EXTRACTION OPTIMIZATION ISOLATION AND APPLICATIONS OF FERULIC ACID FROM *ARACHIS HYPOGAEA* SHELLS

A PROJECT REPORT

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LIST OF ABBREVIATIONS

FA - Ferulic acid
EEPS - Ethanolic Extract of Peanut shells
UV - Ultra Violet
ROS - Reactive Oxygen Species
HPLC - High Performance Liquid Chromatography
TLC - Thin Layer Chromatography
PTLCIC -Preparative Thin Layer Chromatography Isolated Compound
BSI - Botanical Survey of India
DPPH - 1, 1-Diphenyl-2-Picryl-hydrazil
PL - Pancreatic Lipase
LPL - Lipoprotein Lipase
HSL -Hormone Sensitive Lipase

ABSTRACT

ABSTRACT

The extraction optimization of ferulic acid by L_{16} orthogonal design of experiments, inhibitory action of α-amylase and *in vitro* antioxidant activity of *Arachis hypogaea* shells were studied. The maximum yield of ferulic acid was obtained from the microwave irradiation mediated extraction than the ultrasonication method. The yield was found to be 3.572 mg/g at optimum conditions like 45 sec at 540 watts with 1:10 material ratio for microwave mediated extraction and 10.855 mg/g in 15min using 95% ethanol with material ratio of 1:15 for ultrasonication mediated extraction. Presence of ferulic acid related compounds in the optimal extract was identified by Thin Layer Chromatography (TLC) and isolated by preparative thin layer chromatography (PTLC). RP-HPLC fingerprints also revealed the presence of ferulic acid related compounds. EEPS (Ethanolic Extract of Peanut Shells) and PTLCIC (Preparative Thin layer Chromatography Isolated compound) showed α-amylase inhibitory activity of about 78.2% and 33.3% respectively and appreciable antioxidant activity was observed (DPPH for EEPS and PTLCIC are 41.2% and 79.6% respectively).

Keywords: Ferulic acid, *Arachis hypogaea,* α-amylase inhibition, antioxidant activity, Microwave irradiation, Orthogonal design.

INTRODUCTION

1.INTRODUCTION

A medicinal plant is any plant which, in one or more of its parts, contains substances that can be used for therapeutic purpose or which is a precursor for synthesis of drugs. Any plant that possesses therapeutic properties or exerts beneficial pharmacological effects on an animal system is also designated as medicinal plant. According to WHO 80% of the population in the developed countries depent upon traditional herbal medicine. Plant based products are considered to be less or non toxic. It has now been established that the plants which naturally synthesize and accumulate some secondary metabolites, like phenolics, alkaloids, flavonoids, glycosides, tannins, and volatile oils, possess medicinal properties. (Sathish kumar *et al.,* 2009*)*.

Medicinal plants have been considered as a healthy source of life for all people. Therapeutical properties of medical plants are very useful in healing various diseases and the advantage of these medicinal plants is being 100% natural. Nowadays people are being marketed with thousands of unhealthy products, the level of sensibility infront of diseases is very high and here the medicinal plants play a pivotal role as an alternative for synthetic products. Herbalism is a traditional medicinal or folk medicine practice based on the use of plants and plant extracts. Herbalism is also known as botanical medicine, medical herbalism, herbal medicine, herbology, and phytotherapy. The scope of herbal medicine is sometimes extended to include fungal and bee products, as well as minerals, shells and certain animal parts (Acharya *et al.*, 2008).

Many plants synthesize substances that are useful to the maintenance of health in humans and other animals. These include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins. Many are secondary metabolites, of which at least 12,000 have been isolated a number estimated to be less than 10% of the total. In many cases, substances such as alkaloids serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores. Many of the herbs and spices used by humans as season food offerly provides beneficial effects (Lai, 2004).

Despite the increased popularity of herbal treatments, the safety and effectiveness of alternative medicines have not been scientifically corroborated and remain largely unknown. Furthermore, "adulteration, inappropriate formulation, or lack of understanding of plant and drug interactions have led to adverse reactions that are sometimes life threatening or lethal." Herbalists are often trained to take well-established risks into consideration when patients consult them.

Phenolics are considered as potential therapeutic agents against a wide range of ailments including neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunction, inflammatory diseases and in ageing (Soobrattee *et al.*, 2005). They are widely distributed in the plant kingdom and are therefore an integral part of the diet, with significant amounts being reported in vegetables, fruits and beverages (Luximon-Ramma *et al.*, 2005). Although the dietary intake of phenolics varies considerably among geographic regions, it is estimated that daily intake range from about 20 mg to 1 gm, which is higher than that for vitamin E (Hollman and Katan, 1998). Phenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Middleton *et al.*, 2000). Dietary plant phenolic compounds have been described to exert a variety of biological actions such as free radical scavenging, metal chelation, modulation of enzymatic activity and more recently to affect signal transduction, activation of transcription factors and gene expression. They received particular attention in the past 10 years because of their putative role in the prevention of several human diseases, particularly atherosclerosis and cancer (Nardini and Ghiselli, 2004). Phenolic compounds are essential for the growth and reproduction of plants, and are produced as a response for defending in injured plants against pathogens. The importance of antioxidant activities of phenolic compounds and their possible usage in processed foods as a natural antioxidant have reached a milestone both in food biotechnology and health care sector. The absorption and bioavailability of phenolics in humans are controversial. Data on these aspects of phenolics are scarce and merely highlight the need for extensive investigations of the handling of phenolics by the gastrointestinal tract and their subsequent absorption and metabolism.

Phenolic acid compounds seem to be universally distributed in plants. They have been the subject for chemical, biological, agricultural, and medical related studies. Phenolic acids form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Hydroxycinnamic acid compounds occur most frequently as simple esters with hydroxy carboxylic acids or glucose. Hydroxybenzoic acid compounds are present mainly in the form of glucosides. One such prominent phenolic acid is ferulic acid. Ferulic acid is an organic compound that is abundant phenolic phytochemical found in plant cell walls and highly distributed in the seeds of plants such as in rice, wheat, and oats, as well as in coffee, apple, artichoke, peanut, orange and pineapple (Iiyama, 1994). The dehydrodimers of ferulic acid are important structural components in the plant cell wall and serve to enhance its rigidity and strength (Mathew and Abraham, 2004).

LITERATURE REVIEW

Ferulic acid is an antioxidant in the sense that it is reactive toward free radicals such as reactive oxygen species (ROS) and other free radicals that are implicated with DNA damage.

Animal studies and *in vitro* studies suggest that ferulic acid may have direct antitumor activity against breast cancer and liver cancer (Shahadi *et al.,* 2004). Ferulic acid may have pro-apoptotic effects in cancer cells, thereby leading to their destruction. Ferulic acid may be effective at preventing cancer induced by exposure to the carcinogenic compounds ethyl 3-(4-geranyloxy-3-methoxyphenyl)-2-propenpate (Taniguchi, 1999), benzopyrene and 4-nitroquinoline 1-oxide. Ferulic acid, being highly abundant, may be useful as a precursor in the manufacturing of vanillin. Synthetic flavoring agent often used in place of natural vanilla extract. Ferulic acid ethyl ester protects neurons against amyloidal [beta] - peptide and neurotoxin (Sultana *et al.*, 2005). Ferulic acid is used as the starting material for the preparation of reagents having anti-bacterial activity, chemo preventive agents, fragrances, food additives, control of germination and cosmetic (Taniguchi *et al.*, 2003).

2. LITERATURE REVIEW

2.1 *Arachis hypogaea*

The peanut, or groundnut (*Arachis hypogaea*), is a species in the legume "bean" family (Fabaceae) native to South America, Mexico and Central America. In India it is distributed in the southern states viz., Tamil Nadu, Karnataka, Andhra Pradesh. It is an annual herbaceous plant growing 30 to 50 cm (0.98 to 1.6 ft) tall. The leaves are opposite, pinnate with four leaflets (two opposite pairs; no terminal leaflet), each leaflet 1 to 7 cm (⅜ to 2¾ in) long and 1 to 3 cm (⅜ to 1 inch) broad. The flowers are a typical peaflower in shape, 2 to 4 cm (¾ to 1½ in) across, yellow with reddish veining. After pollination, the fruit develops into a legume 3 to 7 cm (1.2 to 2.8 in) long, containing 1 to 4 seeds, which forces its way underground to mature. Peanuts are known by many local names, including earthnuts, ground nuts, goober peas, monkey nuts, pygmy nuts and pig nuts. The term "Monkey nut" is often used to mean the entire pod.

Table 2.1.1 Classification of *Arachis hypogaea*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Subfamily	Faboideae
Tribe	Aeschynomeneae
Genus	Arachis
Species	A. hypogaea
Binomial name	*Arachis hypogaea*

The peanut is perhaps the world's most widely researched oilseed. Advantages over other oilseeds include relatively bland flavour, minor colour problems,and minimal preparation requirements (Lusas, 1979). Products in use throught the world include boiled, partially or completely defatted peanuts, peanut butters grits and flours (full fat or defatted). That finds applications in fortified breads and bakery products, snacks, meat products, extended milks, cheese and curd type products, and various mass-feeding foods in developing countries.

Seeds yield a non-drying, edible oil, used in cooking, margarines, salads, canning, for deep-frying, for shortening in pastry and bread, and for pharmaceuticals, soaps, cold creams, pomades and lubricants, emulsions for insect control, and fuel for diesel engines. The oil cake, a high-protein livestock feed, may be used for human consumption. Other products include dyes, ice cream, massage oil, paints, and peanut milk. Seeds are eaten raw, whole roasted and salted, or chopped in confectioneries, or ground into peanut butter. Young pods may be consumed as a vegetable. Young leaves and tips are suitable as a cooked green vegetable (Martin and Ruberte, 1975). Peanut hulls are used for furfural, fuel, as filler for fertilizers, and for livestock feed, or sweeping compounds. Recent research studies on peanuts and nuts in general have revealed their health benefits. (Yao, 2004).

Fig 2.1.2 ***Arachis hypogea***

Peanuts play a significant role in various folk pharmacopoeias like inflammation, nephritis aphrodisiac, cholecystosis, and decoagulant (Duke and Wain (1981). In China the nuts are considered demulcent, pectoral, and peptic; the oil aperient and emollient, taken internally in milk for gonorrhea, externally for rheumatism (Duke and Ayensu, 1985). It is used in folk remedies for plantar warts. The alcoholic lipid fraction of the seed possesses preventive action against hemophiliac tendencies and for the treatment of some blood related disorders like mucorrhagia, arthritic hemorrhages and hemophilia. Peanut is a potent plant to be induced to synthesize bioactive stilbenoids and it possess potent anti-oxidant, anti- inflammatory activity (Chang *et al.*, 2006). Reports are there for amylase inhibitory effect of peanuts (Irshad *et al.*, 1981).

Peanuts contain high concentrations of antioxidant polyphenols, primarily a compound called p-coumaric acid, and the roasting process can increase the peanuts' p-coumaric acid levels, boosting their overall antioxidant content by as much as 22% of the total content. Peanut (per 100 g) contains Carbohydrates 22 g, Fat 50 g, Dietary fiber 9 g, Protein 24 g and Energy 590 kcal 2450 kJ. Peanuts are among the most common causes of immediate hypersensitivity reactions to foods. In vivo studies suggest *Arachis hypogea* that the ethanolic (95%) extract of *Arachis hypogea* nut shells might be useful as a treatment to reduce the dietary fat absorption, triacylglycerol content in the liver, as well as the serum glucose and insulin. PSE inhibits a number of lipases, including PL (Pancreatic lipase), LPL (Lipoprotein lipase) and, possibly, HSL (Hormone sensitive lipase) (Moreno *et al.*, 2006).

However, there are no scientific reports dealing the extraction optimization of ferulic acid from the plants using a consortium of simple chemical and physical methods with a suitable cost effective design. In this connection, our laboratory is trying to explore a suitable cost effective method for the extraction and identification of ferulic acid.

2.2 Ferulic acid (FA) - Introduction

They are naturally occurring substances found in plants. Ferulic acid (FA) is a phytochemical commonly found in fruits and vegetables such as tomatoes, sweet corn and rice bran. It arises from metabolism of phenylalanine and tyrosine by Shikimate pathway in plants. It exhibits a wide range of therapeutic effects against various diseases like cancer, diabetes, cardiovascular and neurodegenerative. A wide spectrum of beneficial activity for human health has been advocated for this phenolic compound, at least in part, because of its strong antioxidant activity. FA, a phenolic compound is a strong membrane antioxidant and known to positively affect human health. FA is an effective scavenger of free radicals and it has been approved in certain countries as food additive to prevent lipid peroxidation. It effectively scavenges superoxide anion radical and inhibits the lipid peroxidation. It possesses antioxidant property by virtue of its phenolic hydroxyl group in its structure. The hydroxy and phenoxy groups of FA donate electrons to quench the free radicals. The phenolic radical in turn forms a quinone methide intermediate, which is excreted via the bile. The past few decades have been devoted to intense research on antioxidant property of FA. So, the present review deals with the mechanism of antioxidant property of FA and its possible role in therapeutic usage against various diseases.

2.2.1 Sources and structure of FA

FA is a ubiquitous plant constituent that arises from the metabolism of phenylalanine and tyrosine. It occurs in seeds and leaves both in its free form and covalently linked to lignin and other biopolymers. In wheat, FA is ester linked to cell wall carbohydrates and occurs in higher concentration in the alcurone, pericarp and embryo cell walls. The trans-isomer predominates and accounts for 90% of the total phenolic acids in common flour (Fulcher, 1983). FA is also a major constituent of fruits (e.g. orange), some vegetables (e.g. tomato, carrot), and sweet corn (Balasubashini *et al.*, 2003).

FA (4-hydroxy-3-methoxy cinnamic acid) (Fig 2.2.1.1) is a phenolic compound it possesses three distinctive structural motifs that can possibly contribute to the free radical scavenging capability of this compound. The presence of electron donating groups on the benzene ring (3 methoxy and more importantly 4-hydroxyl) of FA gives the additional property of terminating free radical chain reactions. The next functionality-the carboxylic acid group in FA with an adjacent unsaturated C-C double bond-can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. In addition, this carboxylic acid group also acts as an anchor of FA, by which it binds to the lipid bilayer, providing some protection against lipid peroxidation. Clearly, the presence of electron donating substituents enhances the antioxidant properties of FA (Kanaski *et al.,* 2002)

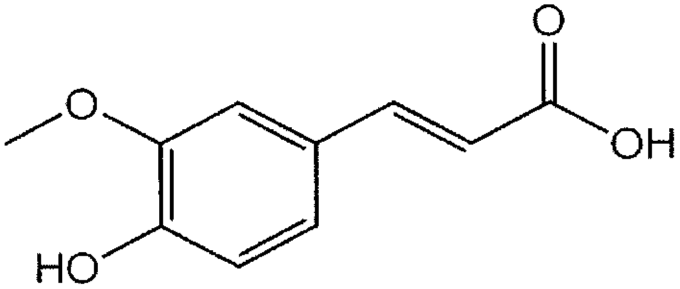


Fig 2.2.1.1 Structure of ferulic acid

2.2.2 Bioavailability of FA

The physiological importance of FA and notably its antioxidant property depends upon its availability for absorption and subsequent interaction with target tissues (Scheline, 1968). It is more bioavailable than other dietary flavonoids and monophenolics so far studied (Beecher, 1998). FA stays in blood for longer than other antioxidants such as vitamin C. FA would therefore be expected to stay in the body long enough to help in keeping the free radicals at bay. Under normal conditions 56.1% of perfused FA enters the enterocytes by a yet unidentified mechanism. In these cells, FA is readily conjugated and the resulting metabolites leave the intestinal cells only towards the serosal side because no conjugated forms of FA are detected in the intestinal lumen. Under such conditions 56.1% of perfused FA, corresponding to the absorption, is recovered in the plasma mesenteric vein as conjugated derivative. A part of these conjugates enters into the hepatocytes and secreted in the bile (6%) and 49.9% of the perfused dose is distributed to the peripheral tissues and may have biological effects (Adam *et al.*, 2002).

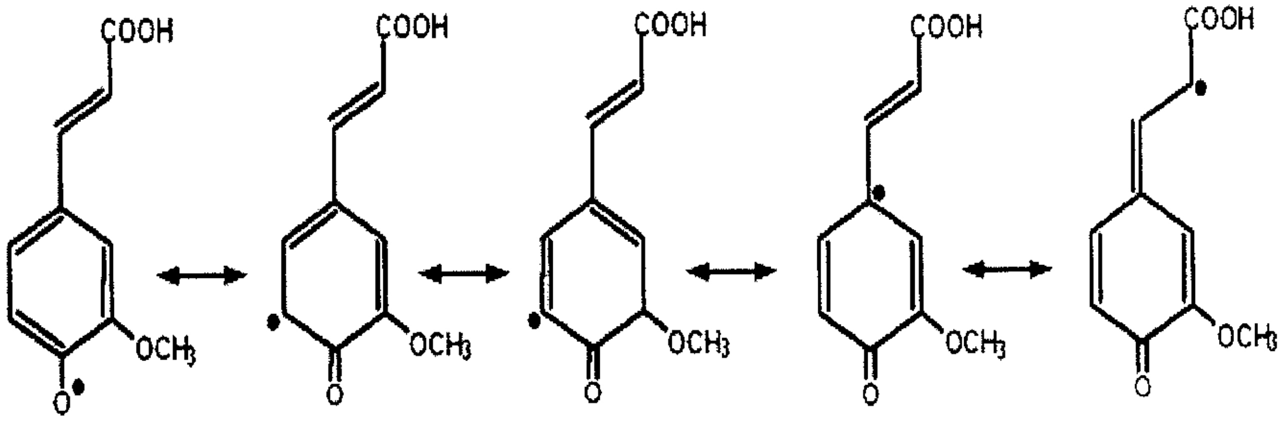
2.2.3 Metabolism and Absorption of FA

The absorption, metabolism and tissue distribution of FA has been extensively studied in rodents and humans. The metabolites of FA and their relative proportions will depend on many factors, including dose, route of administration and animal species. Ingestion of FA into humans is metabolized and excreted in urine as 3-hydroxyphenyl and 3-methoxy-4-hydroxy phenyl derivatives of phenyl propionic acid, hydracrylic acid and glycine conjugates. Feeding studies in rats with FA revealed metabolism to a dehydroxylated compound and the same hydroxy methoxy derivatives, as in the human studies, with FA itself being partly excreted as the glucuronide. Intraperiotneal administration of FA to the rats is excreted as 3-hydroxy phenyl propionic acid a major urinary metabolite.

2.3 Applications of FA

2.3.1 Antioxidant Action of FA

The antioxidant potential of FA can usually be attributed to its structural characteristics (Fig. 2.3.1.1) FA, because of its phenolic nucleus and unsaturated side chain can readily form a resonance stabilized phenoxy radical, which accounts for its potent antioxidant activity. Any reactive radical colliding with FA easily abstracts a hydrogen atom to form phenoxy radical. This radical is highly resonance stabilized since the unpaired electron may be present not only on the oxygen but it can be delocalized across the entire molecule. Additional stabilization of the phenoxy radical is provided by the extended conjugation in the unsaturated side chain. This resonance stabilization accounts for the effective antioxidant potential of FA. Moreover this phenoxy radical is unable to initate or propagate a radical chain reaction, and its most probable fate is a collision and condensation with another ferulate radical to yield the dimer curcumin. Such coupling may lead to a host of products, all of which still contain phenolic hydroxyl groups capable of radical scavenging. The presence of a second phenolic hydroxyl group substantially enhances the radical scavenging activity due to additional resonance stabilization and o-quinone formation (Graf, 2000).



2.3.1.1 Resonance Stabilization of FA radical

2.3.2 Anti-inflammatory Effect of FA

Chronic or acute inflammation is a multiple process, which is mediated by activated inflammatory or immune cells. From the immune system, macrophages play a central role in managing many different immunopathological phenomena such as the overproduction of pro-inflammatory cytokines and inflammatory mediators (reactive oxygen species (ROS), nitric oxide (NO) and prostaglandin E_2) generated by activated inducible nitric oxide synthase (iNOS) and cyclooxygenase (Walsh, 2003). It has been reported that a number of antioxidants including FA and related ester derivatives decrease the levels of some inflammatory mediators, e.g., prostaglandin E2 and tumor necrosis factor-alpha (Ou *et al.*, 2003) and iNOS expression and function (Tetsuka *et al.*, 1996) in cells stimulated by the bacterial endotoxin lipopolysaccharide. There is evidence that hydrophobic ester derivatives of FA have enhanced inhibitory activity on iNOS protein expression in lipopolysaccharide/interferon-γ (LPS/IFNγ) activated RAW 264.7 cells (Murakami *et al.*, 2002). (Hosoda *et al.,* 2002) reported that feruloyl-myo-inositois the derivatives of FA suppressed the cyclooxygenase-2 promoter activity through the β-galactosidase reporter gene assay system in human colon cancer DLD-1 cells. It was also reported that FA inhibits the production of murine MIP-2, one of the members of chemokine superfamily, in LPS-stimulated RAW 264.7 cells in a dose dependent manner. These findings suggest that FA might have potential as an anti-inflammatory drug and reveals at least in part the mechanisms of its anti-inflammatory effect (Sakai *et al.*, 1997).

2.3.3 Pulmonary Protective Effect

Nicotine is one of the major hazardous compounds of cigarette smoke and thus mimics most of the deleterious effects of cigarette smoking. It increases lipid peroxidation and thus causes oxidative cellular injury, which is believed to play a major role in the pathogenesis of several smoking-related diseases (Yildiz *et al.*, 1998) Administration of FA, reversed the damage induced by nicotine and increases the endogenous antioxidant defense system and protects the cells from oxidative damage. FA effectively quenches the free radicals, prevents them from attacking the membrane, protects the membrane, inhibits the leakage of marker enzymes into circulation, and improves the antioxidant status in circulation (Sudheer *et al.*, 2005).

2.3.4 Anti-Diabetic Effect

Diabetes is the most common endocrine disorder characterized by the hyperglycemia, which causes over production of free radicals thereby result in oxidative stress (Aragno *et al.*, 2000). This stress is defined as an imbalance between the levels of prooxidants and antioxidants in the biological systems, leading to cellular injury (Villa-caballero *et al.*, 2000). It has been reported that the blood glucose level in streptozotocin induced diabetic animals is reduced by the administration of FA. FA, which has been shown to have antioxidant properties, helps to neutralize the free radicals produced by streptozotocin in the pancreas and thereby decrease the toxicity of streptozotocin. This decreased oxidative stress/toxicity on the pancreas may help the beta cells to proliferate and secrete more insulin, which may have been reduced due to streptozotocin treatment. This increased insulin secretion can cause increased utilization of glucose by the extra hepatic tissues and thereby decrease the blood glucose level (Balasubashini *et al.*, 2004). Nomura *et al.* (Noumura *et al.*, 2003) have also been reported that amide compounds of FA exhibited their stimulatory abilities on insulin secretion in rat pancreatic RIN-5F cells. Administration of FA at a dose of 0.01% and 0.1% of basal diet showed it can suppress the blood glucose levels in streptozotocin induced diabetic mice. In KK-Ay mice 0.05% of FA suppressed the blood glucose level effectively (Ohnishi *et al.*, 2004).

2.3.5 Anti-Cancer Effects

Free radicals are considered as important factors in the etiology of cancer. Dietary components with antioxidant activity have been receiving particular attention as potential inhibitors of several cancers (Dedoussis *et al.*, 2005). Phytochemicals can exert anti-cancer activities, partially based on their ability to quench ROS and thereby protecting critical cellular molecules (i.e. DNA, proteins and lipids) from oxidative insult (Roy *et al.*, 2003). Phytochemicals may also interfere with intracellular signaling pathways, such as those, which regulate proliferation, induction of apoptosis and response to oxidative stress (Loo, 2003). Important mechanisms for the anticarcinogenic effects of polyphenols include the reduction of proliferative activity and the induction of apoptosis in cancer cells (Vermeulen *et al.*, 2003). Studies have shown that FA exhibits anticarcinogenic effects against azoxymethane-induced colon carcinogenesis in F344 rats (Kawabata *et al.*, 2000). It has also been reported to depress 12-O-tetradecanoylphorbol-13-acetate (TPA)-promotion of skin tumorgenesis (Asanoma *et al.*, 1993) as well as to inhibit occurrence of pulmonary cancers in mice (Lesca, 1983). Stich *et al.* have reported that there was a significant decrease in urinary N-nitrosoproline levels in humans on treatment with FA. The mechanism suggesting that inhibition of nitrosation and endogenous formation of carcinogenic nitrosamines. Several plant phenolics are known to be potent inhibitors for mutagenesis and carcinognesis by polycyclic aromatic hydrocarbon (Newmark, 1987). They act as effective electrophilic trapping agents (Newmark, 1984) and are also known to be blockers of nitrosamine formation (Kuenzing *et al.*, 1984).

2.3.6 Anti-Apoptotic Effect

Apoptosis is a certain type of cell death in multicellular organisms and involves a cascade of closely regulated intracellular events leading to cell suicide (Jacobson, 1996). Reports suggest that phenolic compounds generally bring about the normal homeostasis by inducing apoptosis in various cancer cells (Taraphder *et al.*, 2001). Studies have shown the cytotoxic effects of these dietary polyphenols against different tumors, mediated through apoptosis (Inoue *et al.*, 1994). Cells undergoing programmed cell death express phosphatidyl serine on their surface, which aids in their recognition and phagocytosis by macrophages, thereby limiting inflammation (Martin *et al.*, 1995). Externalization of phosphatidyl serine by hydrogen peroxide (H_2O_2) indicates pre-apoptotic stage of the peripheral blood mononuclear cells (PBMCS). The inhibition of externalization of phosphatidyl serine by FA indicates the anti-apoptotic activities of these polyphenols in human PBMCS. The decrease in H_2O_2-induced externalization of phosphatidyl serine in peripheral blood mononuclear cells pretreated with polyphenols indicates the involvement of scavenging of radicals by the phenolics and/or dissociation of phenolic-translocase enzyme binding due to oxidative stress (Khanduja *et al.*, 2006) have reported that phenolic compounds like FA significantly exhibit anti-apoptotic activity in normal PBMCS exposed to H_2O_2 induced oxidative stress.

2.3.7 Anti-Ageing Effect

Acute and chronic exposure to sun rays promotes premature skin ageing, erythema, inflammation, immunodepression and photo-carcinogenesis (Anselmi *et al.*, 2004). Exposure of ultra violet (UV) radiation results in the generation of reactive oxygen/nitrogen species resulting in oxidative damage. These events can ultimately lead to diseases related to UV-radiation, such as irritation or sunburn, photoallergy, immunosuppression, photoageing and skin cancer (Black *et al.*, 1997). Recently a great deal of focus has been shed on the antioxidant potentialities of FA. This is due to its phenolic nucleus and an extended side chain conjugation, FA readily forms a resonance stabilized phenoxyl radical, which accounts for its potent antioxidant potential. UV absorption by FA catalyses stable phenoxyl radical formation and thereby potentiates its ability to terminate free radical chain reactions. By virtue of effectively scavenging chain reactions and deleterious radicals and suppressing radiation-induced oxidative reactions, FA may also serve as an important antioxidant in preserving physiological integrity of cells exposed to both air and the impinging UV radiation (Graf, 2000).

2.3.8 Hepatoprotective Effect

Chronic alcohol ingestion is associated with a variety of pathological conditions varying from simple intoxication to severe, life threatening derangement of metabolism in liver (Lindi *et al.*, 1998). Intake of alcohol results in excessive generation of free radicals (Rouach *et al.*, 1997), which alter the biomembrane and cause severe damage. FA works well in herbal antioxidant formula, vitamin and herbal health supplement, and body's immune system can be benefited from FA. These reports heavily favour the idea that regular ingestion of FA may provide substantial protection against alcohol and polyunsaturated fatty acid (PUFA) induced toxicity and may provide the body with the ability to triumph over the deleterious effects of alcohol and PUFA (Rukkumani *et al.*, 2004). Treatment with FA significantly decreased the activities of these enzymes in plasma. FA is shown to preserve physiological integrity of the cells exposed to various stress. This can be attributed to the effective antioxidant property of FA. Normally phenolic compounds act by scavenging free radicals and quenching the lipid peroxidative side chain. Phenolic compounds can act as free radical scavengers by virtue of their hydrogen donating ability and forming aryloxyl radicals (Gil *et al.*, 2000). It has been proposed that hydroxyl and hydroperoxyl radicals initiate H^+ abstraction from a free phenolic substrate to form phenoxyl radical that can rearrange to quinonemethide radical intermediate (Pan *et al.*, 1999), which is excreted via bile. (Srinivasan *et al.*, 2005) have also been reported FA protects against carbon tetrachloride (CCl_4) induced toxicity in an experimental animal model, which ascribed to antioxidant potential.

OBJECTIVES

3. OBJECTIVES

1. To estimate the amount of ferulic acid present in the Peanut shells.
2. To optimize the extraction of ferulic acid from peanut shells by using ultra sonication and microwave irradiation.
3. To detect the presence of ferulic acid related compounds from optimal extract using thin layer chromatography (TLC).
4. To isolate ferulic acid using preparative thin layer chromatography (PTLC).
5. To purify ferulic acid using High Performance Liquid Chromatography (HPLC).
6. To compare the applications of crude extract with the PTLC isolated ferulic acid related compound (*in vitro* antioxidant & pancreatic α-amylase inhibitory activity).

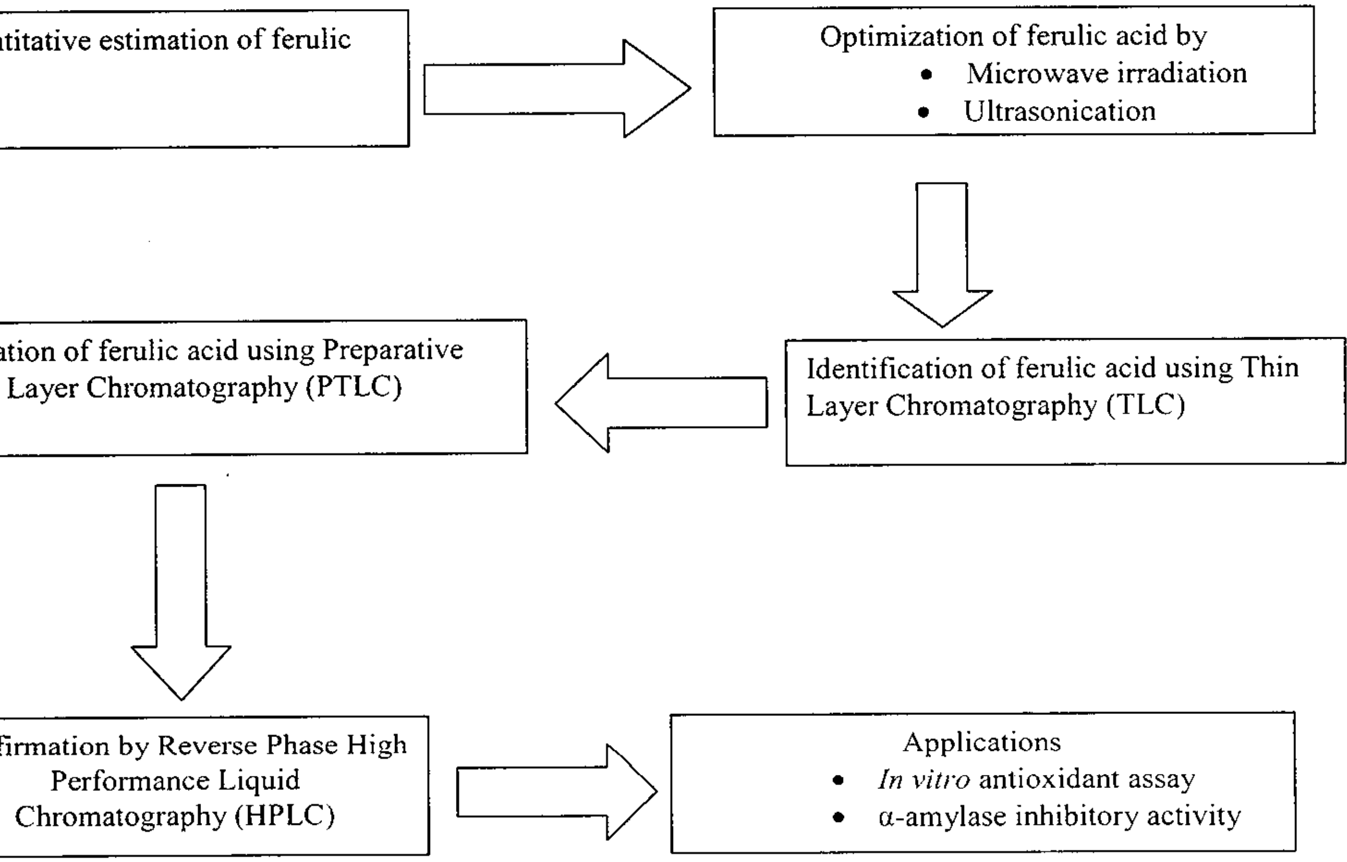
MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Chemicals

All chemicals used were of analytical grade. Ethyl ferulate was obtained from Sigma Chemicals, USA. Ferulic acid, quercetin and ethanol were obtained from HiMedia Chemicals, Mumbai, India. Titanium tetra chloride was obtained from loba chemicals Chennai.

4.2 Methodology



भारत सरकार / **GOVERNMENT OF INDIA**
पर्यावरण एवं वन मंत्रालय / **MINISTRY OF ENVIRONMENT & FORESTS**
भारतीय वनस्पति सर्वेक्षण / **BOTANICAL SURVEY OF INDIA**

दक्षिणी परिमण्डल / **Southern Regional Centre**
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सं. भावस/ दप
No. BSI/SC/5/23/09-10/Tech. – 1696

दिनांक/Date: 11 March 2010

सेवा में/To

Mr. M. Sampath
Mr. S.V. Sivachandran
Final Year B. Tech. (Biotechnology)
Kumaraguru College of Technology
Coimbatore – 641 006

महोदय/Sir,

The plant specimen brought for identification is identified as *Arachis hypogaea* L. – FABACEAE.

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

GVS Murthy
11/3/1

(जि.वि.एस. मूर्ति/Dr. G.V.S. MURTHY
संयुक्त निदेशक /Joint Directo

4.3 Plant material

The peanut shells were collected from the Department of oil seed at Tamilnadu agricultural university, Coimbatore, India. The species was identified, confirmed by Botanical Survey of India (BSI), Southern Circle, and Coimbatore, India. The shells of *Arachis hypogaea* dried in a hot air oven for 2-3 days at 35-45°C and made into powdered form. Dried shells were used for the analysis.

4.4 Optimization of aqueous extraction of ferulic acid by microwave irradiation (Khizar Hayat *et al.*, 2009)

Extraction of ferulic acid could be obtained by $L_{16}(4)^3$ orthogonal design i.e., four levels and three different variables (Microwave power, Extraction time, Solid liquid ratio) Power varying from 180 to 720. Extraction time varies from 15 to 45 sec and the solid liquid ratio varying from 1:5 to 1:20.

Table 4.4.1.1 $(4)^3$ orthogonal design parameters

Levels	**Power (A)**	**Sol:Liq (B)**	**Microwave time (sec) (C)**
1	180	1:5	15
2	360	1:10	25
3	540	1:15	35
4	720	1:20	45

Table 4.4.1.2 (4) ^{3} L_{16} orthogonal design

Experiments	A	B	C
1	1	1	1
2	1	2	2
3	1	3	3
4	1	4	4
5	2	1	2
6	2	2	1
7	2	3	4
8	2	4	3
9	3	1	3
10	3	2	4
11	3	3	1
12	3	4	2
13	4	1	4
14	4	2	3
15	4	3	2
16	4	4	1

4.5 Estimation of ferulic acid (Michael Eskin *et al.*, 1978)

Principle

Ferulic acid is extracted with alcohol, dried and dissolved in acetone. It is reacted with titanium ion to form a coloured complex which is measured at 450nm.

Materials

- Titanium Reagent: 20% $TiCl_4$ in con. HCl
- Standard 25-200 µg/ml Ferulic acid in Acetone
- Acetone
- 80% Ethanol
- 2.5N HCl

Procedure

1. Reflux twice a known quantity of dried peanut shells powder in 80% ethanol (adjusted to pH 4.0 with 2.5N HCl) for 30 min (125ml to 1g peanut shells).
2. Discard the precipitate and collect 250ml of the extract.
3. Remove 0.5ml of samples and dry in a hot air oven at 50^0C and 700mm pressure for 2 hours.
4. Dissolve dried extract in 4.75ml of acetone.
5. Add 0.25ml of $TiCl_4$
6. Read the colour at 450nm against reagent blank (acetone plus $TiCl_4$).
7. Similarly treat the standards with $TiCl_4$ and read the colour intensity.
8. Draw a standard curve and find out the ferulic acid content in the sample.

Calculation

Express the ferulic acid content as g per 100g sample.

4.6 Optimization of ethanolic extraction of ferulic acid by ultrasonication (Lan Zhang *et al.*, 2009).

Extraction of ferulic acid could be obtained by $L_{16}(4)^3$ orthogonal design i.e., four levels and three different variables (Solvent percentage, Extraction time, Solid Liquid ratio). Solvent percentage varied from 65 to 95. Extraction time varied from 10 to 25 min and the solid liquid ratio varied from 1:5 to 1:20. The optimal extract was analyzed for ferulic acid content **(See section 4.5)**

Table 4.6.1 $(4)^3$ orthogonal design parameters

Levels	Con. ethanol (%) (A)	Sol:Liq (B)	Ultrasound time (min) (C)
1	65	1:5	15
2	75	1:10	25
3	85	1:15	35
4	95	1:20	45

Table 4.6.2 $(4)^3$ L_{16} orthogonal design

Experiments	A	B	C
1	1	1	1
2	1	2	2
3	1	3	3
4	1	4	4
5	2	1	2
6	2	2	1
7	2	3	4
8	2	4	3
9	3	1	3
10	3	2	4
11	3	3	1
12	3	4	2
13	4	1	4
14	4	2	3
15	4	3	2
16	4	4	1

4.7 Identification methods

4.7.1 Identification of ferulic acid by Silica gel mediated Thin Layer Chromatography

Chromatographies of the optimized extracts from shells were run one dimensionally in the mobile phase solvent (Chloroform-Ethanol-Formic acid, 85:15:1, v/ v/ v) at room temperature of 20-25°C. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 5mm. Authentic markers of ferulic acid and ethyl ferulate obtained commercially were co-chromatographed. Identification of the ferulic acid in the extracts was identified under UV light after the application of 1% ferric chloride. (Leonard Vuyani Mabinya *et al.*, 2006).

4.7.2 Identification of flavonoids by Thin Layer Chromatography

Chromatographies of the optimized extracts from leaves and flowers were run one dimensionally in the mobile phase solvent (ethyl acetate - ethanol - water, 5:1:5, v/ v/ v) at room temperature of 20-25°C. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 5mm. Authentic markers of flavonol (quercetin) and flavanoid glycoside (rutin) obtained commercially were co-chromatographed. Identification of the flavosnoids in the extracts was identified under UV light after the application of Ammonia (Sathish kumar *et al.*, 2009).

4.8 Isolation method

The Preparative Thin Layer Chromatography (PTLC, 20 X 20 cm, 2mm thickness of Silica gel) was performed as similar as TLC to isolate ferulic acid related compounds.

4.9 RP-HPLC analysis

HPLC analysis was performed using isolated fragments. Separation was carried out at 25°C on a C_{18}-agilentcolumn (25 cm length). Ten microlitres of the isolated compounds from shells was injected and eluted radiantly at 1 ml/min with the mixture of acetic acid and water (50:50). Peaks were detected by UV absorbance at 320 nm and qualitative analysis was performed by comparison with standard ferulic acid retention time (Mustafa Tuzen, 2003).

5. APPLICATIONS

5.1 *In vitro* antioxidant assays

5.1.1 Determination of ferric ion reducing/Antioxidant power assay (FRAP):

A method developed by (Oyaizu, 1986) was adopted for the determination of reducing power. 2.5ml of different concentrations of the EEPS (100-500 µg/ml) and PTLCIC 0f (0.5-2.5 ml) were mixed with 2.5 ml phosphate buffer (0.2 M, P^H 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20minutes, then rapidly cooled, mixed with 2.5ml of 10% trichloroacetic acid and centrifuged for 10minutes. To the 2.5ml of supernatant, added 2.5ml of distilled water and 0.5ml of 0.1% ferricholoride. Mixed well and allowed to stand for 10minutes. The absorbance was measured at 700nm.

5.1.2 Determination of DPPH- radical scavenging assay

DPPH- radical scavenging activity was determined according to the (Park and Kim, 2004). To 0.5ml of DPPH radical solution, 1.0ml of the EEPS (100-500 µg/ml) and PTLCIC of (0.5-2.5 ml). The reaction mixture was vortexed for 10s and allowed to stand at room temperature for 30 minutes. The absorbance was recorded at 517nm by using (Beckman bu-530) UV-Vis spectrophotometer and compared with the 75 % ethanol which acted as control solution. The percentage of DPPH scavenging activity was expressed in percentage [1 - (test sample absorbance/blank sample absorbance)] X 100(%).

5.1.3 Determination of nitric oxide scavenging activity

Sodium nitroprusside (5mM) was prepared in Phosphate buffer saline.1 ml of this was mixed with 1 ml of EEPS in different concentrations (1 - 10 mg/ml) and PTLCIC of (0.5-2.5ml) in methanol (Green *et al.*, 1982). The mixture was incubated at 25°C for 30 min. After 30 min, an equal volume of Griess reagent was added to the incubated solution. The absorbance of the chromophore formed due to diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine was measured at 546 nm.

5.1.4 Determination of super oxide scavenging activity

The scavenging activity towards the superoxide radical (O2.$^{-}$) was measured in terms of inhibition of generation of O2.$^{-}$(Okamura *et al.*, 1993). The reaction mixture consisted of 2.0 ml of phosphate buffer (50 mM, pH 7.6), 0.2 ml of riboflavin (20 µg / 0.2 ml), 0.2 ml of EDTA (12 mM), 0.2 ml of NBT (0.1 mg / 3ml) and 0.2 ml of sodium cyanide (3µg / 0.2 ml) Test compounds of various concentrations of EEPS (1- 10 mg / ml) and PTLCIC of (0.5-2.5ml) were added to make a total volume of 3.0 ml. The absorbance was read at 530 nm before and after illumination under UV lamp for 15 minutes against a control with buffer instead of sample and 3.0 ml of buffer as blank

5.2 Determination of α -amylase inhibitory activity

Bernfeld method (1955) with a little modification was used to analyze the α-amylase inhibitory activity . To 100µl of EEPS and PTLCIC, 200µl porcine pancreatic α-amylase enzyme and 400µl of 2mM of phosphate buffer (pH 7.5) was added. After 20 minutes incubation, 100µl of 1% starch solution was added. The same was performed for the control where 200µl of enzyme was replaced by distilled water. After 15 minutes incubation 1000μl of dinitrosalicylic acid reagent was added to both control and test. The tubes were kept in a boiling water bath for 5 minutes. The absorbance was recorded at 540nm using Beckman DU530 UV/Vis spectrophotometer and the percentage inhibition of α amylase enzyme was calculated using the formula, Inhibition = ((control - test) / control) x 100%.

RESULTS AND DISCUSSION

6. RESULT AND DISCUSSION

6.1 Optimization ferulic acid from *Arachis hypogaea* shells using microwave irradiation through L_{16} orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table (6.1.1) and Table (6.1.2). The order of the effect of factors on ferulic acid extraction was B>C>A. The (Solid: liquid) had the greatest effect on the extraction procedure and it was found to be significantly different at 1% level. The optimum extraction conditions obtained from the statistical analysis were microwave power 540 watts, Solid: liquid 1:10, 45 sec extraction duration, a material ratio 1:10 and the maximum yield was found to be 346.840 mg/g.

Table 6.1.1 One way ANOVA in microwave irradiation

Levels	Sum of square	Degrees of freedom	Mean square	F-value
A	2369.018	3	1184.509	0.202
B	38915.487	3	19457.743	10.914
C	21406.857	3	10703.428	1.752

Table 6.1.2 Experimental results and range analysis in microwave irradiation

Experiments	A	B	C	Ferulic acid (mg/g)
1	1	1	1	1.15
2	1	2	2	1.94
3	1	3	3	1.94
4	1	4	4	1.11
5	2	1	2	1.71
6	2	2	1	1.71
7	2	3	4	2.92
8	2	4	3	0.64
9	3	1	3	1.67
10	**3**	**2**	**4**	**3.57**
11	3	3	1	0.97
12	3	4	2	0.83
13	4	1	4	1.39
14	4	2	3	2.08
15	4	3	2	1.32
16	4	4	1	1.11
K_1	599.072	576.569	481.962	
K_2	680.110	905.380	565.290	
K_3	684.672	695.870	617.102	
K_4	574.305	360.340	873.805	
K_1	149.768	144.142	120.490	
K_2	170.0275	226.345	141.322	
K_3	171.168	173.967	154.275	
K_4	143.576	90.085	218.451	
R	21.4	136.26	97.96	

6.2 (a) Effect of material ratio (W/V) in the extraction of ferulic acid

Maximum yield of ferulic acid was obtained at 1:10 materials ratio (Fig 6.2.1). Further increase in the material ratio leads to a gradual decrease in the ferulic acid content. This decrease might be due to the fact that when the material ratio reached a certain level, the extract has well dissolved in the solution that may lead the contents of the extract become saturated and prevent further increase (Yaqin Xu *et al.*, 2005).

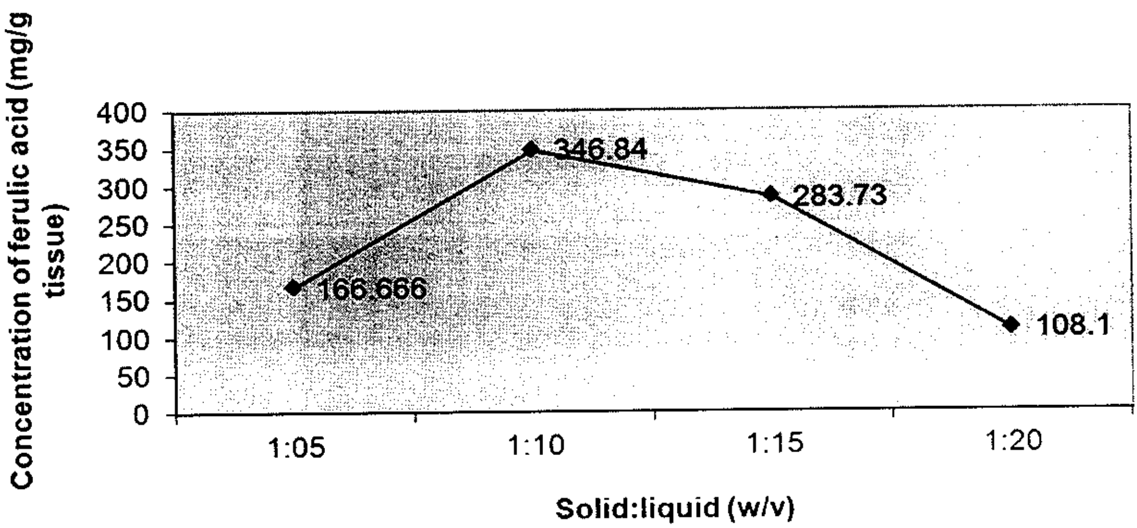


Fig 6.2.1 Effect of solid:liquid (w/v) in the extraction of ferulic acid

6.2 (b) Effect of microwave power (watts) in the extraction of ferulic acid

The ferulic acid were extracted at different microwave power (180 to 720 watts). Yield of ferulic acid was maximum at 540 watts (Fig 6.2.2). A gradual increase in the ferulic acid content was noticed when the microwave power increases from 180 to 540 watts but suddenly decreased at 720 watts. High microwave power increases the dipole rotation and the cell rupture occurs by sudden rise in temperature and internal pressure inside the cells of plant sample, which promotes the destruction of cell surface and in turn exude out the chemical substance within the cells into the surrounding solvents (Zhang *et al.*, 2008).

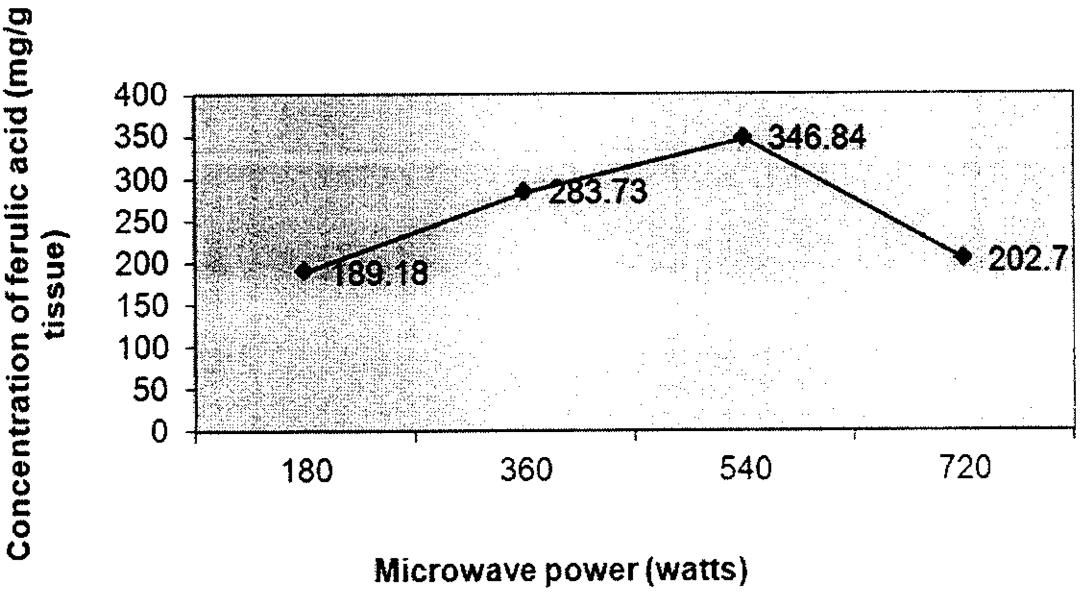


Fig 6.2.2 Effect of microwave power in the extraction of ferulic acid

6.2 (c) Effect of extraction time in the extraction of ferulic acid

The ferulic acid were extracted at different time intervals (15 to 45 sec). The maximum yield was obtained at 45 sec (Fig 6.2.3). With the increase in time period, the cell rupture rate get increased and the intracellular ferulic acid compounds released drastically. This increase in the ferulic acid content is mainly due to the rise in the electromagnetic field of the dipole movement in the plant material.

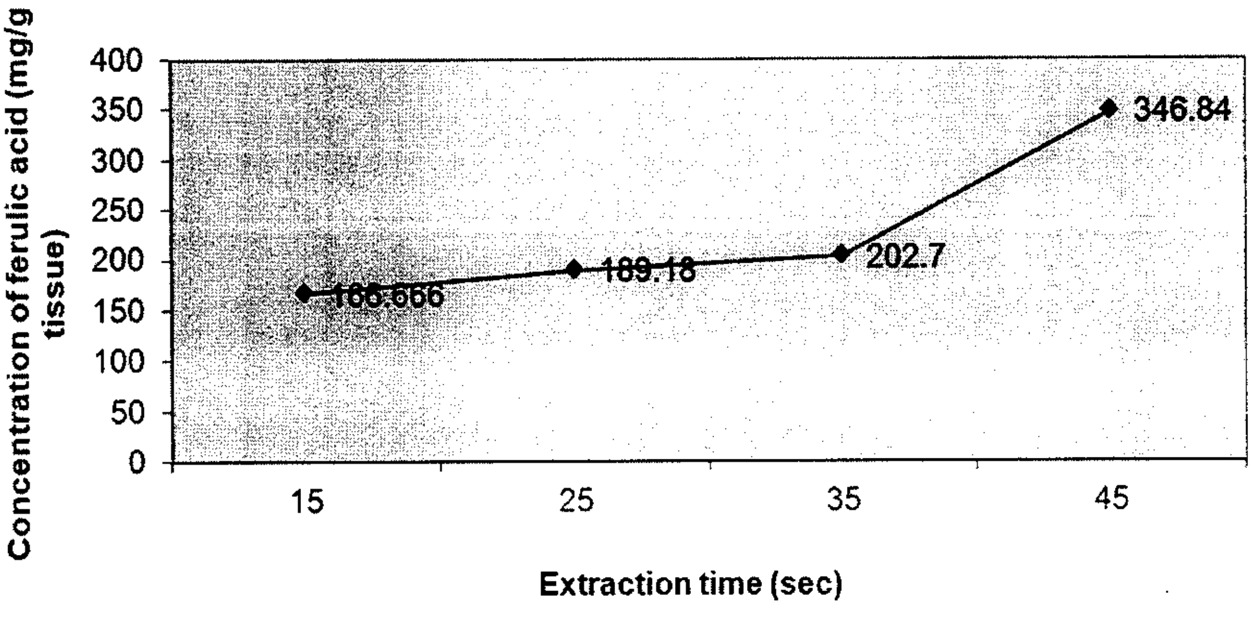


Fig 6.2.3 Effect of time in the extraction of ferulic acid

6.3 Optimization of extraction of ferulic acid from *Arachis hypogaea shells* (Ethanol) using ultrasonication through L_{16} orthogonal design

The results were made in the form of range analysis and one way ANOVA by SPSS software. The results were depicted in Table (6.3.1) and Table (6.3.2). The order of the effect of factors on ferulic acid extraction was A>B>C. The concentration of ethanol % had the greatest effect on the extraction procedure and it was found to be significantly different at 1% level. The optimum extraction conditions obtained from the statistical analysis were 95% 0f ethanol concentration, 15min extraction duration, a material ratio 1:20 and the maximum yield was found to be 1054.050 mg/g.

Table 6.3.1 One way ANOVA in ultrasonication

Levels	Sum of square	Degrees of freedom	Mean square	F-value
A	108668.211	3	36229.403	1.905
B	103650.595	3	34550.198	1.777
C	61781.317	3	20593.772	0.898

Table 6.3.2 Experimental results and range analysis in ultrasonication

Experiments	A	B	C	Ferulic acid (mg/g)
1	1	1	1	5.56
2	1	2	2	8.02
3	1	3	3	7.23
4	1	4	4	6.12
5	2	1	2	7.97
6	2	2	1	8.58
7	2	3	4	10.71
8	2	4	3	8.81
9	3	1	3	6.49
10	3	2	4	8.4
11	3	3	1	6.19
12	3	4	2	8.07
13	4	1	4	6.00
14	4	2	3	7.74
15	**4**	**3**	**2**	**10.85**
16	4	4	1	7.51
K_1	2617.12	2529.27	2700.43	
K_2	3499.98	3185.16	3391.87	
K_3	2840.58	3398.64	2941.43	
K_4	3119.35	2963.94	3043.28	
k_1	654.28	632.317	675.107	
k_2	874.995	796.29	847.967	
k_3	710.145	849.66	735.357	
k_4	779.837	740.98	760.82	
R	220.71	217.34	172.86	

6.3 (a) Effect of extraction time in the extraction of ferulic acid

Maximum extraction yield of ferulic acid was found at 15 min Fig (6.3.1) and then decreased with prolonging ultrasound time. Considering that ferulic acid were located into the cytoplasm of the cells, it was clear that ultrasound led to a kind of tissue permeabilization by the disruption of important cellular structures such as cell walls and cell membranes, which are of great importance for mass transfer control. Some studies showed that, in contrast to conventional extractions, plant extracts diffused across cell walls due to ultrasound, causing cell rupture over a shorter period (Chemat *et al.*, 2004; Schinor *et al.*, 2004; Albu *et al.*, 2004). In addition, this might increase the amount of impurities with the rise of ultrasound time.

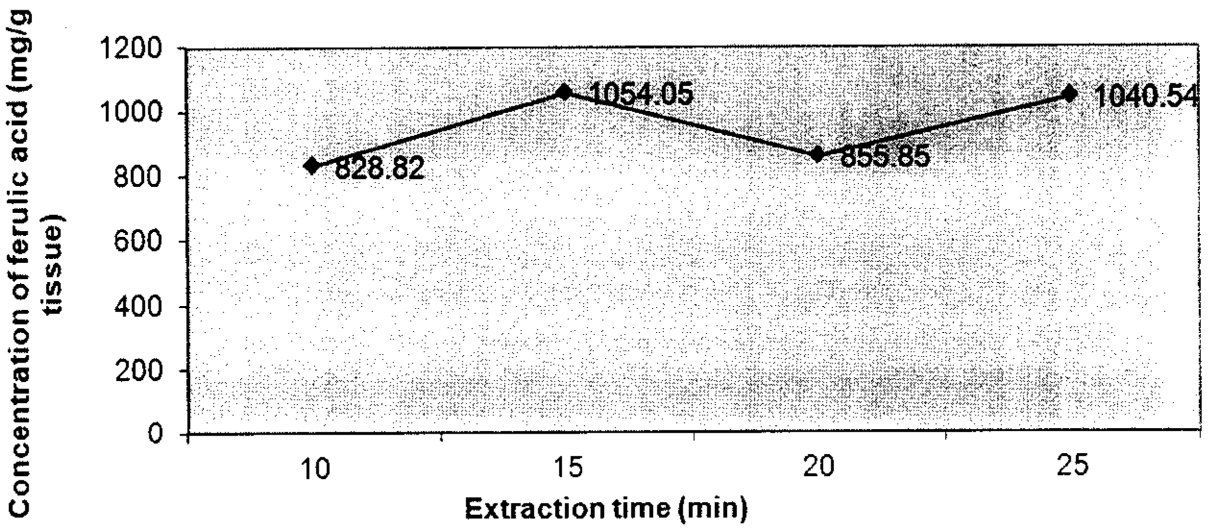


Fig 6.3.1 Effect of time in the extraction of ferulic acid

6.3 (b) Effect of material ratio (W/V) in the extraction of ferulic acid

Extraction yield of ferulic acid increased significantly with the ratio of solvent to raw material in a range of 1:5 ~ 1:20 and decreased insignificantly when the ratio 1:20 (Fig 6.3.2). Recent report indicates that (Sathishkumar *et al.*, 2009) when the ratio of solvent to raw material was between 1:5 and 1:20 flavonoids were extracted fully with the rise of volume the extracting agent so that extraction yields increased. Otherwise, when the ratio of solvent to raw material reached a certain level, the extract gets saturated in the solution. This lead to extraction yield become steady and wouldn't increase significantly. The optimum ratio of solvent to raw material was 1:15.

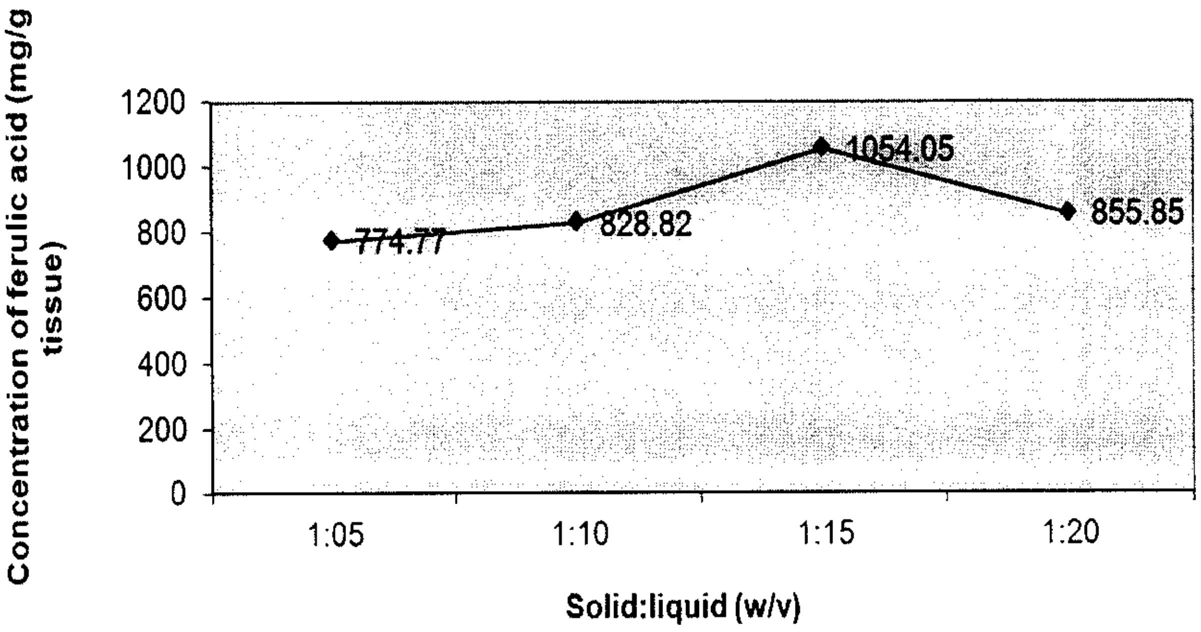


Fig 6.3.2 Effect of solid:liquid (w/v) in the extraction of ferulic acid

6.3 (c) Effect of extracting agent (ethanol) in the extraction of ferulic acid

Fig 6.3.3 showed the extraction yield of ferulic acid with the ethanol concentration ranging from 65 - 95%. A increase in extraction yield of ferulic acid was noticed at 75% and 95%. Ethanol was used for extracting the raw ferulic acid among various solvents, since it is environmentally benign and relatively safe to human health (Zhang *et al.*, 2003; He *et al.*, 2005). Ethanol interacts with the ferulic acid probably through noncovalent interactions and promotes a rapid diffusion into the solution (Li *et al.*, 2008; Luque de Castro and Tena, 1996). Various concentration of ethanol used exhibited different effect in changing the fluid polarity and thus had diverse effect on the solubility enhancement of the flavonoids (He *et al.*, 2005; Sathishkumar *et al.*, 2008). The optimal extraction yield might be fulfilled when the polarity of the fluid and its ferulic acid were coincident. The results indicated that the optimal ethanol concentration for extraction ferulic was found to be 95%.

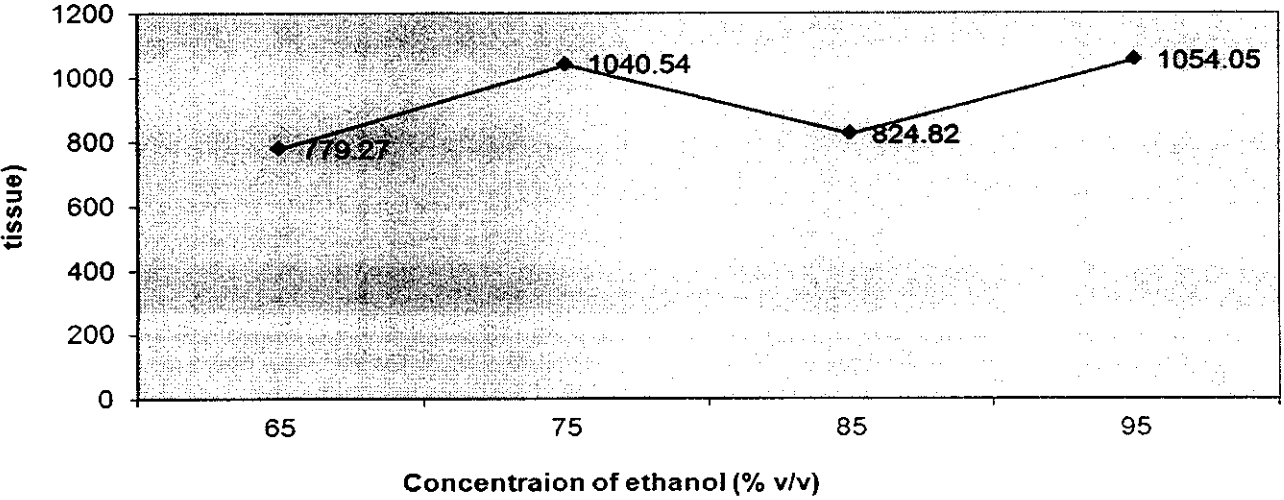


Fig 6.3.3 Effect of solvent ratio in the extraction of ferulic acid

6.4 Identification and isolation of ferulic acid from optimal extract using and Preparative Thin Layer Chromatography (PTLC) and Thin Layer Chromatography (TLC)

6.4.1 TLC and PTLC

The results of Fig (6.4.1.1, 6.4.1.2, and 6.5.1.3) showed that *Arachis hypogaea shells* revealed the presence of ferulic acid related compound in the optimized extracts.

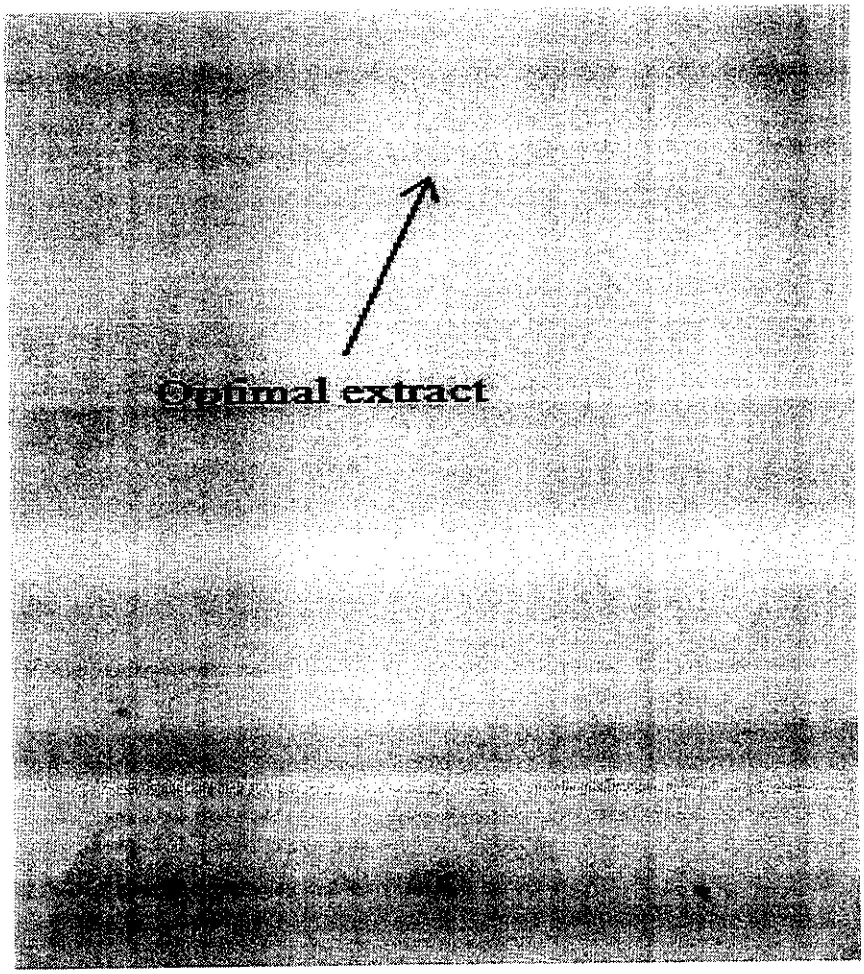
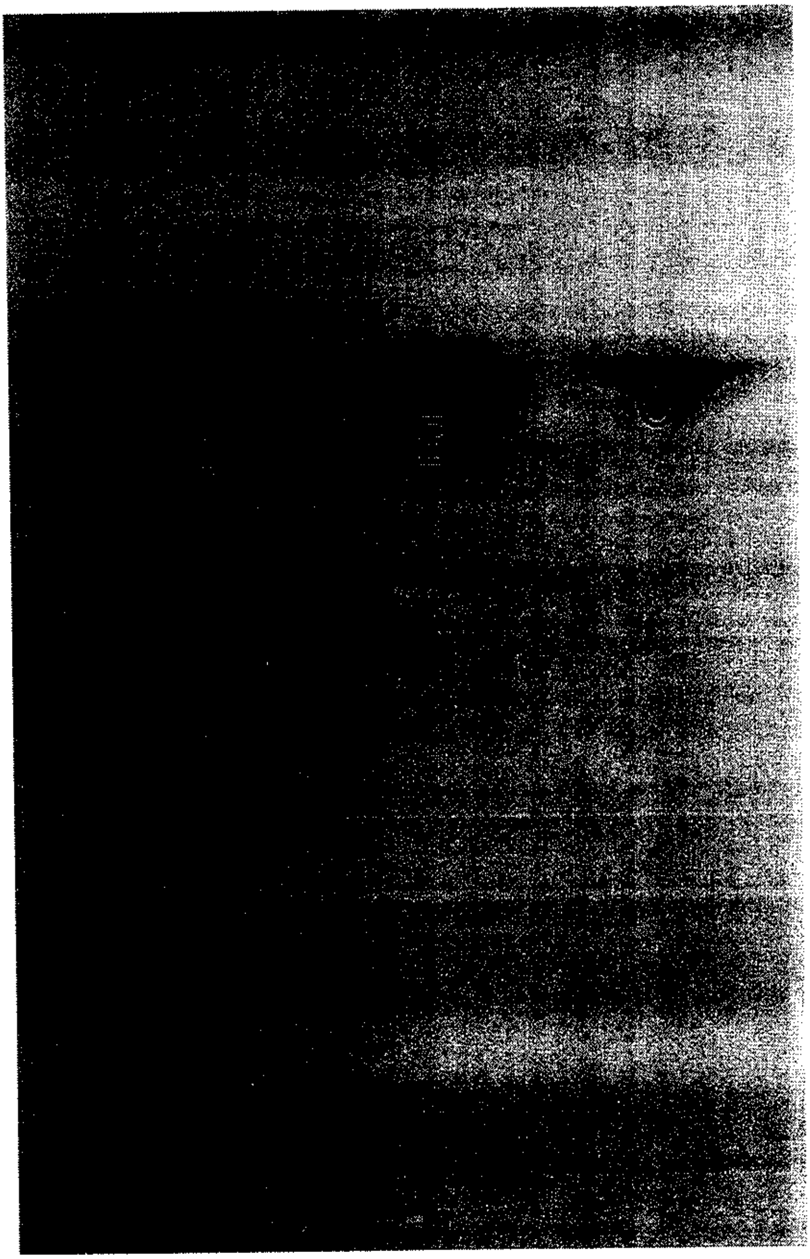
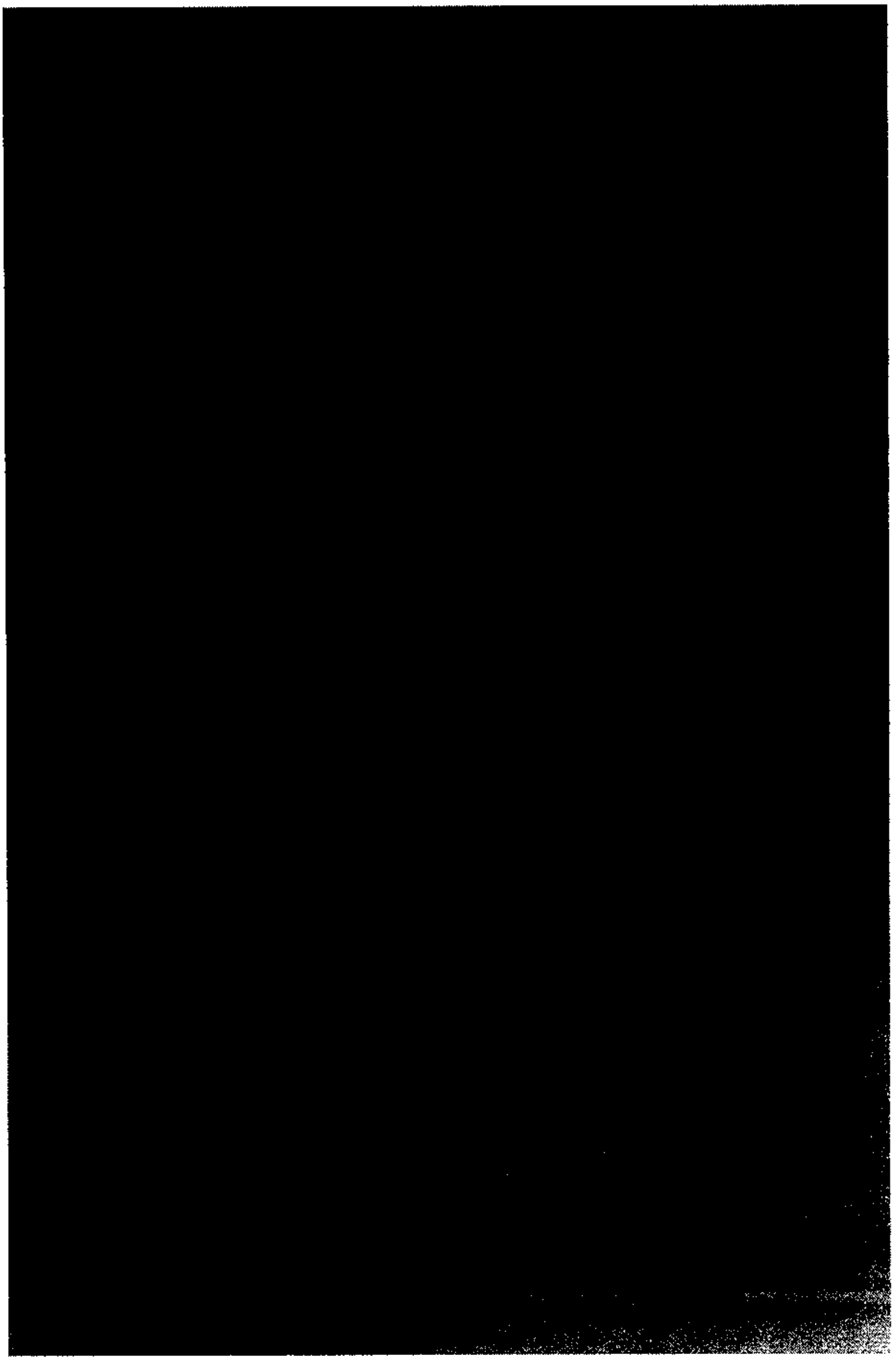


Fig 6.4.1.1 Identification of ferulic acid related compounds by PTLC



A-Ferulic acid; B-Optimal extract; C-Ethyl ferulate

Fig 6.4.1.2 Identification of ferulic acid related compounds by TLC under visible light



A-Ferulic acid; B-Optimal extract; C-Ethyl ferulate

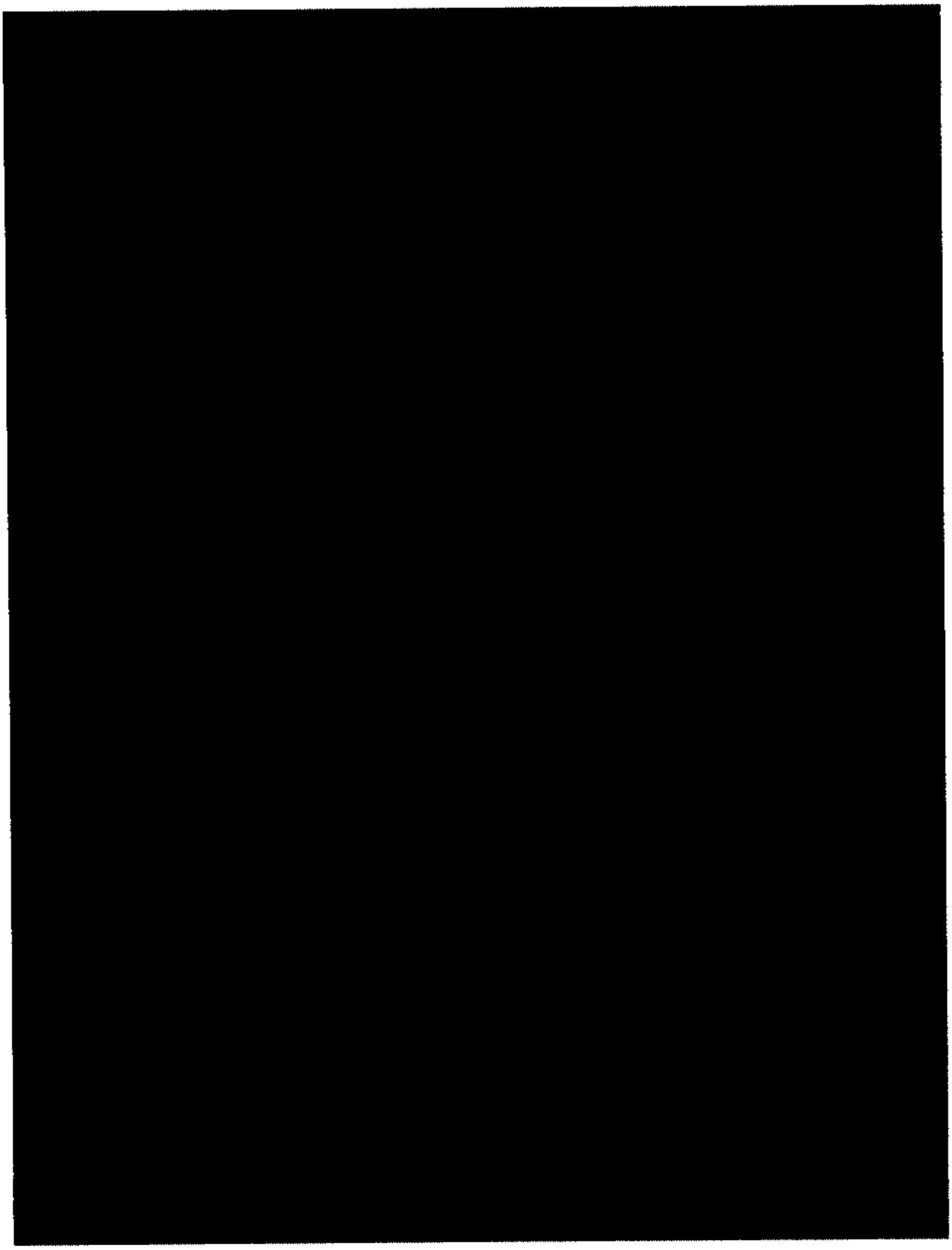
Fig 6.4.1.3 Identification of ferulic acid related compounds by TLC under UV light

6.5 Identification of flavonoids from optimal extract using Thin Layer Chromatography (TLC)

The results of Fig (6.5.1, 6.5.2) showed that *Arachis hypogaea shells* revealed the presence of flavonoids related compound in the optimized extracts.

X-Rutin; Y-Optimal extract; Z-Quercetin

Fig 6.5.1 Identification of flavonoids related compounds by TLC under visible light



X-Rutin; Y-Optimal extract; Z-Quercetin

Fig 6.5.2 Identification of flavonoids related compounds by TLC under UV light

6.6 High Performance Liquid Chromatography (HPLC)

6.6.1 HPLC analysis of peanut shells

Fig (6.6.1.1), Fig (6.6.1.2) shows typical HPLC chromatograms obtained by analysis of the PTLC fragments. Single peaks were observed at *R*t = 2.977 min for ferulic acid, and Rt = 2.957 min for the unknown compound.

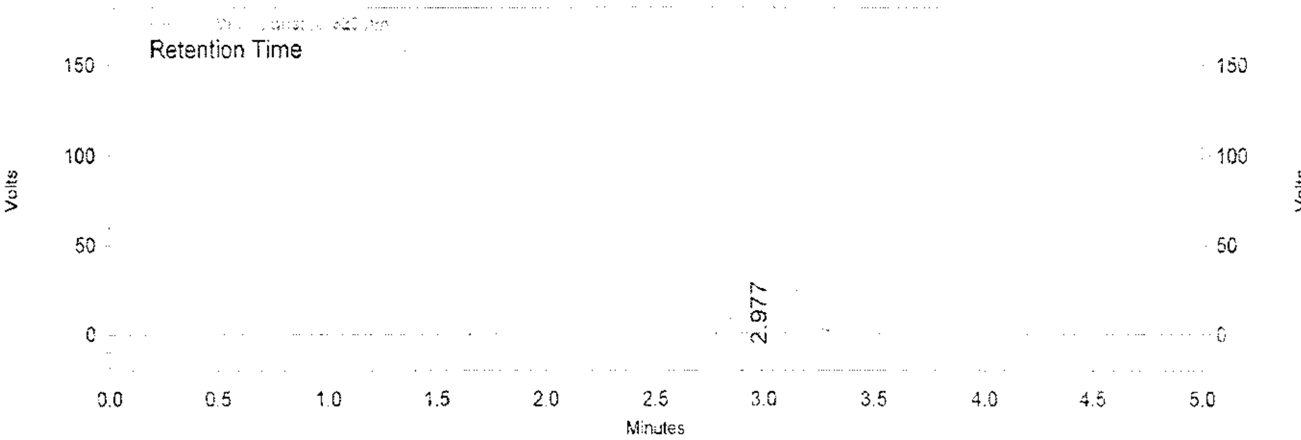


Fig 6.6.1.1 HPLC analysis for the standard ferulic acid

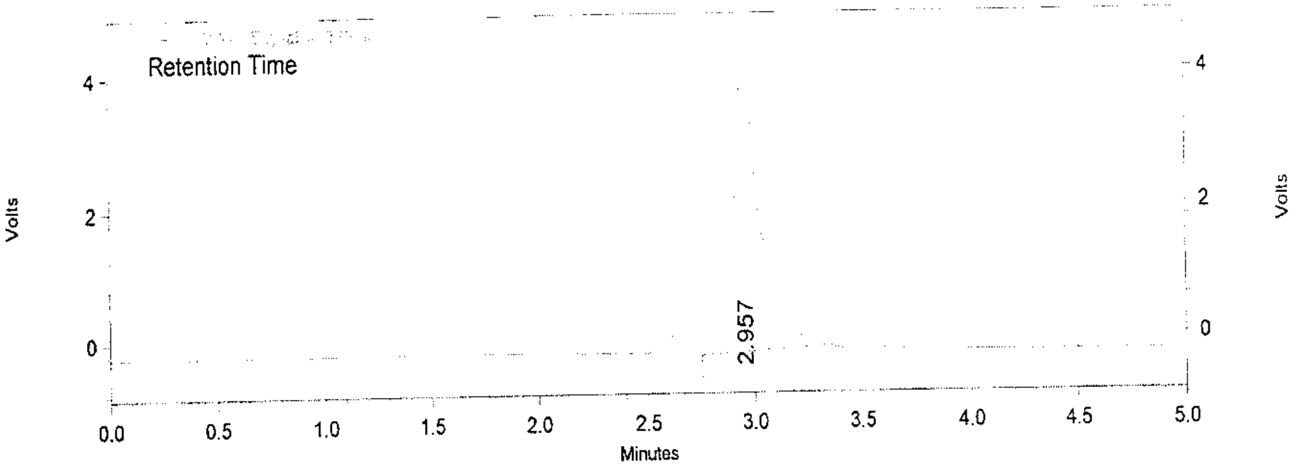


Fig 6.6.1.2 HPLC analysis of isolated compounds from PTLC

6.7 Antioxidant assay

6.7.1 Ferric ion reducing/Antioxidant power assay (FRAP)

Fe^{3+}/Fe^{2+} transformation was investigated in the presence of samples for the measurements of the reductive ability. Absorbances for 100µg/ml to 500µg/ml of extract (Fig 6.7.1.1) were noted. The reducing power of EEPL ranged from 0.030 to 0.270 and for PTLCIC ranges from 0.025 to 0.102. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Awika *et al.*, 2003). The IC_{50} value of EEPS and PTLCIC were estimated as 2.969 g/ml and 2.976 g/ml respectively. The significant difference at 5% level ($p<0.05$) was observed between rutin, quercetin, vitamin C and EEPL/ PTLCIC using one way ANOVA. The students t-Test analysis proved a significant difference at 5% level ($p<0.05$) between Vitamin C and EEPL/ PTLCIC. The results revealed that EEPL and PTLCIC possessed a moderate reducing power than Vitamin C

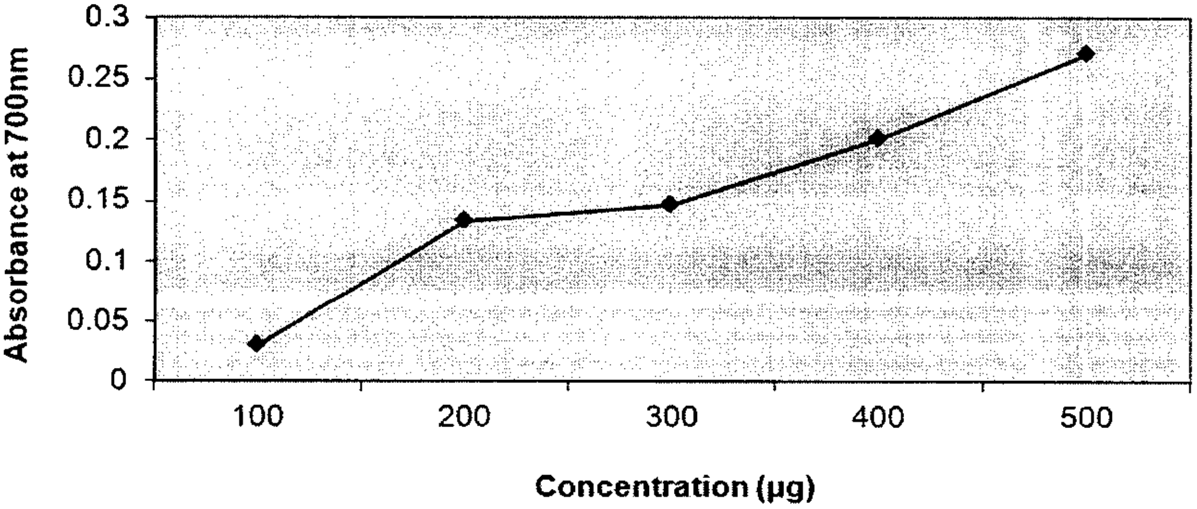


Fig 6.7.1.1 FRAP assay for EEPS

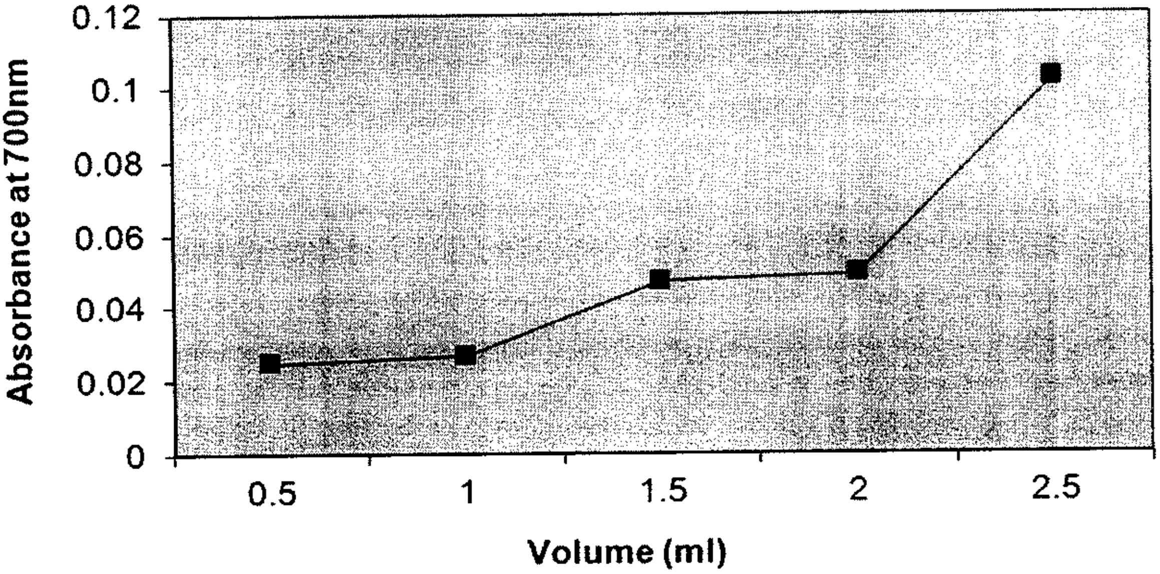


Fig 6.7.1.2 FRAP assay for PTLCIC

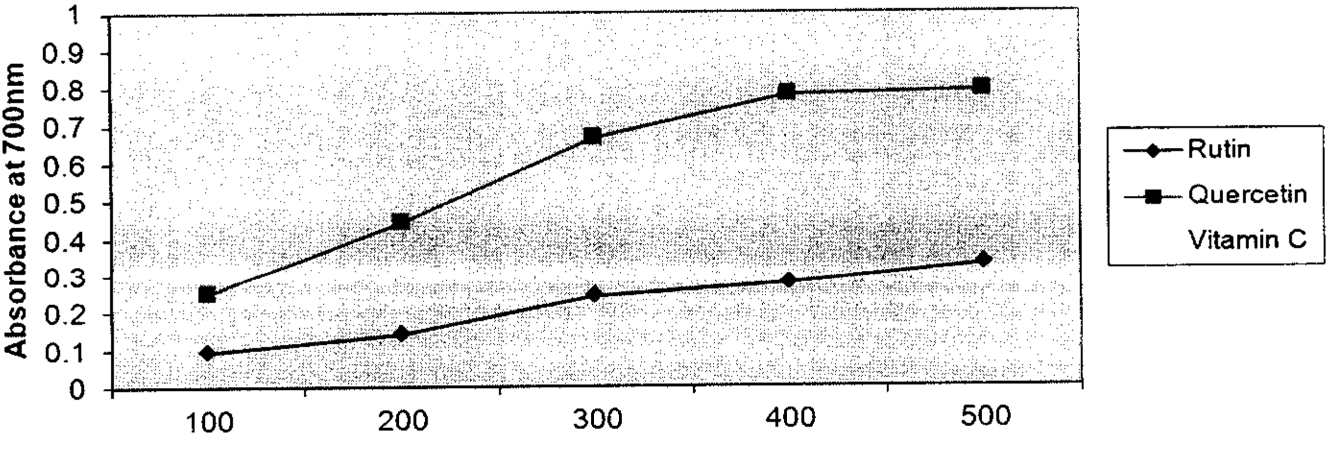


Fig 6.7.1.3 FRAP assay for some flavonoids and Vitamin C

6.7.2 DPPH (1, 1-Diphenyl-2-Picryl-hydrazil) scavenging assay

Phenolic compounds in plants are viewed as powerful *in vitro* antioxidants due to their ability to donate hydrogen or electrons and to form stable radical intermediates (Calbert *et al.*, 2005). DPPH (a stable nitrogen centered free radical) is used to evaluate natural antioxidants for their radical quenching capacities in a relatively short time compared with other methods. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced. Based on the principle, the antioxidant activity of the substance can be expressed as its ability in scavenging the DPPH- radical (Park and Kim, 2004). The DPPH scavenging activity of EEPS and PTLCIC was found to be 41.20 % and 79.64% respectively. As shown in Fig. 6.7.2.1, PTLCIC exhibited a strong scavenging activity on the DPPH radical in *In vitro* assay. There is no significant difference at 5% level ($p<0.05$) between rutin, quercetin and EEPS/ PTLCIC using one way ANOVA. The students t-Test analysis proved no significant difference at 5% level ($p<0.05$) between Vitamin C and EEPL/PTLCIC. All these results proved that PTLCIC possessed a significant and equivalent DPPH-radical scavenging power along with rutin and quercetin which proved a strong antioxidant activity of PTLCIC.

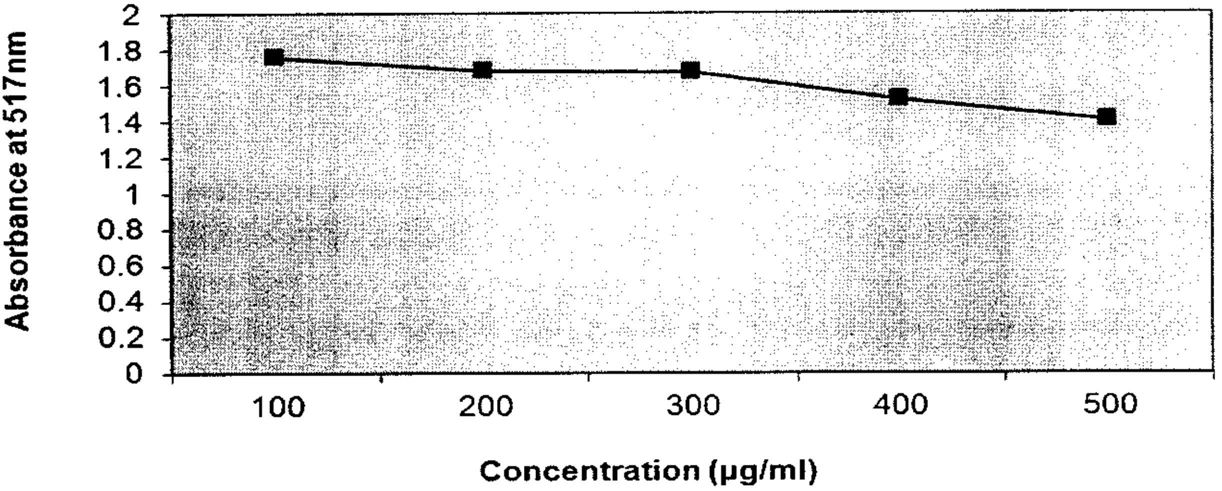


Fig 6.7.2.1 DPPH assay for EEPS

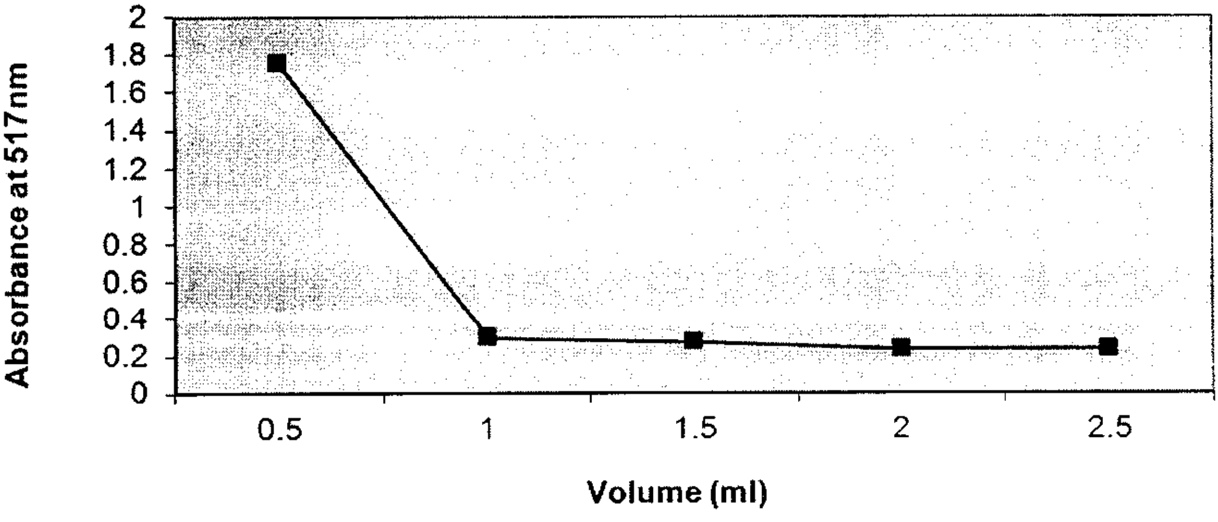


Fig 6.7.2.2 DPPH assay for PTLCIC

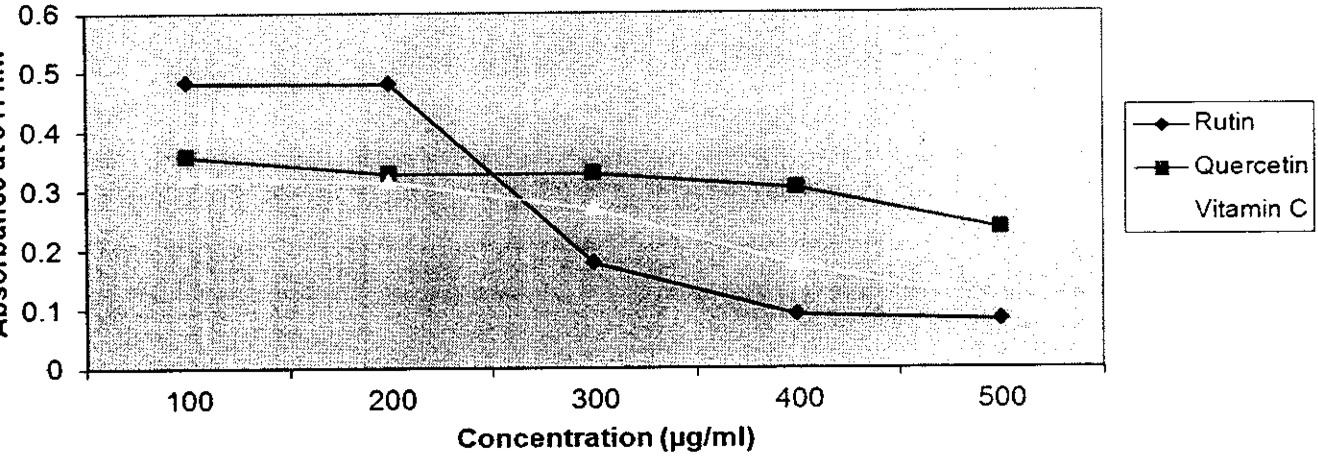


Fig 6.7.2.3 DPPH assay for some flavonoids and vitamin C

6.7.3 Super oxide scavenging activity

Superoxide anion is produced by a number of enzyme systems in auto oxidation reactions and by nonenzymatic electron transfers that univalently reduce molecular oxygen. In cellular oxidation reactions, superoxide radical is normally formed first, and its effects can be magnified because it produces other types of cell-damaging free radicals and oxidizing agents, which has been implicated in the initiating oxidation reactions associated with aging (Liu and Ng, 2000). It has been reported that peanut skin extract was apparently more potent than the nonenzymatic superoxide scavenger Butylated Hydroxyanisole (BHA). The superoxide scavenging activity of EEPS and PTLCIC was found to be 67.94% and 78.23% respectively (Fig. 6.7.3.1 and 6.7.3.2). There is no significant difference at 5% level ($p<0.05$) between quercetin and EEPS/ PTLCIC using one way ANOVA. This proved a potent superoxide scavenging activity of PTLCIC than EEPS. From these results we infer that EEPS might be possibly advantageous in the micromolar range for preventing injury induced by superoxide anion radicals in pathological conditions.

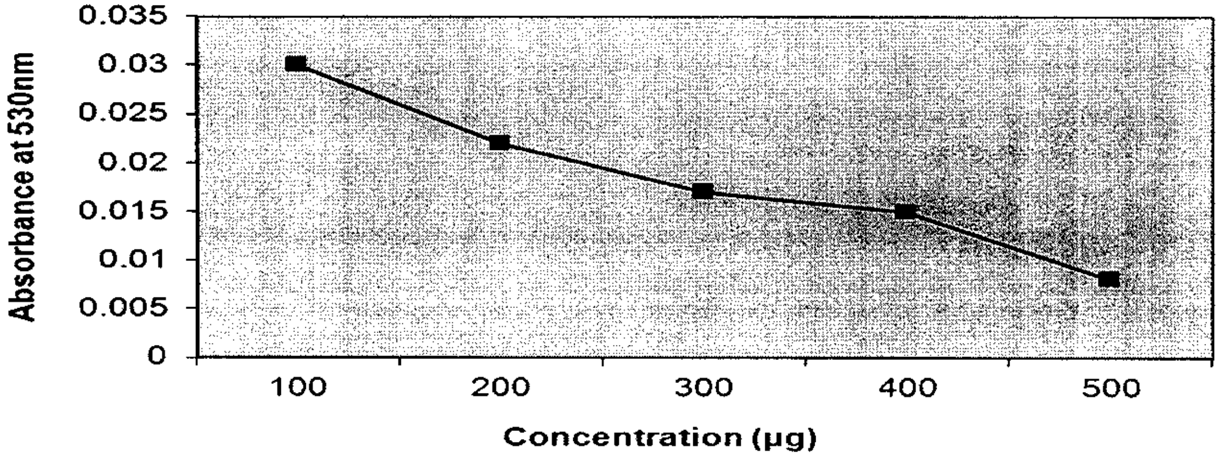


Fig 6.7.3.1 Super oxide scavenging activity for EEPS

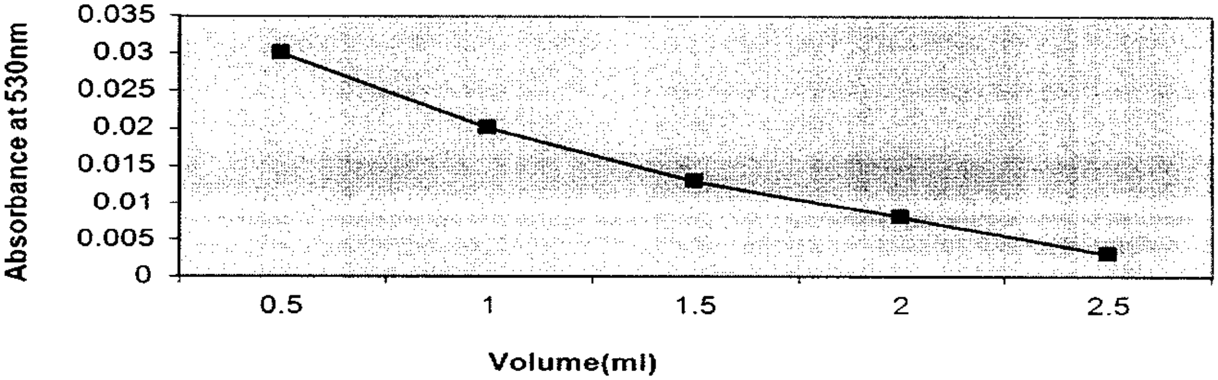


Fig 6.7.3.2 Super oxide scavenging activity for PTLCIC

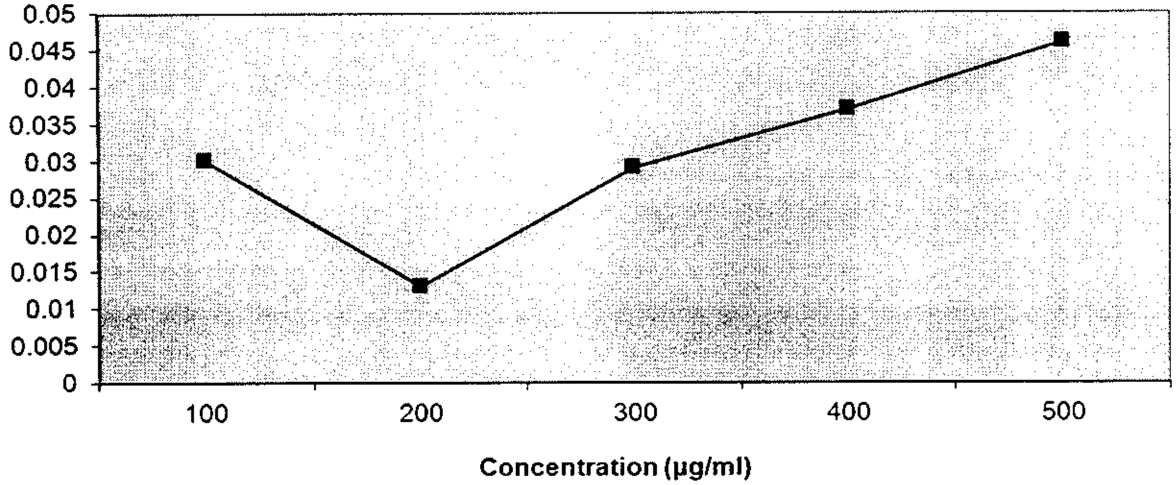


Fig 6.7.3.3 Super oxide scavenging activity for quercetin

6.7.4 Nitric oxide scavenging activity

Nitric oxide (NO) is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities (Hagerman *et al.*, 1998). Suppression of released NO may be partially attributed to direct NO scavenging, as the extracts of EEPS and PTLCIC decreased the amount of nitrite generated from the decomposition of Sodium Nitro Prusside (SNP) *In vitro.* The scavenging of NO by the extracts was increased in dose dependent manner. Figure (6.7.4.1, 6.7.4.2) illustrates a significant decrease in the NO radical due to the scavenging ability of EEPS and PTLCIC. The EEPS and PTLCIC showed maximum scavenging activity of 64.74% and 42.83% respectively. The activity may be related to the flavonoids and phenolic compounds in this plant extract (Balakrishnan *et al.*, 2009).

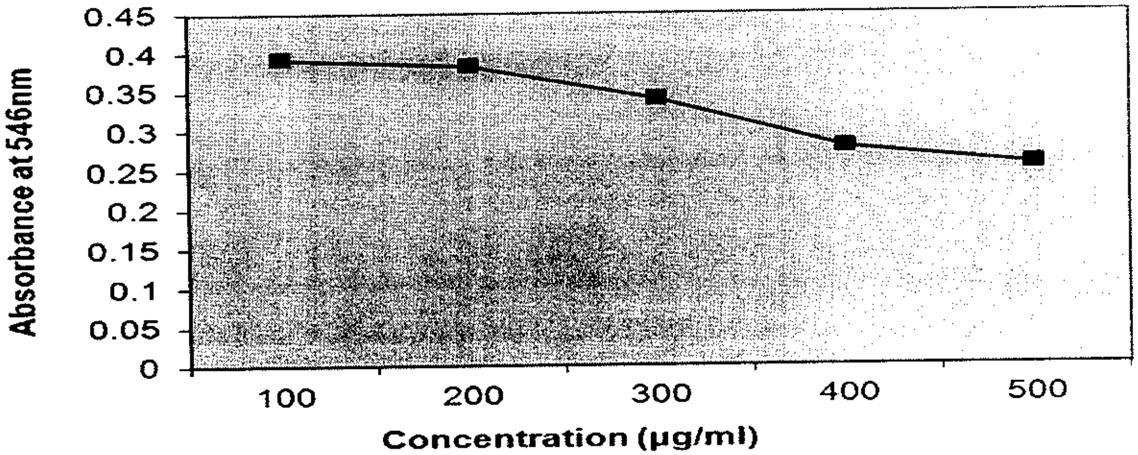


Fig 6.7.4.1 Nitric oxide scavenging activity for EEPS

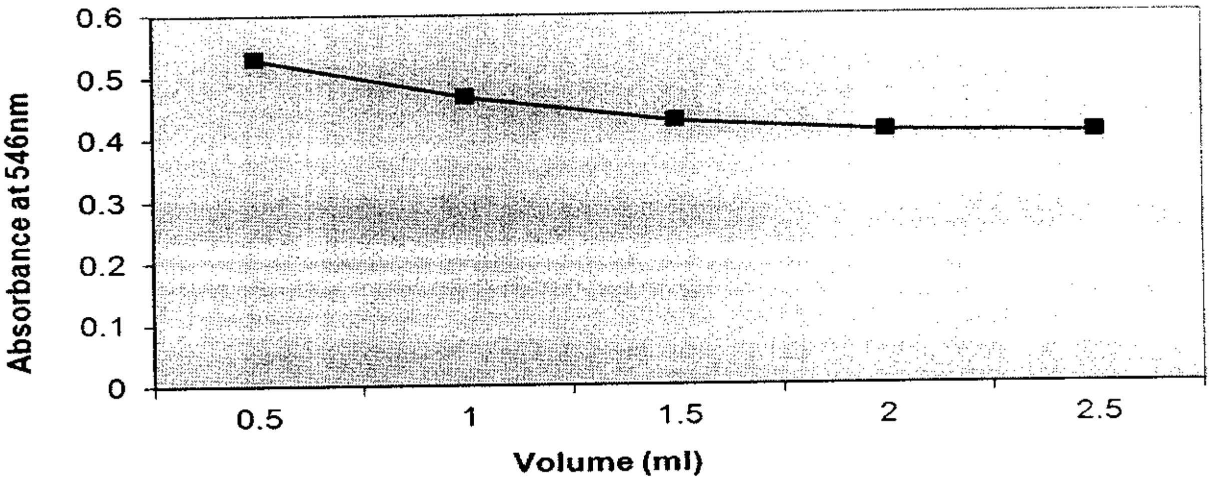


Fig 6.7.4.2 Nitric oxide scavenging activity for PTLCIC

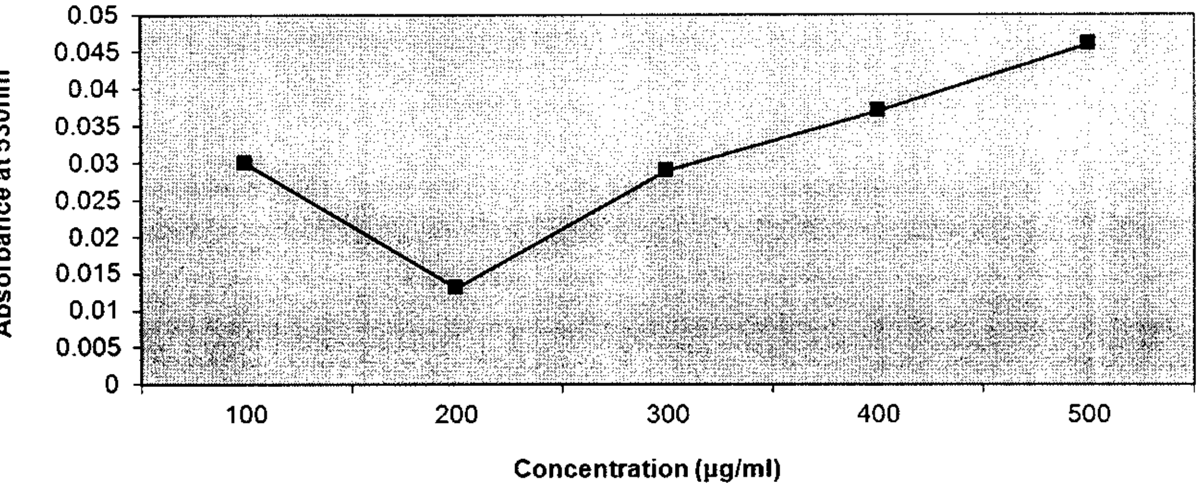


Fig 6.7.4.3 Nitric oxide scavenging activity for quercetin

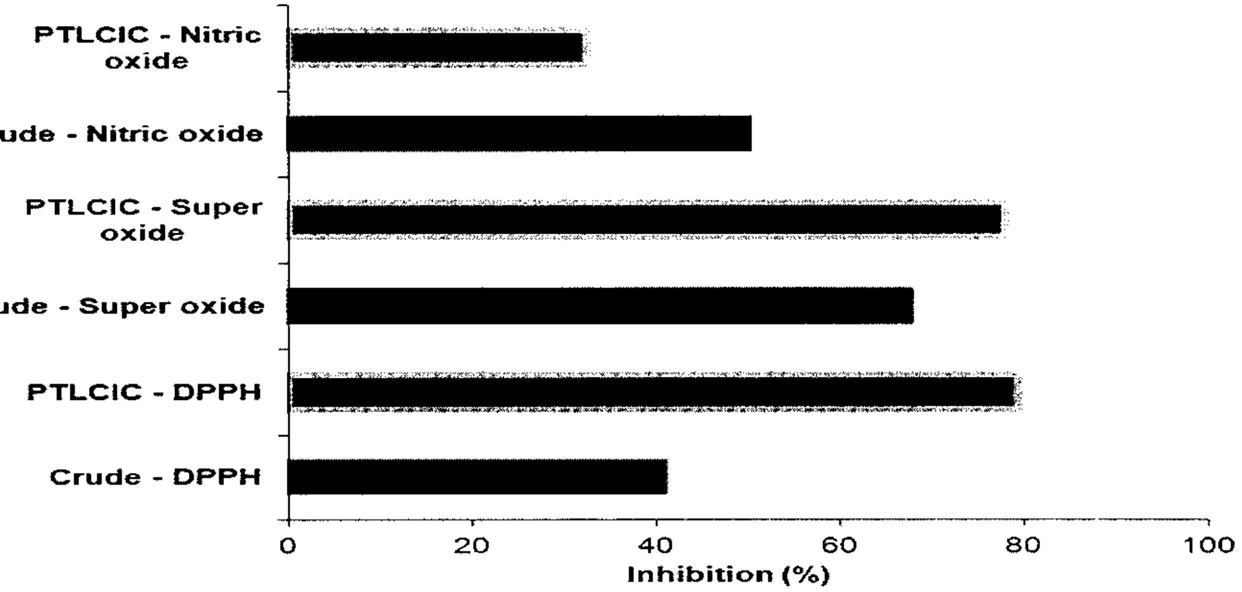


Table 6.7.4.4 over all scavenging activity of EEPS and PTLCIC

6.8 α-amylase inhibitory activity

Majority of human food consists of polysaccharides i.e. starch, which ultimately gets broken down into monosaccharide (glucose) by α-amylase and other digestive enzymes. In spite of various other metabolic pathways and energy consumption, high glucose levels in blood can cause diabetes which can further lead to many other undesirable consequences like glucoma and heart related disorders. Therefore α-amylase inhibitors are required to regulate the blood glucose levels. The α-amylase inhibitory activity of EEPS and PTLCIC were found to be 78.2 % and 30.3% respectively (Fig 6.9.1). This was mainly due to the presence of flavonoid related compounds in the extract. Thus the consumption of minimal quantities of EEPS would inhibit α-amylase activity.

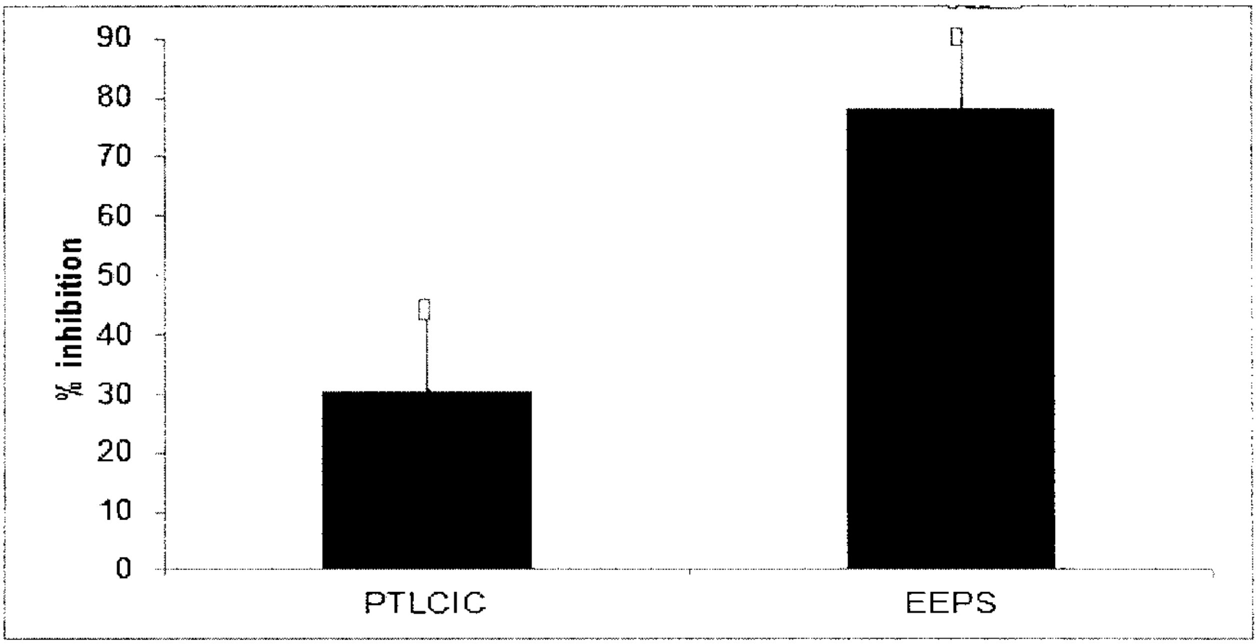


Fig 6.8.1 α-amylase inhibition for EEPS and PTLCIC

CONCLUSION

7. CONCLUSION

Ferulic acid has many applications like antidiabetic effect, antioxidant activity, anticancer effect, etc. In the present study, microwave irradiation showed maximum yield than the ultrasonic mediated extraction. Microwave irradiation showed many advantages, such as shorter time, less solvent, higher extraction rate, savings of energy and better products with lower cost compared to ultrasonication method. The disadvantage of ultrasonic method is that the ultrasonic extraction mainly depends on the ultrasonic effects of acoustic cavitations and the uneven distribution of which leads to low yield of ferulic acid. Ferulic acid related compounds present in the optimal extract were identified by TLC and PTLC which was confirmed in HPLC by comparing with the standard ferulic acid retention time. Also the *Arachis hypogaea* shells have potent α-amylase inhibitory activity and appreciable antioxidant property.

APPENDICES

8. APPENDICES

8.1 Appendix

REDUCING POWER ASSAY – OYAIZU METHOD

REAGENTS

a) Sample preparation

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

b) 0.2M Phosphate buffer (pH = 6.6)

Mixed 26.5ml of Na_2HPO_4 with 73.5ml of NaH_2PO_4.

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

c) 1% potassium ferricyanide

Dissolved 1g of potassium ferricyanide in 100ml of distilled water.

d) 10% TCA

Dissolved 10g of TCA in 100ml of distilled water.

e) 0.1% $FeCl_3$

Dissolved 0.1g of Ferric chloride in 100ml of distilled water.

8.2 Appendix

DPPH ASSAY

REAGENTS

a) Sample preparation

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

b) Preparation of DPPH solution

Take 4 mg of DPPH and make up to 100 ml with ethanol.

Keep in the dark for 30 minutes.

8.3 Appendix

SUPER OXIDE RADICAL

REAGENTS

a) Sample preparation

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

b) Phosphate buffer (0.2 mM, pH 7.4)

Mixed 3.6 ml of KH_2PO_4 with 1.4 ml of $Na_2H_2PO_4$ both in 100ml 0f distilled water.

($0.2M\ KH_2PO_4 = 2.7.2$ g/ 100ml, $0.2M\ Na_2H_2PO_4 = 28.2$g/100 ml)

c) 0.2 ml Riboflavin

20 μg of riboflavin in 0.2 ml of distilled water

d) 0.2 ml EDTA

0.1 mg of EDTA in 3ml distilled water

e) 0.2 ml NBT

0.1 mg of NBT in 3ml distilled water

f) 0.2 ml Sodium cyanide

3μg of sodium cyanide 0.2 ml in distilled water

8.4 Appendix

NITRIC OXIDE SCAVENGING

REAGENTS

a) Sample preparation

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

b) Phosphate buffer (50 mM, pH 7.6)

Mixed 81 ml of KH_2PO_4 with 19 ml of $Na_2H_2PO_4$ both in 100ml 0f distilled water.

0.2M KH_2PO_4 = 890mg/ 100ml, 0.2M $Na_2H_2PO_4$ = 692 mg/100 ml)

b) Sodium Nitroprusside (5mM)

0.148 gm in 100 ml distilled water.

d) Griess reagent

1 gm of Sulphanamide, 2gm of Phosphoric acid (H_3PO_4) and 0.1gm of Napthyl Ethylene Diamine (NED) in 100 ml distilled water.

8.5 Appendix

α- AMYLASE INHIBITION

REAGENTS

a) Sample preparation

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

a) Phosphate buffer (20 mM, pH 6.9)

Mixed 45 ml of KH_2PO_4 with 55 ml of $Na_2H_2PO_4$ both in 100ml 0f distilled water.

(KH_2PO_4 = 2.85 gm/ 100ml, $Na_2H_2PO_4$ = 3.12gm/100 ml)

b) α- amylase

Dissolve 50 mg of amylase in 5 ml of phosphate buffer (6.9 pH)

d) 1% Starch

1 gm of Starch in 100 ml of phosphate buffer (6.9 pH)

e) 4% Rochelle salt

4g of Rochelle salt in 100 ml distilled water.

f) Dinitro salicylic acid (DNS)

1gm of DNS, 200 mg of crystalline phenol, 50 mg of sodium sulphite in 100 ml of distilled water.

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