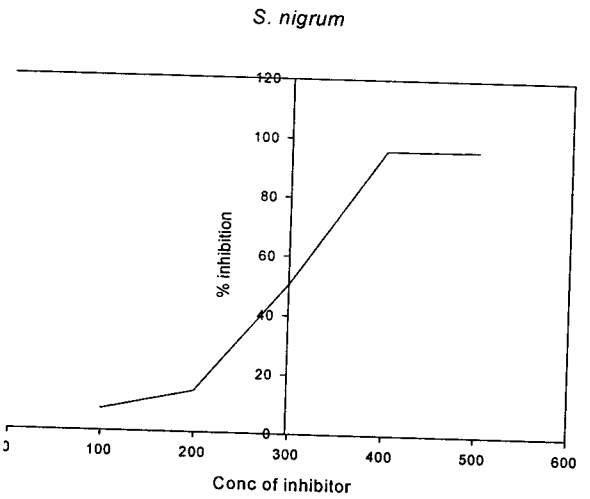


H.cannabinus



Sigma plot for IC_{50} values continued





4.2 Antioxidant activity

4.2.1 Frap Assay

The ferric reducing antioxidant power assay is carried out to determine the ability of the plant extracts to scavenge free radicals by donating electrons. The greater the absorbance, the greater the reducing potential of the plant extract.

The antioxidant activity obtained for all the twelve green leafy aqueous extract are presented in table 4.2.1.1 and figure 4.2.1.1. Among the twelve plants tested, *Amaranthus blitum* showed relatively higher antioxidant activity followed by the order shown below

A. blitum> P. oleracea> H. cannabinus> M.quadrifolia> S. nigrum> M. oleifera.

The antioxidant activity was lower in *B. alba, A. sessilis, S. grandiflora* and *C.asiatica.*

4.2.2 DPPH radical scavenging activity:

DPPH is a relatively stable free radical. The assay is based on the measurements of the antioxidants' ability to scavenge the stable radical DPPH. DPPH radicals react with suitable reducing agents. The electrons become paired off and the solution loses color stoichiometrically depending on the number of electrons taken up (Blois, 1958).

The proton radical scavenging action is known as an important mechanism of auto-oxidation. DPPH was used to determine the proton radical scavenging action of the methanol and acetone fractions of the plants. It shows a characteristic absorbance at 517 nm. The purple color of the DPPH solution fades rapidly when it encounters proton radical scavengers (Yamaguchi *et al.*, 1998).

The dose- dependent increase in antioxidant activity from DPPH radical scavenging is shown in table 4.2.2.1 and figure 4.2.2.1. The methanol extract of *Portulaca oleracea* showed higher antioxidant activity by DPPH assay method.

The order of activity is shown below

P. oleracea> *S. nigrum*> *A. blitum*> *C. halicacabum*> *H. cannabinus*> *M. quadrifolia*> *A. sessilis*> *S. grandiflora*> *M. oleifera*> *C. asiatica*> *B. alba*> *E. prostrata.*

The Indian green leafy vegetables were screened by Gupta and Prakash (2009) for their antioxidant variety. Among the vegetables screened, the total antioxidant was highest in *Murraya koenigii* and least in *Centella asiatica.* In the present investigation, *Centella asiatica* recorded comparatively lower values but higher than *Eclipta prostrata* and *Basella alba* (DPPH method).

Gupta and Prakash (2009) have also shown that the extract concentration causing 50% inhibition of DPPH (IC_{50}) was of the order,

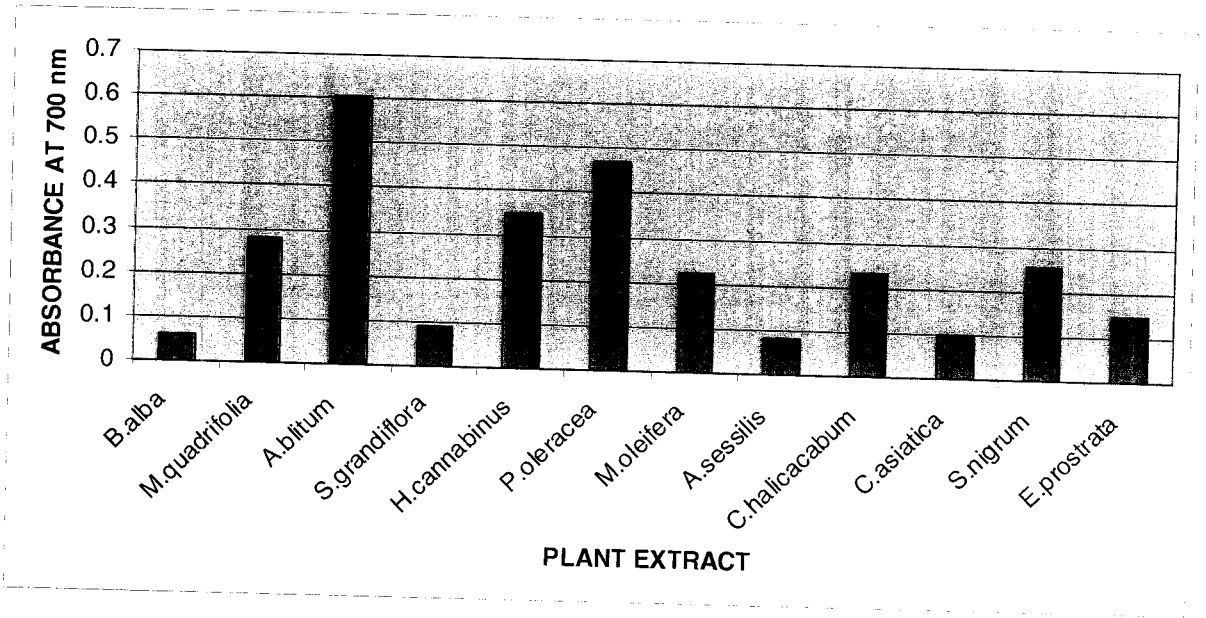
M. koenigii< *C. asiatica*< *Amaranthus* sp< *T. graeceum*

Multiple regression analysis showed that the relationship of total antioxidant activity and free radical scavenging activity was highly significant. But in the present study only less significant r values. The antioxidant properties of different cultivars of *Portulaca oleracea* was reported .They have obtained a good correlation between the total phenol content and FRAP values ($r^2 = 0.9$) for all the cultivars.

Table 4.2.1.1 Absorbance of aqueous extract of plant species for antioxidant activity

PLANTS	ABSORBANCE AT 700 nm				
	50µg/ml	**100µg/ml**	**150µg/ml**	**200µg/ml**	**250µg/ml**
B.alba	0.015	0.042	0.053	0.058	0.059
M.quadrifolia	0.075	0.122	0.140	0.194	0.282
A.blitum	0.069	0.117	0.241	0.259	0.604
S.grandiflora	0.029	0.049	0.066	0.076	0.091
H.cannabinus	0.093	0.127	0.199	0.221	0.349
P.oleracea	0.304	0.323	0.378	0.392	0.469
M.oleifera	0.065	0.098	0.101	0.146	0.223
A.sessilis	0.025	0.036	0.066	0.075	0.080
C.halicacabum	0.011	0.026	0.070	0.111	0.235
C.asiatica	0.045	0.063	0.077	0.082	0.099
S.nigrum	0.030	0.040	0.062	0.101	0.147
E.prostrata	0.012	0.040	0.062	0.101	0.147

Fig 4.2.1.1 Absorbance of aqueous extract of plant species for antioxidant activity



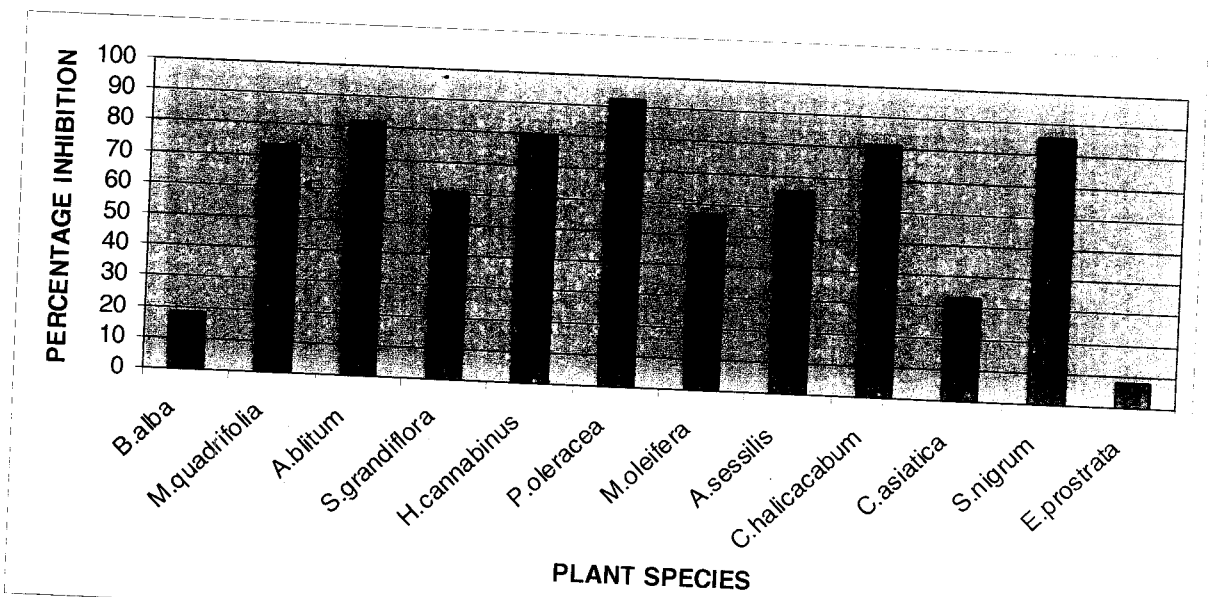
The absorbance values at 250µg/ml were observed and it was found that the extract of *Amaranthus blitum* exhibited maximum reducing power having absorbance value of 0.604.

Table 4.2.2.1 Percentage inhibition of DPPH radical by aqueous extract of plant species.

PLANTS	% INHIBITION ACTIVITY					
	100µg/ml	200µg/ml	300µg/ml	400µg/ml	500µg/ml	IC_{50}µg/ml
B.alba	5.29	11.21	13	15	18.68	-
M.quadrifolia	46.8	56.9	60.8	64.39	73.48	135.0
A.blitum	37.06	45.03	54.96	65.03	82.37	256.4
S.grandiflora	28.68	36.7	46.51	52.69	60.19	358.0
H.cannabinus	75.7	76.4	77.4	78.1	80.4	-
P.oleracea	54.5	78.9	80.6	85.60	92.80	-
M.oleifera	24.64	48.31	54.0	54.8	56.96	226.0

A.sessilis	26.14	29.69	39.65	43.7	65.13	429.2
C.halicacabum	41.15	63.84	70.7	79.42	81.3	139.6
C.asiatica	20.6	21.8	27.03	29.69	33.5	-
S.nigrum	68	74.2	77.09	83.66	85.5	-
E.prostrata	4.47	5.6	5.73	5.8	8.1	-

Fig 4.2.2.1 Percentage inhibitions of DPPH radicals by aqueous extracts of plant species.



The percentage inhibitions at 500µg/ml concentration were observed and it was found that the plant ***Portulaca oleracea*** is more potent in neutralizing DPPH free radical. The methanol extract shows a slightly better scavenging activity.

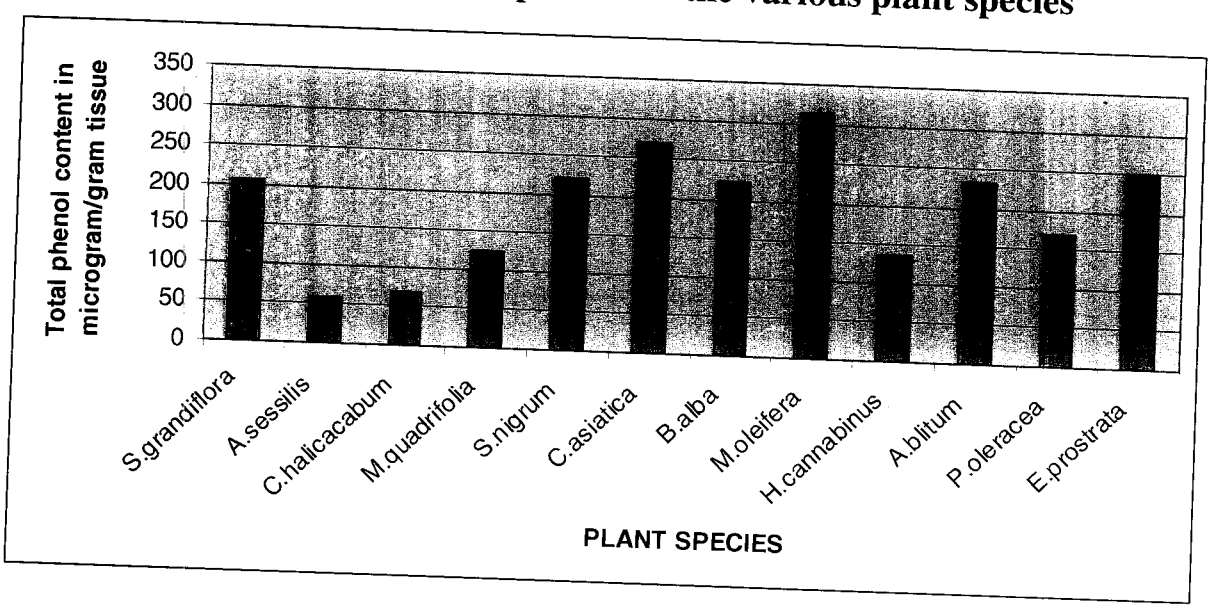
4.3 Estimation of total phenols

The amount of total phenols in extract was determined with Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965). The antioxidative properties of some vegetables and fruits are partly due to the low molecular weight phenolic compounds, which are known to be potent as antioxidants (Huda et al, 2009).

Table 4.3.1 Levels of total phenols in plant species

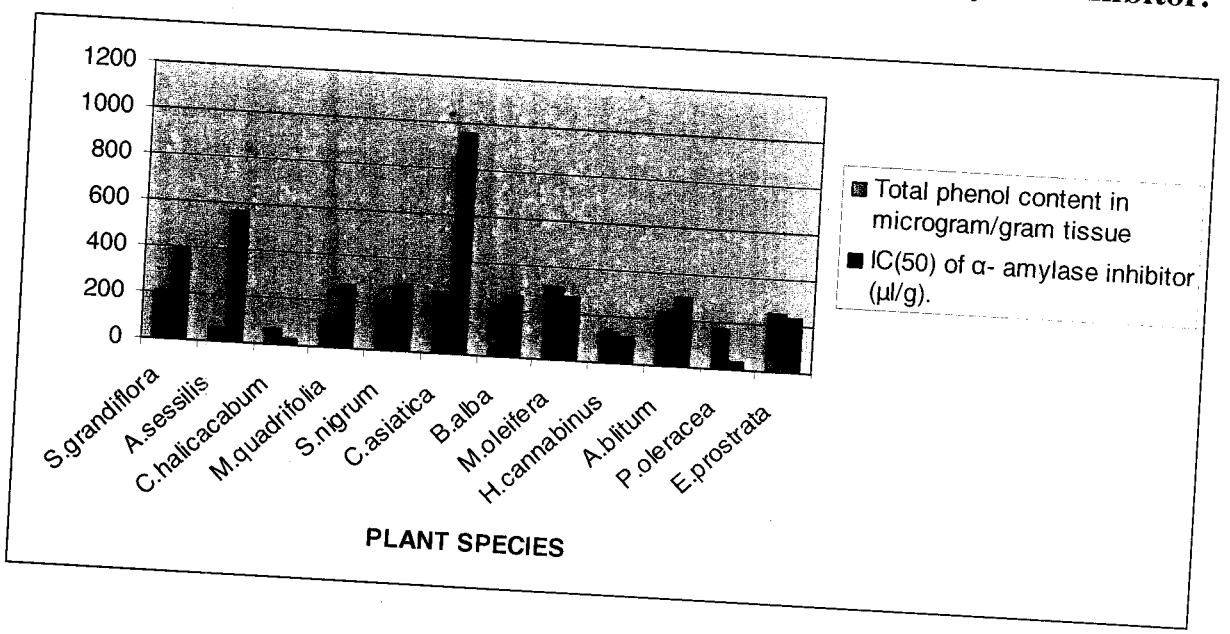
PLANTS	ABSORBANCE AT 650 nm	CONCENTRATION OF PHENOLS(µg)	CONCENTRATION OF PHENOLS(µg/g tissue)
S.grandiflora	0.350	32.74	205.4
A.sessilis	0.275	10.65	59.08
C.halicacabum	0.318	11.42	68.3
M.quadrifolia	0.276	26.65	123.3
S.nigrum	0.558	33.77	220.0
C.asiatica	1.255	42.15	269.6
B.alba	1.037	34.27	222.8
M.oleifera	1.464	52.28	314.5
H.cannabinus	0.628	20.56	134.9
A.blitum	1.082	60.07	232.4
P.oleracea	0.796	26.90	171.0
E.prostrata	1.163	37.57	249.4

Fig4.3.1 Levels of total phenols present in the various plant species



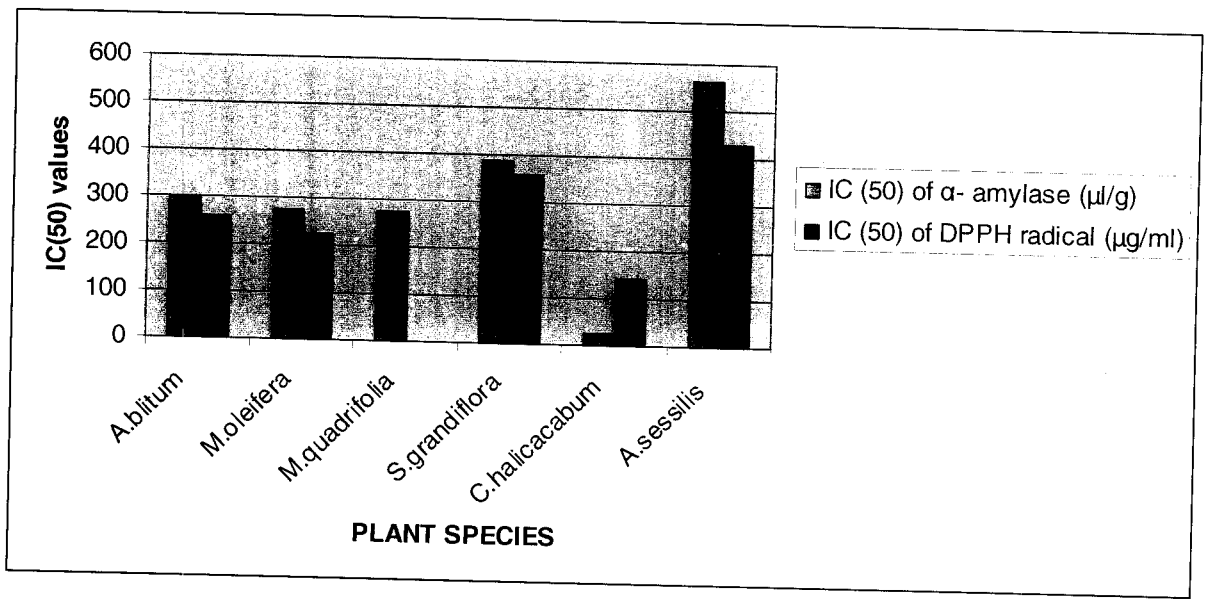
The results obtained indicate that the level of total phenols is highest in *M.oleifera* having total phenol content of 314µg/g tissue. This suggests total phenols may be responsible for the free radical scavenging activity of the plant and amylase inhibitor activity. On the other hand, *Althernanthera sessilis* has the lowest level of phenols having total phenol content of 59.08µg/g tissue.

Fig 4.4 Comparison of Total phenol content and IC_{50} α-amylase inhibitor.



The graph illustrates the comparison of IC_{50} values between α-amylase inhibitor and phenol content of plant species. It was observed that *C. halicacabum*, *P.oleracea* and *E.prostrata* possess maximum α- amylase inhibitor activity with increase in phenol content and all other plants showed less activity.

Fig 4.5 Comparison of inhibition activity between DPPH radical and α-amylase.



The graph illustrates the comparison of IC_{50} values between α-amylase inhibitor and DPPH radical inhibitor of certain plant species. The remaining plant species did not attain 50% inhibition. It was observed that *Althernanthera sessilis* possess maximum activity in inhibiting the DPPH radical and α-amylase. *Cardiospermum halicacabum* and *Marsilea quadrifolia* showed similar activity in inhibiting DPPH radical.

Inhibition of the activity of carbohydrate-hydrolyzing enzymes plays an important role in the prevention and treatment of diabetes (Truong *et al.*, 2007). Synthetic α-glucosidase inhibitors such as acarbose and miglitol are known to reduced post prandial hyperglycemia primarily by blocking the action of the enzyme in the small intestine, thereby delaying glucose absorption. Stabilization of blood glucose is important for diabetic patients, because it prevents hyperglycemia and the complications associated with diabetes. In recent years, research on traditional plants for the management of diabetes has attracted the

interest of scientist. More than 400 kinds of plants with blood glucose-lowering potential are known. A number of plants are known to exert their anti-hyperglycemic activity via the inhibition of carbohydrate-hydrolyzing enzymes in the small intestine.

Oxidative stress has been implicated in the pathology of many diseases, inflammatory conditions, cancer and aging (Marx, 1987). Literature suggests that antioxidant activity and inhibition of α-amylase is high on herbal plants and vegetables (Asmah *et al.*, 2003). Fruits and vegetables are rich in many nutrients. Studies carried out by researchers have however shown that low consumption of vegetables is associated with an increased risk of cancer and diabetes (Tavani and Vecchia, 1995). The antioxidant defence system represents a complex network with interactions, synergy and specific tasks for a given antioxidant. Recent studies show that majority of the plasma antioxidants are depleted in Type 2 diabetes patients. This depletion is a major cause of diabetes-related complications and onset of other disease conditions like atherosclerosis and coronary heart disease (Ashok and Madhususana, 2002).

On the other hand, traditional medicinal plants with various active principles and properties as discussed in this article have been used since ancient times by physicians and laymen to treat a great variety of human diseases such as diabetes, coronary heart disease and cancer. The beneficial multiple activities like manipulating carbohydrate metabolism by various mechanisms, preventing and restoring integrity and function of β-cells, insulin-releasing activity, improving glucose uptake and utilization and the antioxidant properties present in medicinal plants offer exciting opportunity to develop them into novel therapeutics.

Some of the green leafy vegetables are under exploited, but are potent sources of natural antioxidants and α-amylase inhibitor.

Each plant species has a potent antioxidant and α-amylase inhibiting activity and its beneficial effects have been proved by experiments both *in vivo*

and *in vitro*. The present study was carried out in aqueous conditions. Compared to the previous literatures, the assays gave lower results due to less solubility of the compounds.

CONCLUSION AND FUTURE PERSPECTIVES

5. CONCLUSION

Diabetes is becoming something of a pandemic and despite the recent surge in new drugs to treat and prevent the condition; its prevalence continues to soar. The multiple activities of plant-based medicinal preparations meant for diabetes offer enormous scope for combating the threat of the diabetic epidemic. α-Amylase inhibitors are those compounds which can inhibit the activity of the enzyme thereby preventing the degradation of starch into sugar molecules. Antioxidants are compounds in fruits and vegetables, which help in avoiding chronic diseases. They act as a defence system against oxidative damage in our bodies and may help in avoiding chronic diseases. The present work was undertaken to study about the free radical scavenging ability of twelve species of green leaf vegetables, namely, *Centella asiatica, Sesbania grandiflora, Marsilea quadrifolia, Eclipta prostrata, Althernanthera sessilis, Cardiospermum halicacabum, Solanum nigrum, Hibiscus cannabinus, Moringa oleifera, Basella alba, Amaranthus blitum and Portulaca oleracea.* The primary study carried out confirmed the presence of α-amylase inhibitors and free radical scavenging potential in the plant extracts. On the whole, all the plants exhibited good α-amylase inhibiting activity as well as antioxidant potential. The plants were also analysed for total phenol content. Correlation analysis carried out between total phenol content with α-amylase inhibitory activity, antioxidant activity, separately revealed less significant values.

Our result demonstrated that *Cardiospermum halicacabum* and *Portulaca oleracea* possessed strong inhibitory activity (low IC_{50} values) against porcine pancreatic α-amylase.

DPPH radical scavenging activities of different plant extracts revealed that *Portulaca oleracea* and *Solanum nigrum* have relatively antioxidant activities among the plants tested.

It was also found that *Portulaca oleracea* and *Cardiospermum halicacabum* contain both antioxidant capacity and α-amylase inhibitory activity.

6. FUTURE PERSPECTIVES

The plants which possessed higher antioxidant and α-amylase inhibitor activities will be further investigated for the active compound(s) responsible for these properties. The compound(s) exhibiting these bioactivities will be isolated, purified by chromatographic methods and identified with the help of LC-MS, NMR, IR and other spectroscopic techniques. Animal experiments will be carried out to confirm the bioactivity *in vivo* condition.

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