Antioxidant Potential and Free Radical Scavenging Activities of *Pergularia deamia*

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Thesis submitted to

Avinashilingam Institute for Home Science and

Higher Education for Women

Coimbatore-641043

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

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Signature of the
Head of the Department

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Signature of the Guide

ACKNOWLEDGEMENT

It is with the choice of blessings and the divine grace of **LORD ALMIGHTY** that any human endeavour is achieved. In addition to the will of supreme divinity, the willingness of many subject experts and erudite scholars to extend their assistance and help for the completion of a work plays, indeed a vitally important role.

The Author takes take the opportunity of express her sincere thanks to **Dr**. (**Thiru) P.R. Krishna Kumar**, Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and infrastructure to undertake this investigation.

The author immensely thank **Dr. Premavathy Vijayan**, Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the entire facilities essential to carry out and complete the study.

She records her sincere thanks to **Dr. S. Kowsalya**, Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for timely help rendered to carry out the work.

She also express her sincere thanks to **Dr. A. Parvathi**, Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for the valuarable guidance and support.

I author her special gratitude to **Dr. S. Annapurani,** Former Professor and Head, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her sustained support throughout the study.

She also her sincere thanks to **Dr. P.R. Padma**, Professor and Head of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and timely help rendered to carry out the work successfully.

The author owes her indebtedness, profound and deepest thanks to her guide **Dr. S.Gayathri Devi** Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her excellent guidance and

meticulous care, throughout the research and motivation right from the selection of topic to the compilation and completion of the work efficiently and effectively.

She also submit her sincere thanks to all **The Staff Members**, of Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for lending a helping hand and invaluable guidance during the course of the thesis work.

She also expresses her sincere thanks to family and friends for their kind help during the course of the study.

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1.0 INTRODUCTION

Herbs can be defined as a plant, plant part or extract which are used for flavor, fragrance or medicinal purposes (Rajan and Averal, 2016). They have been used as well as food as well as for medicinal purposes for centuries. Herbal medicine is now in great demand in the developing world for primary health care because of their better acceptability and better compatibility with the human body (Kalimuthu *et al.,* 2014).

Herbal treatment includes Ayurveda, Siddha and Homeopathy which has been using plant and plant formulations to treat various ailments. According to World Health Organization, more than 80 percent of people in poor and underdeveloped countries depend on plant based medicines for their primary health care needs. India is a home of 45, 000 plant species. Among these, many of the plants have medicinal properties and their scientific validation is very much essential. Plant based products have been used extensively because of its efficacy, safety, no side effects, easily available and low cost (Rajan and Averal, 2016).

The use of herbs has been reported in Indian traditional systems of medicine and modern application are receiving wide spread attention day by day (Sethi *et al.,* 2004). South Indian tribes are blessed with rich biological diversity of plants and a high degree of traditional knowledge about medicinal plants and their uses for various ailments of human being (Anjalam and Premalatha, 2017).

Free radicals are chemically unstable atoms that cause damage to lipid cells, proteins and DNA, as a result of imbalance between the generations of reactive oxygen species (ROS) and the antioxidant enzymes. Free radicals and reactive oxygen species are well known inducers of cellular and tissue pathogenesis leading to several human diseases, asthma, cancer, cardiovascular disease, cataract, diabetes, gastrointestinal inflammatory disease, liver disease, muscular degeneration and other inflammatory process. Reactive oxygen species are continuously produced during cell metabolism and under normal conditions they *are* scavenged and converted to nonreactive species by different intracellular enzymatic and non-enzymatic antioxidant system (Vijayakumari *et al.,* 2013).

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. The most common reactive oxygen species (ROS) include superoxide (O2·) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO·) radicals and reactive hydroxyl (OH·) radicals. The nitrogen derived free radicals are nitric oxide (NO·) and peroxynitrite anion (ONOO·) (Kabel, 2014).

Free radicals are generated continuously in the body due to metabolism and cause oxidative stress, is known to result in the oxidation of biomolecules, thereby leading to cellular damage and therefore plays a key pathogenic role in the ageing process. They attack the unsaturated fatty acids in the biomembranes resulting in membrane fluidity. The oxidative stress, due to the presence or generation of free radicals, especially reactive oxygen and their activity plays a major role in human diseases. Alzheimer's disease, Cancer, Diabetes, Sclerosis, Parkinson's disease, Inflammation, stroke and other heart diseases are major diseases reported due to oxidative stress. To maintain healthy biological system, maintenance of balance between oxidation and antioxidation is important. Thus, intake of antioxidant rich nutrition is highly recommended. This results in accelerated research on identifying the natural resources for antioxidant molecules (Prakash *et al.,* 2014). However, natural products represent a rich source of biologically active compounds and are an example of molecular diversity (Yakubu *et al.,* 2015).

Antioxidants have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. Most plants have protective biochemical functions of naturally occurring antioxidants in the cells (Moattar *et al.,* 2017). They are a group of secondary metabolites created by aerobic organisms to frustrate oxidative stress initiated by reactive oxygen species (ROS). A number of ROS such as superoxide anion radicals and the hydroxyl radicals are generated during normal oxidation/reduction reactions as a result of aerobic metabolism. An antioxidant system is naturally present to scavenge such radicals. However, when the balance between creation and elimination of radical is altered due to various disorders, the use of external antioxidants is necessary. The constant use of artificial antioxidants may initiate some health hazards and prompt toxicity level in the body system (Moattar *et al.,* 2016).

The antioxidants present in food can limit the damage of somatic cells and pathogenesis of diseases by acting directly on reactive species or by stimulating endogenous defence systems. The importance of antioxidant constituents of plant materials in maintenance of health and

protection from ageing related diseases has attracted interest in natural antioxidants (Yasodamma *et al*., 2013).

Pergularia daemia is a perennial twining herb, foul-smelling when bruised. Stems bears milky juice and covered with longer stiff erect hairs 1mm. Leaves are thin, broadly ovate and heart-shaped 2-12 cm long, covered with soft hairs. Greenish yellow or dull white, sweet-scented flowers born in axillary, double white corona at the base of a stamina column, long-peduncled, umbellate or corymbose clusters tinged with purple fruits paired with follicles 5.8 cm long and 1 cm in diameter, reflexed, beak long, covered with soft spinous outgrowth and release many seeds with long white hairs when they split open. Seeds are densely velvety on both sides. The entire plant constitutes the drug and is used as a medicine (Karthishwaran *et al.,* 2010).

With this background of information, since no scientific study has undergone in exploring this plant, the present study "Antioxidant potentialand free rdical scavenging activities of *Pergularia deamia*" was done with the following objectives

- To assess the enzymic and non-enzymic antioxidants in the leaves of the *Pergularia deamia*
- To study the *in vitro* free radical scavenging activity of the leaves of *Pergularia deamia*

2.0 REVIEW OF LITERATURE

The review of literature pertarning to the present study entitled **"Antioxidant potential and free radical scavenging activities of *Pergularia deamia*"** is discussed under the following headings.

2.1 Herbal medicines

Herbs are widely exploited in the traditional medicine and their curative potentials are well documented. Plants have been a rich source of medicines because they produce wide array of bioactive molecules, most of which probably evolved as chemical defense against predation or infection. The use of medicinal plants is increasing worldwide (Karthishwaran and Mirunalini, 2012). They are an important source for the therapeutic remedies of various ailments. Now a days there are widespread interest in evaluating drugs derived from plant source (Doss and Anand, 2012).

The wealth of medicinal plants and the traditional knowledge drastically recede in the wake of burgeoning population pressure, acculturation, rapid modernization, multifarious human activities and various developmental activities. Much of the current work in ethnobotany is concerned with the loss of traditional knowledge and the preservation of biological diversity in remote parts of the world where cultures and their ecosystems are being destroyed by development (Senthil kumar *et al.,* 2005).

Ayurveda an ancient system of Indian medicine has recommended in number of drugs from indigenous plant/animal sources for the treatment of several diseases or disorders. Herbal remedies are unpurified plant extracts containing several constituents, which often work together synergistically. The use of medicinal parts is accepted as the most common form of traditional medicine (Rageeb and Usman, 2012).

In India, traditional healers are reported to use 2500 species, in which 100 species of plants are found to serve as regular source of medicine. In recent years, use of ethnobotanical information in medicinal plant research has gained considerable attention in segments of the scientific community. Recently plants were important sources for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants. Numerous wild and cultivated plants play a key role among tribal cultures and this relationship has been continuing from one generation to another
(Sindhu and Manorama, 2014)

In industrialized countries, herbal medicine are becoming increase in popular, however the expanded use of herbal medicine has lead to concerns relating to assurance of safety, quality and efficacy. Secondly many of the antibiotics and synthetic drugs have shown sensitization reaction and other undesirable side effects and there is a feeling that the herbal drugs are comparatively safer (Chandak, 2010).

The importance of medicinal plants have been discovered in ancient days itself. At that time there was no synthetic medicines, they have been using only the herbal medicines to treat all diseases. From this it can be understood the plants are rich in medicinal properties and they are very useful in human health and wellbeing. Biological studies are essential to find more medicinal properties of the plants. But still the many medicinal plants and their medicinal properties are unexplored. Recently many of the research were being carried out in medicinal plants. The main reason was that the synthetic drugs which was now taken up by the human have many side effects that often lead to serious complications. Comparing to modern medicine the herbal medicine was the life saving drug. Among 4,00,000 plant species only 6% of the plants are studied for their biological activity and only few have been phytochemically investigated. This shows that the investigation is needed for many medicinal plants for its activity and pharmacological properties (Rukshana, 2017).

2.2 Free radicals and reactive oxygen species

Oxygen, an element indispensable for life, can under certain circumstances, adversely affect the human body. It is produced by plants during photosynthesis, and is necessary for aerobic respiration in animals. The oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals (O2 . -), hydroxyl radicals (OH·) and non free-radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen (1O2). ROS are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides (Chauhan *et al.*, 2016). Free radicals are derived from two sources: endogenous sources, e.g., nutrient metabolism, ageing process and exogenous sources, e.g., tobacco smoke, ionizing radiations, air pollution, organic solvents, pesticides (Upadhyay, 2014).

The effects of free radicals on human beings are closely related to toxicity, disease and aging. Most living species have an efficient defense system to protect themselves against the oxidative stress induced. Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process. The antioxidants can interfere with the oxidation

process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Shareef *et al.,* 2014).

Oxidative stress associated with the production of reactive oxygen species (ROS) is believed to be involved not only in the toxicity of xenobiotics but also pathophysiology in various tissue damages and organs such as liver, heart, kidney and brain. It is associated with the metabolic activation of foreign compounds to form free radicals or with the production of ROS such as superoxide anion, hydroxyl radicals, hydrogen peroxide and radicals. These are responsible for tissue damaging effects such as lipid peroxidation and DNA damage are resulted (Raj, 2016). A potent broad spectrum scavenger of these species may serve as a possible preventive intervention for free radical mediated cellular damage and diseases
(Zahin *et al.,* 2009).

ROS are formed under normal physiological conditions, but becomes deleterious when not being eliminated by the endogenous system and are known to be the major cause of various chronic and degenerative diseases. ROS related to lipid peroxidation has been considered as one of the main causes of these diseases. Under conditions of elevated ROS production or when the antioxidant system is compromised, cells are unable to scavenge the free radicals efficiently, leading to ROS accumulation (Jahan *et al.,* 2014).

It is possible to reduce the risk of chronic diseases and prevent disease progression by either enhancing the body's natural antioxidant defenses or by supplementing with dietary antioxidants. Antioxidants offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by many other mechanisms and thus prevent the disease progression (Pavithra and Vadivukkarasi, 2015).

2.3 Oxidative stress and oxidative damage

Oxidative process is one of the most important routes for producing free radicals in foods, drugs and even in living systems. The most effective path to eliminate and diminish the action of free radicals which cause the oxidative stress is antioxidative defense mechanisms (Prakash Veeru *et al.,* 2009). It is an important risk factor in the pathogenesis of numerous chronic disesases (Mahdi *et al.,* 2012).

Oxidative stress is a state of imbalance between generation of rective oxygen species like hydroxyl and superoxide radicals and the level of antioxidant defence system. It also results in

the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates (Karthikeyen, 2002).

Oxidative stress caused by reactive oxygen species (ROS) results in an increased risk for many diseases such as inflammatory disease, cardiovascular disease, cancer, diabetes mellitus, liver disease, Alzheimer's disease and cataracts. Antioxidants may directly react with the reactive radicals to destroy them by accepting or donating electrons to eliminate the radical, or they may indirectly decrease the formation of free radicals (Attanayake and Jayatilaka, 2016).

Chemical compounds and reaction capable of generating potential toxic oxygen species/free radicals are referred to as 'pro-oxidants'. They attack macromolecules including protein, DNA and lipid causing to cellular/tissue damage on the other hand, compounds and reactions disposing of these species, scavenging them suppressing their formation or opposing their actions are called antioxidants. In a normal cell there is an appropriate
pro-oxidant, antioxidant balance. However, this balance can be shifted towards the pro-oxidant when production of oxygen species is increased or when levels of antioxidants are diminished. This state is called 'oxidative stress' and can result in serious cell damage if the stress is massive or prolonged (Chirag *et al.*, 2013).

Oxygen free radicals and other reactive oxygen species (ROS) such as superoxide anion radical O2 •−, hydrogen peroxide (H_2O_2), alkoxyl (RO·), peroxyl (ROO·), hydroxyl radical (OH·), and hypochlorous acid (HOCl·), as well as reactive nitrogen species (RNS) such as nitric oxide (NO·) and peroxynitrite, are known to damage living tissues and cellular components. In biological systems this process is called oxidative stress or oxidative damage and has become a significant topic in the field of environmental toxicology. Many environmental pollutants are shown to initiate oxidative damage, for example heavy metals, polycyclic aromatic hydrocarbons, pesticides, polychlorinated biphenyls, dioxins and other xenobiotics. Free radical reactions and the production of toxic ROS/RNS are known to be responsible for a variety of adverse health effects and diseases (Kevin *et al.*, 2013).

The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease. Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS continuous exposure to chemicals and contaminants may lead to an

increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage (Sahaa, 2008).

Oxidative damage has been suggested to occur as a consequence of reactive oxygen species (ROS) produced as a byproduct of electron transport chain in mitochondria. A number of studies have been suggested that ROS can affect critical events associated with many disorders. It gets special attention due to many factors such as drought, cold, heat, herbicides and heavy metals, because they harm the cell by raising the oxidative level through loss of cellular structure and function (Starlin *et al.*, 2013).

2.4 Disease caused by free radical damage and oxidative stress

Free radicals have been claimed to play a key role, affecting human health by causing severe diseases, such as cancer and cardiovascular diseases by cell degeneration. These free radicals can be generated during normal body function, and can be acquired from the environment (Sindhu and Manorama, 2013).

2.4.1 Cancer

Free radicals act as second messengers in the intracellular signalling cascades, which induce and maintain the oncogenic phenotype of cancer cells. ROS can also induce cellular apoptosis and can therefore function as anti-tumourigenic species. Oxidative stress is common for many types of cancer cells that are linked with altered redox regulation of cellular signalling pathways. Oxidative stress was found in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating such damage in the etiology of cancer. The role of enzymatic and non-enzymatic antioxidants in the process of carcinogenesis as well as the antioxidant interactions with various regulatory factors, including NF-kB and AP-1 suggest a strong relationship between reactive oxygen species and the development of cancer (Kabel, 2014). Cancer chemoprevention by using antioxidant approaches has been suggested to offer a good potential in providing important fundamental benefits to public health, and is now considered by many clinicians and researchers as a key strategy for inhibiting, delaying, or even reversal of the process of carcinogenesis (Vikas, 2017).

2.4.2 Cardiovascular Diseases

Cardiovascular disease (CVD) is a multifactorial etiology, associated with a variety of

risk factors for its development including hypercholesterolemia, hypertension, smoking, diabetes, poor diet, stress and physical inactivity. Oxidative stress is a primary or secondary cause of many cardiovascular diseases (Antonio, 2008). It has been linked to atherosclerosis, hypertension, cardiomyopathy and chronic heart failure in humans. Increase in oxidant catalysts such as copper, iron and cadmium have been associated with hypertension, coronary artery disease, atherosclerosis and sudden cardiac death. Both endogenous and exogeneous antioxidants decrease the risk of cardiovascular diseases (Videan *et al.,* 2009).

2.4.3 Ageing

The various pathological conditions have been implicated in human beings due to ROS and oxidative stress, involving cardiovascular disease, cancer, neurological disorders, diabetes and above all ageing . The process of ageing may be defined as a progressive decline in the physiological functions of an organisms after the reproductive phase of life. The major mechanism of ageing attributes to DNA or the accumulation of cellular and functional damage (Husain *et al.,* 2012).

2.4.4 Diabetes mellitus

Diabetes mellitus is a metabolic disorder with increasing incidence throughout the world. Insulin is a key player in control of glucose haematosis. Lack of insulin affects metabolism of carbohydrates, fats and proteins. It was proposed that inhibition of α-amylase and α-glucosidase delay the degradation of carbohydrate, which would in turn cause a decrease in the absorption of glucose, as a result the reduction of postprandial blood glucose level elevation (Elayaraja *et al.,* 2015).

2.4.5 Neurodegenerative disease

Oxidative stress is a harmful condition that occurs when there is an excess of ROS and/or a decrease in antioxidant levels, this may cause tissue damage by physical, chemical, psychological factors that lead to tissue injury in human and causes different diseases Neurodegenerative disorder like Alzheimer's disease, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, memory loss and depression (Mahantesh *et al.,* 2012).

2.5 Antioxidant defence system

Antioxidative components of natural origin have attracted special interest because they can protect human body from free radicals (Deepika and Rajagopal, 2014). Antioxidant is a molecule, which terminate the chain reaction by removing free radical intermediates. Plants and

animals maintain complex system of multiple type of antioxidant, the natural plant based antioxidants are playing an important role in the maintenance of human health for the past three decades. This has attracted a great deal of research interest in natural antioxidants (Mety and Mathad, 2011).

Antioxidant enzymes are capable of stabilizing or deactivating free radicals before they oxidize the components of cells. There are various mechanisms for antioxidant enzyme preservation they could reduce the energy of the free radicals or give up some of their electrons for their use, thereby causing them to become stable. Moreover, they may also interrupt the oxidizing chain reaction to minimize the damage caused by free radicals. Our body's potential of overcoming free radicals related health problems could be made more substantial by reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements. Consequently, antioxidant enzymes play a critical role for maintaining optimal cellular and systemic health and wellbeing (Alici and Arabaci, 2016).

Hence the rationale for the use of antioxidants is well established in prevention and treatment of diseases where oxidative stress plays a major aetiopathological role. Antioxidants may protect the body against ROS toxicity either by preventing the formation of ROS, by bringing interruption in ROS attack by scavenging the reactive metabolites or by converting them to less reactive molecules. The antioxidant capacity gives information about the duration while the activity describes the starting dynamics of antioxidant action. Therefore the uses of antioxidants, both natural and synthetic are gaining wide importance in prevention of diseases (Saumya and Basha, 2011).

Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are effective in their role as antioxidants, are commercially available and currently used in industrial processes (Li, 2011).

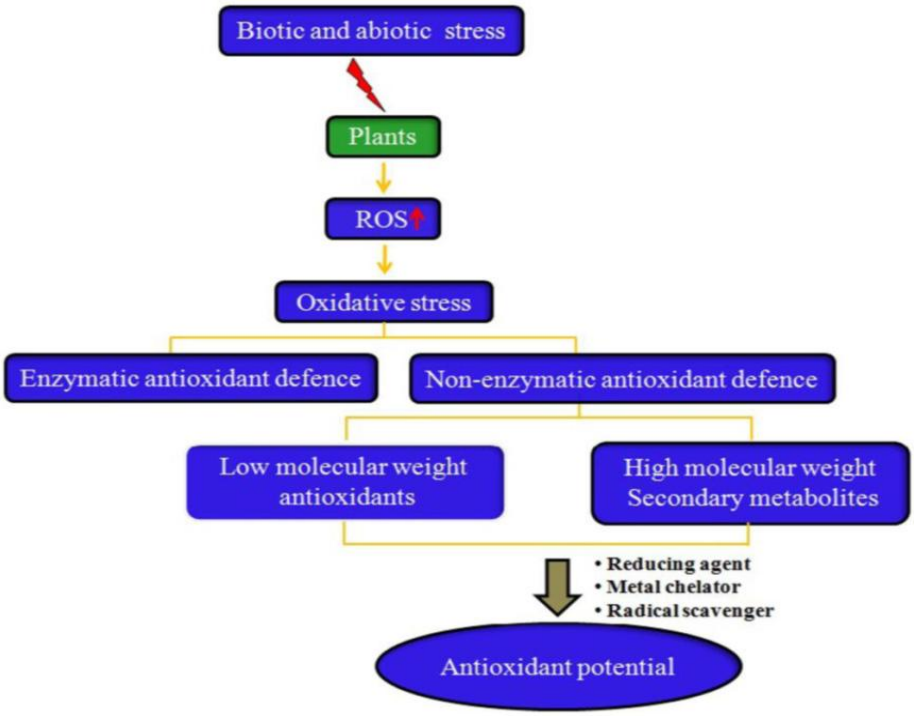
Almost all organisms are protected from free radical attack by defense mechanisms such as a preventive antioxidant system that reduces the rate of free radical formation, and another is a system to produce chain-breaking antioxidants that scavenge and stabilize free radicals. But, when free radical production rate exceeds the capacity of the antioxidant defense mechanisms substantial tissue injury results. Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of free radical mediated diseases (Hasan, 2009).

Antioxidants are molecules that inhibit or quench free radical reactions and delay or inhibit cellular damage. Though the antioxidant defenses are different from species to species, the presence of the antioxidant defense is universal. Antioxidants exists both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment. The other way of categorizing the antioxidants is based on their solubility in the water or lipids. The antioxidants can be categorized as water-soluble and lipid-soluble antioxidants. The water-soluble antioxidants (e.g. vitamin C) are present in the cellular fluids such as cytosol, or cytoplasmic matrix. The lipid-soluble antioxidants (e.g. vitamin E, carotenoids, and lipoic acid) are predominantly located in cell membranes (Nimse and Pal, 2015).

Figure1 represent the schematic representation of antioxidant activity of plants.

Figure 1

Schematic representation of antioxidant activity of plants



(Kasote *et al.*, 2015).

2.5.1 Enzymic antioxidants

2.5.1.1 Catalase

Catalase (H_2O_2 oxidoreductase) is a tetramer of four polypeptide chains, each over 500 amino acids long, contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. Catalase has one of the highest turnover rates of all enzymes one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second. It is present in all prokaryotes and eukaryotes. With the exception of erythrocytes, it is predominantly located in peroxisomes of all types of mammalian cells where H_2O_2 is generated by various oxidases. Since H_2O_2 serves as a substrate for certain reaction that generate the highly reactive hydroxyl radical, catalase is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2. The role of catalase in defending cells and tissues against oxidative stress has been studied extensively. Overexpression of catalase renders cells more resistant to toxicity of H_2O_2 and oxidant-mediated injury (Kabel, 2014).

2.5.1.2 Peroxidase

In the presence of peroxide, peroxidases from plant tissues are able to oxidize a wide range of phenolic compounds such as guaiacol, pyrogallol, chlorogenic acid, catechin and catechol (Onsa *et al.,* 2004). Glutathione peroxidase acts as a radical scavenger, membrane stabilizer and precursor of heavy metal binding peptides. Peroxides are abundant in plants and are characterised according to isoelectric points as acidic or basic peroxidises. They are ubiquitous enzymes involved in the detoxification of hydrogen peroxide to water at the expense of electrons obtained from reducing substrate (Muthukrishnan *et al.,* 2014).

2.5.1.3 Polyphenol Oxidase

Polyphenol oxidase catalyses the rapid polymerization of O-quinones to produce black,brown or red pigments (polyphenols) that is the cause of fruit browning. The aminoacid tyrosine contains a single phenolic ring that may be oxidized by the reaction ofpolyphenol oxidase to form O-quinones. Hence, polyphenol oxidase may also be referred to as tyrosinase (Mayer, 2006).

2.5.2 Non enzymic antioxidants

Non enzymic antioxidants also play an important role in second line defense mechanism against damage induced by oxidative stress. Antioxidant vitamins that are derived from the diet can be considered under non enzymic antioxidants (Muthu and Durairaj, 2015).

2.5.2.1 Ascorbic acid

Vitamin C is a water soluble antioxidant and most effective scavenger of oxygen free radicals and other oxygen derived species. Vitamin C is the most important vitamin for human nutrition. L-ascorbic acid is the main biologically active form of vitamin C. It is then reversibly oxidized to form L-dehydroascorbic acid. It can act as an oxidant and pro-oxidant and protect DNA from free radical damage (Raj *et al.,* 2016). It act as a chain breaking antioxidant impairs with the formation of free radicals in the process of formation of intracellular substances throughout the body, including collagen, bone matrix and tooth dentine (Veeru, 2009).

2.5.2.2 Reduced Glutathione

Glutathione reductase reduces oxidized glutathione to glutathione, a substrate for glutathione peroxidase. Glucose-6-phosphate dehydrogenase is the first enzyme in the pentose phosphate pathway. The main physiological function of glucose-6-phosphate, which are essential for reductive biosynthesis and nucleic acid synthesis (Muthukrishnan, 2014)

2.5.2.3 α–Tocopherol

α–tocopherol interact with the polyunsaturated acyl groups of lipids, stabilize membranes, scavenge and various reactive oxygen species (ROS) and lipid soluble by products of oxidative stress (Deepika and Rajagopal, 2014).

2.5.2.4 Polyphenols

Polyphenols are one of the most numerous groups of substances in plant kingdom ranging from simple molecules, such as phenolic acids, to complex compounds, such as tannins. In addition, polyphenols function in trapping and scavenging free radicals due to their antioxidant properties. Other functions of polyphenols are regulating nitric oxide, decreasing leukocyte immobilization, inducing apoptosis, inhibiting cell proliferation and angiogenesis and exhibit phytoestrogenic activity (Hakiman and Maziah, 2009).

2.6 Free radical scavenging activity

Free radicals generated in aerobic metabolism are involved in a series of regulatory processes such as cell proliferation, apoptosis, and gene expression. When generated in excess, free radicals can counteract the defense capability of the antioxidant system, impairing the essential biomolecules in the cell by oxidizing membrane lipids, cell proteins, carbohydrates, DNA, and enzymes (Pisoschi *et al.,* 2016).

2.6.1 1,1'-diphenyl 2- picryl hydrazyl hydrate (DPPH)

DPPH is a stable nitrogen-centered free-radical, and their color change from violet to yellow when is reduced by either the process of hydrogen- or electron- donation (Hakiman and Maziah 2009).

2.6.2 Nitric oxide

Nitric oxide (NO), synthesized from L-arginine by nitric oxide synthase, is a relaxing factor for the vascular endothelium that may mediate hepatic injury from reactive oxygen species and lipid peroxidation products (Koruk *et al.,* 2004).

2.6.3 Hydrogen peroxide

Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can probably react with Fe2+ and possibly Cu2+ ions to form hydroxyl radicals and this may be the origin of many of its toxic effects (Hazra *et al* ., 2008).

2.6.4 Superoxide generation

Superoxide radical, known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributes to tissue damage and various diseases. In a biological system, its toxic role can be eliminated by superoxide dismutase. The radicals may also play an important role during the peroxidation of unsaturated fatty acids and other potential susceptible substances (Hussein, 2011).

2.6.5 Hydroxyl radical

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage (Hazra *et al.,* 2008).

2.7 Medicinal plant as sources of pharmaceuticals

2.7.1 *Pergularia deamia*

The plant *Pergularia daemia* (Asclepiadaceae) known as "Veliparuthi" in Tamil. Traditionally the plant *P.daemia* is used as anthelmintic, laxative, antipyretic and expectorant, also used to treat infantile diarrhoea and malarial intermittent fevers. Latex of this plant used for toothache. Stem bark remedy for cold and fever. Aerial parts of this plant reported the various pharmacological activities like hepatoprotective, antifertility, anti-diabetic, analgesic, antipyretic and anti-inflammatory. Aerial parts of the plant used for snake bite. Entire plant used as an anthelmintic, emmenagogue, emetic, antiseptic, emetic expectorant, expectorant and antivenin and used to facilitate parturition, while used in Ayurvedic medicine for delayed childbirth, amenorrhea, asthma, snakebite, rheumatic swellings and used to treat post-partum hemorrhage. Latex of this plant used for boils and sores. Dried leaf used as an emetic, anti rheumatic and used for bronchitis , amenorrhea,

dysmenorrheal, asthma, healing cuts and wounds, while used to treat whooping cough and

to facilitate parturition. Fresh leaf used as fish poison, while leaf juice used for amenorrhea, dysmenorrheal, catarrhal infections, infantile diarrhea and used reduce the body pain. Dried root used as an abortifacient, emetic, bronchitis and used for cough, asthma and constipation, while fresh root used as an abortifacient and used to treat gonorrhea. Shoots used to treat whooping cough. Stem bark has been used to treat malaria and twig used as an antipyretic and appetizer (Bhaskar and Balakrishnan, 2009).

3.0 EXPERIMENTAL PROCEDURE

The methodology adoped for the present study "Antioxidant potential and free radical scavenging activities of *Pergularia deamia"* is described below:

3.1 Collection of the plant samples

3.2 Preparation of the plant extract

3.3 Assessment of the antioxidants of *Pergularia deamia*

3.4 Determination of *in vitro* free radical scavenging activities of *Pergularia deamia*

3.1 COLLECTION OF THE PLANT SAMPLE

The plant sample (Plate 1) was collected from Telungu palayam, Coimbatore District. Fresh leaves ware collected and cleaned to remove adhering dust particles, washed under tap water and gently blotted dry between filter paper. The present study was done to compare the antioxidant activities and in vitro free radical scavenging activities of *Pergularia deamia* leaves of ethanolic extract of fresh leaves (ELFP) and dry leaves (ELDP).

Plate 1. *Pergularia deamia*

3.2 PREPARATION OF THE PLANT EXTRACT

Two different set of plant extracts of *Pergularia deamia* was considered for the experiment .

1. The fresh leaves of *Pergularia deamia* was extracted with ethanol as such and used for the assay.
2. The shade-dried leaves of *Pergularia deamia* were blended and made into a fine powder. 10 g of fine leaf powder was immersed in 100 ml of ethanol solvent. It was incubated at room temperature for 72 hrs. The sample ware filtered by whattman No1 filter paper. The filtrate was evaporated to dryness and stored.

3.3 ASSESSMENT OF ANTIOXIDANTS

An antioxidant systems are very vital for protecting cellular membranes and organelles from the injurious effects of active oxygen species. Plants with high amounts of antioxidants, either constitutive or induced, possess more resistance to the oxidative injury. This type of protection comprises both enzymatic and non-enzymatic antioxidants (Fatemeh Shams Moattar *et al*., 2017)

3.3.1 ENZYMIC ANTIOXIDANTS

The enzymic antioxidant such as catalase, peroxidase, superoxide dismutase, polyphenol, oxidase, and glutathione peroxidase were analysed in the leaves of *Pergularia deamia.*

3.3.1.1 ESTIMATION OF CATALASE ACTIVITY

Catalase is a tetrahedral protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen. Catalase activity was estimated by the method of Luck, (1974) and it is explained in appendix I.

3.3.1.2 ESTIMATION OF PEROXIDASE ACTIVITY

Peroxidases refer to heme containing enzymes which are able to oxidize organic and inorganic compounds using hydrogen peroxide as co-substrate .The activity of peroxidase was determined by the method of Reddy *et al.*,(1995) and it is detailed in appendix II .

3.3.1.3 ESTIMATION OF SUPEROXIDE DISMUTASE

Superoxide dismutase (SOD), a metallo-protein that catalyzes the conversion of superoxide radical into hydrogen peroxide.The superoxide dismutase activity was estimated by the method of Misra and Fridovich, (1972) and it is explained in appendix III.

3.3.1.4 Estimation of poly phenol oxidase

The activity of polyphenol oxidase was estimated by the method of Esterbauer *et al.*(1974) The detailed procedure is given in appendix IV.

3.3.1.5 Estimation of glutathione peroxidase activity

GPx plays a significant role in peroxyl scavenging mechanism and it maintains functional integration of the cell (Raj *et al.,* 2016*).* Glutathione peroxidase activity was determined by the method of Rotruck *et al.*(1973). The procedure is described in appendix V.

3.3.2 NON-ENZYMIC ANTIOXIDANTS

The non-enzymatic antioxidant includes ascorbic acid, α tocopherol, vitamin A, polyphenols and reduced glutathione.

3.3.2.1 Estimation of ascorbic acid

Ascorbic acid acting as a chainbreaking antioxidant impairs with the formation of free radicals in the process of formation ofintracellular substances throughout the body, including collagen, bone matrix and tooth dentine (Balaji *et al.,* 2011*).* Ascorbic acid of the plant sample was estimated by the method followed by Roe and Keuther, (1953) and the procedure is given in the appendix VI.

3.3.2.2 Estimation of α-tocopherol

Vitamin E also belongs to a group of lipid-soluble antioxidants.The biological activity of vitamin E exhibit tocopherol and tocotroenols,especially α-tocopherol. The predominant reaction

for tocopherol antioxidant activity is hydrogen atom donation, where a tocopheroxyl radical is formed (Suriyavathana Muthukrishna *et al.,* 2014*).* The activity of the α-tocopherol content in the plant sample was estimated by Rosenberg, (1992) and the procedure is presented in appendix VII.

3.3.2.3 Estimation of vitamin A

The vitamin A content of the plant sample used in the study was estimated by Bayfield and Cole.(1994) as given in the appendix VIII.

3.3.2.4 Etimation of polyphenols

The total polyphenol content of the plant sample used in the study was estimated using the method proposed by Malick and Singh. (1980) and it is presented in appendix IX.

3.3.2.5 Estimation of reduced glutathione

The activity of redusced glutathione was estimated by Moron *et al.*(1979) and it is described in appendix X.

3.4 DETERMINATION OF *in vitro* FREE RADICALS SCAVENGING ACTIVITY

3.4.1 Determination of DPPH radicals

The antioxidant activity of the extracts, on the basis of the scavenging activity of the stable 1, 1-diphenyl -2-picrylhydrazyl (DPPH) scavenging activity was measured by the method of Mensor *et al.* (2001) as given in the appendix XI.

3.4.2 Determination of inhibition of nitric oxide generation

Inhibition of *in vitro* nitric oxide generation was measured by the method of Green and Hill.(1984) as described in appendix XII.

3.4.3 Determination of hydrogen peroxide scavenging radicals

Hydrogen peroxide scavenging activity was measured by the method of Ruch *et al.*(1989) and it is described in appendix XIII.

3.4 .4 Determination of inhibition of superoxide generation

Inhibition of superoxide generation was measured by the method of Mc Cord and Fridovich (1969) and it is given in appendix XIV.

3.4.5 Determination of hydroxyl radicals scavenging activity

Inhibition of hydroxyl radicals of the sample was measured by the method of Elizabeth and Rao. (1990) and the procedure is given in appendix XV.

3.4.6 Determination of ABTS radical scavenging activity

Inhibition of ABTS radical scavenging activity was measured by the method of Shiwaikar *et al.*, (2006) and the procedure is given XVI.

4.0 RESULTS AND DISCUSSION

Plants are widely used also as nutritional supplements of special interest is their value as a source of natural antioxidants. Due to increasing safety concerns involved with consumption of synthetic antioxidants, exploitation of cheaper and safer sources of antioxidants from natural origins and especially from plants, is of interest nowadays (Stankovi *et al.,* 2016).

Spices and herbs are added to food, not only for flavour, but also for preservation. Research has shown that constituents of these aromatic plants can function as natural antioxidants and thereby prevent/retard rancidity of food lipids, improve sensory scores and offer greater consumer acceptance of food products (Amarowicz *et al.,* 2004). The antioxidants depending on their origin and the way of their preparation can be synthetic and natural. Plant kingdom in general and aromatic, medicinal herbs and spices in particular are an important source of natural antioxidants. Plants biosynthesise a great number of antioxidant compounds which are present in different concentrations and possess various chemical and physical properties (Venskutonis and Gruzdienė, 2005).

Therefore, *Pergularia deamia* has been evaluated for its antioxidant and free radical scavenging activities *in vitro* to get remedy for a remedy of antioxidants. The results obtained are discussed below the following headings.

4.1 Assessment of antioxidant potential of *Pergularia deamia* leaves

4.1.1 Enzymic antioxidants

4.1.2 Non-enzymic antioxidants

4.2 Free radical scavenging activities of *Pergularia deamia*

4.2.1 DPPH radical scavenging activity

4.2.2 Nitric oxide radical scavenging activity

4.2.3 Hydrogen peroxide radical scavenging activity

4.2.4 Superoxide radical scavenging activity

4.2.5 Hydroxyl radical scavenging activity

4.2.6 ABTS radical scavenging activity

4.1 ASSESSMENT OF ENZYMIC AND NON-ENZYMIC ANTIOXIDANTS

Antioxidants are a substance capable of frustrating or restricting the oxidation of other oxidizable molecules by suppressing the free radical-caused oxidation chain reaction. Antioxidants exhibit their activities by being involved in the oxidation process themselves rather than the biological targets (Ghawi ***et al.*, 2012).** The use of natural antioxidants for treating diseases and as food additives has better consumer acceptability and a trend over the use of the available synthetic products. Many research groups therefore have taken the responsibility of screening and quantifying of the antioxidant activities of the medicinal plant (Pothagar *et al.*, 2017).

Antioxidants dealing with an important role in the prevention and treatment of a variety of diseases by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. The antioxidants in the human diet are of great interest as possible protective agents to help human body to reduce oxidative damage (Menaga *et al*., 2013).

They can be categorized in multiple ways, based on their activity as enzymic and non enzymic antioxidants. Enzymic antioxidants work by breaking down and removing free radicals and non-enzymic antioxidants work by interrupting free radical chain reactions (Nimse and Pal, 2015).

4.1.1 ENZYMIC ANTIOXIDANTS

Reactive oxygen species inhibit the chain reaction by donating an electron to the free radical. The antioxidant defence system, supported by dietary antioxidants, protects the body from free radicals, however, during oxidative stress, antioxidants are insufficient to maintain homeostasis and antioxidants may be given as supplements (Verma *et al.*, 2015). Antioxidants are agents which scavenge the free radicals and prevent the damage caused by them (Umamaheswari and Chatterjee, 2008).

The enzymic antioxidants are produced endogenously and include catalase, peroxidase, superoxide dismutase, polyphenol oxidase and glutathione peroxidase. The activities of various antioxidative enzymes in the leaves of *Pergularia deamia* were assessed and presented in Table 1.

TABLE -1

ACTIVITIES OF ENZYMIC ANTIOXIDANTS IN *Pergularia deamia* LEAVES

ENZYMIC ANTIOXIDANTS (U/g)	***Pergularia deamia***	
	Fresh leaves extract	**Ethanol extract of dry leaves**
Catalase	2.60 ± 0.05	3.80 ± 0.07
Peroxidase	0.45 ±0.05	0.22 ± 0.03
Superoxide dismutase	5.68± 0.03	3.07± 0.01
Polyphenol oxidase	4.61 ± 0.01	1.98 ± 0.01
Glutathione peroxidase	59.56 ± 0.57	44.03 ±0.03

The values are mean ± SD of triplicates

1. Amount of SOD that causes 50 per cent reduction in the extent of nitroblue tetrazolium oxidation

2. Amount of enzyme that brings about decrease in absorbance of 0.05 at 240nm

3. 1 µmole of pyrogallol oxidized per minute

4. µmoles of CDNB-GSH conjugate per minute per gram sample

5. µmoles of GSH consumed per minute per gram sample

6. Amount of enzyme that transforms 1 µmole of dihydrophenol to 1 mole of quinines per minute.

Complicated antioxidant systems are very vital for protecting cellular membrane and organelles from the injurious effect of active oxygen species. Plant with higher amount of antioxidants,either constitutive or induced, possess more resistance to the oxidative injury. This type of protective comprises both enzymic and non enzymic antioxidants (Moattar *et al.*, 2016).

The activity of catalase, peroxidase, superoxide dismutase, polyphenol oxidase and glutathione peroxidase was evaluvated in both the fresh leaves of *Pergularia deamia* and ethanolic extract of leaves of of *Pergularia deamia* . The above table clearly reveals that the

activities of these enzyme were found to be high in fresh leaves extract rather then that of the ethanolic extract of the leaves .

In this study the fresh leaves extract were found to exhibit 2.603± 0.05, 5.680 ± 0.03, 4.616 ± 0.01 and 59.56±0.51 of catalase, superoxide dismutase, polyphenol oxidase and glutathione peroxidase respectively. A marked increased in the glutathione peroxidase activity was observed in the leaves of both the extracts of *Pergularia* which was followed by superoxide dismutase, polyphenol oxidase and catalase.

The antioxidant enzyme catalase and superoxide dismutase by virtue of their ability to catalyse disproportionation reaction of their substrates hydrogen peroxides and superoxide radical, have an enormous theortical advantage over exogenous antioxidant that are stoichometrically consumed (Nelson *et al.,* 2006).

The activity of Superoxide dismutase in the sample was estimated by Misra and Fridovich (1972) method and the results revealed that the superoxide dismutase content in the samples of *Pergularia* species ranged from 5.680- 3.077 U/g of the plant samples (Table 1). *Pergularia deamia* had the superoxide dismutase content of about 3.077 ± 0.01 in ethanol extract. The highest activity of superoxide dismutase 5.680 U/g protein was found in fresh leaf sample of *Pergularia deamia*.

Alici and Arabaci, (2016) reported that edible plant was found to have 11.59±0.01600 U/g of superoxide dismutase activity.

From Table 1, it was clear that activity of glutathione peroxidase was found to be 59.56 ± 0.57 U/g in the fresh leaves and 44.03 ± 0.03 U/g in ethanol extract of *Pergularia deamia*. The higher amount of glutathione peroxidase was present in fresh leaves of *Pergularia deamia*.

Our results are in accordance with that of the Sterlin and Gopalakrishnan, (2013) who reported that the glutathione peroxidase content of *Tylophoro pauciflora* was found to be 261.53± 2.54 U/g. Kukric *et al.*(2012) found that the leaves of *Uritica dioica* contain 1.174±90.14U/g of peroxidase and Muthu and durairaj, (2015) reported 0.33±0.002 U/g of polyphenol oxidase activity for *Annona muricata* leaves.

In this study leaves and ethanol extract of *Pergularia deamia* are found to exhibit 4.614 ± 0.01U/g and 1.982 ± 0.01U/g of polyphenol oxidase activity. A marked increase in the polyphenol oxidase activity was observed in fresh leaves of *Pergularia deamia.*

The activities of these enzymic antioxidant might be due to the presence of bioactive compounds present in *Pergularia deamia.* Glutathione peroxidase is an important enzyme which has the ability to detoxify hydrogen peroxide and lipid peroxidation and therefore protect the cells from damage caused by oxidative stress

4.1.2 NONENZYMIC ANTIOXIDANTS

Antioxidants control and reduce the oxidative damage in foods by delaying or inhibiting oxidation caused by reactive oxygen species (ROS), ultimately increasing the shelf-life and quality of these foods. Ascorbic acid, and many phenolics play dynamic roles in delaying aging, reducing inflammation and preventing certain disorder caused by free radical damage. Increasing consumption of fruits and vegetables has been recommended by many agencies and health care systems throughout the world (Altemimi *et al.*, 2017). Table 2 indicate the levels of the non-enzymic antioxidants namely, ascorbic acid, α-tocopherol, vitamin A, polyphenols and reduced glutathione in the leaves of *Pergularia deamia.*

TABLE -2

LEVELS OF NON ENZYMIC ANTIOXIDANTS IN *Pergularia deamia* LEAVES

NON-ENZYMIC ANTIOXIDANTS (mg/g)	*Pergularia deamia*	
	Fresh leaves extract	**Ethanol extract of dry leaves**
Ascorbic acid	6.67 ± 0.15	3.86 ± 0.45
α-Tocopherol	0.19 ± 0.03	0.04 ± 0.01
Vitamin A	49.8 ± 0.52	22.3 ± 0.98
Reduced glutathione	69.7 ± 0.65	88.6 ± 1.24

Vitamin C, which includes ascorbic acid and its oxidative product dehydroascorbic acid, has many biological activites in human body have food that vitamin C can reduce levels of C-reactive proteins a mark of inflammation and possibly a prediction of heart disease. More than 85% of vitamin C in human diets is supplied by fruits and vegetables (Thevasundai and Rajendran, 2011)

Vitamin E also belongs to the group of lipid soluble antioxidants.The biological activity of vitamin E exhibit tocopherols and tocotrienol. The predominant reaction reported for tocopherol antioxidant activity in hydrogen atom donated, where the tocopheroxyl radical is formed (Muthukrishnan *et al.,* 2014)

Vitamin A is found in the main forms: retinol (Vitamin A_1) 3,4 didetroretinol (Vitamin A_2) and 3-Hydroxy retinol (Vitamin A_3) (Hamid *et al.,* 2010)

Polyphenols are a large and diverse class of compounds, many of which occur naturally in a wide range of food and plants. A range of plant polyphenols is either being actively developed or already currently sold as dietary supplements and/or herbal, derived medicines (Nishaa *et al.,* 2012).

Glutathione is a cysteine containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites an enzyme systems, such as ascorbate in the glutathione ascorbate cycle, glutathione peroxidases and glutaredoxins, as well as reacting directly with oxidants. Due to its high concentration and its central role in maintaining the cells redox state, glutathione is one of the most important cellular antioxidants. (Hamid *et al.,* 2010)

It is evident from the above Table 2, that both the extracts of fresh leaves and ethanol extract of dry leaves exhibit strong antioxidant levels.

The vitamin C content in fresh leaves and dry leaves ranges from 6.67 ±0.015 and 3.86 ±0.45 respectively. Ascorbic acid is found to be correlated with better scavenging activities *in vivo* than the antioxidant enzymes because they are present with intracellular as well as in extracellular. As an antioxidant it is reported that ascorbic reacts with superoxide, hydrogen peroxide or the hydroperoxy radical and the oxidized are recycled back (Karthikeyan and Rani, 2003).

The estimated vitamin E levels in the fresh leaves and the dry leaves of different extracts from 0.192±0.3 and 0.041±0.01.The only protective agent that can act against the toxic effect of oxygen radicals. Higher levels of GSH was found in both the leaves extract of *Pergularia deamia* and the antioxidant activity of phenolics is mainly because of their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quencher and metal chelators. The consumption of food and beverages rich in phenolics can reduce the risk of heart disease by showing the progression of arteriosclerosis (Oboh and Akindhunsi, 2004). The levels of phenol for fresh leaf of *Pergularia deamia* and ethanol leaf extract of *Pergularia deamia* was found to be 6.85± 0.4 and 11.04±0.03 respectively.

The maximum levels for reduced of glutathione were found in the ethanol extract in the *Pergularia deamia* rather than the fresh leaves extract. Our results are in accordance with that of (Oboh *et al*., 2004) that *Telfairia occidentalis* showed maximum levels of non enzymatic anti oxidants.

The present study revealed that the polyphenol content of *Pergularia deamia* was (11.44 ± 0.03mg/g) in the ethanol extract and (6.848 ± 0.14mg/g) in the freash leaf sample. The higher amount of polyphenol content in ethanol extract of *Pergularia deamia* leaves. Adedapo *et al.,* (2009) reported that leaves of *Celtis africana* was found to be *(*14.0 ± 0.11 mg /g) thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized.

The activity of reduced glutathione was highly found to be (88.6 ± 1.24mg/g) in the ethanol extract and (69.7 ± 0.65mg/g) the lower amount of reduced glutathione content in the fresh leaf sample of *Pergularia deamia*.

Moyo *et al.* (2012), reported that the *Moringa oleifera* leaves/sunflower seed cake was found to be 86.0±1.00 and 35.0±2.00b of reduced glutathione respectively.

Since *Pergularia deamia* was found to contain all the non enzymatic antioxidants in moderate quantities, it may protect the cell membrane against oxidative damage by regulating the redox state of protein membrane.

4.2 Free radical scavenging activities of *Pergularia deamia*

Oxidation process is one of the most important routs for producing free radicals in food, drugs and even living systems (Pourmorad *et al.,* 2006). Reactive oxygen species (ROS), including free radicals such as superoxide anion radicals (O_2), hydroxyl radical species (OH.), singlet oxygen (1O2) and hydrogen peroxide, are often generated by biological oxidation reactions of exogenous factors. These ROS are known to cause severe damage to biological molecules (Motlhanka *et al.*, 2008).

In the present investigation 20-100µg/ml of the ethanol extract of fresh and dry leaves of *Pergularia deamia* were used for the *in vitro* free radical scavenging activity. Ascorbic acid was used as a standard.

4.2.1 DPPH radical scavenging activities of *Pergularia deamia*

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule which is widely used to investigate radical scavenging activity (Babu *et al.,* 2013). This assay provides the information on the activity of leaf extract with a stable free radical. The percent inhibition of DPPH scavenging activity of the ethanolic extracts of both fresh and dry leaves *Pergularia deamia* were carried out and the results are presented in Figure 2.

Figure 2

DPPH radical scavenging activity of *Pergularia deamia*

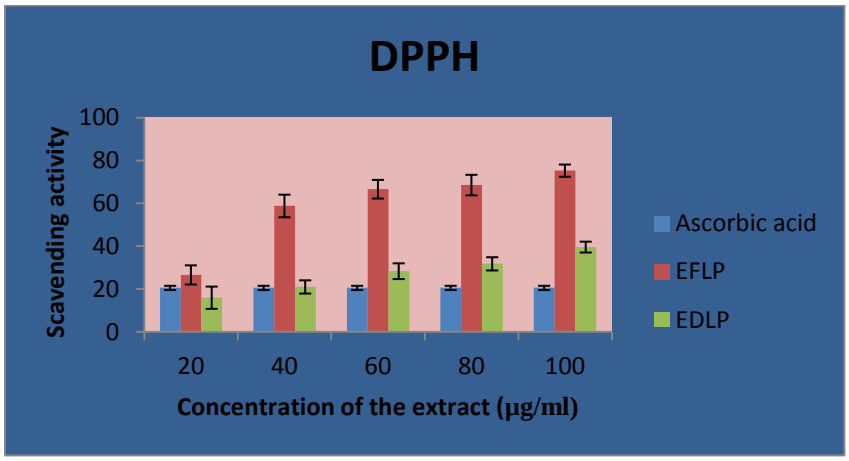


Figure 2 revealed that the DPPH free radical scavenging effect of fresh ethanolic extract of leaves and ethanol extract of dry leaves of *Pergularia deamia*. Ethanol extract of fresh leaves of *Pergularia deamia* exhibits higher activity 75.33 ± 2.85 at a concentration of 100 µg/ml when compared with ethanolic extract of dry leaves of *Pergularia deamia.* The reduction in the DPPH molecule can be correlated with the available number of hydroxyl group, present in the extract and might be due to their hydrogen donating activity.

Chittam (2016), have reported that the *Chlorophytum tuberosum* tested against DPPH stable radicals spectrophotometrically reveals it possessed excellent antioxidant capacity.

Babu *et al. (*2013) showed the increased concentration of DPPH scavenging activity in *Triphala* and *Turnera ulmifolia* leaves was found to be exhibit excellent capacity of DPPH scavenging activity (Kalimuthu *et al.*, 2014).

Chauhan *et al*. (2016) reported the DPPH activity of *Tinospora cordifolia* and *Glycyrrhiza glabra.* Among them *Tinosora cordifolia* was found to exhibit maximum scavenging activity.

DPPH scavenging activity is one of the best methods to study the free radical scavenging activity by medicinal plants and its compounds. Thus the present study confirms that the leaves of *Pergularia deamia* exhibit good DPPH scavenging activity.

4.2.2 Nitric oxide radical scavenging activity of *Pergularia deamia*

Nitric oxide (NO) has an important role in various types of inflammatory processes in the animal body (Shareef *et al.,* 2014). It plays a important role in neurotransmitters in the central nervous system and also involved in vascular homeostasis, antimicrobial and anti tumour activity. Oxidative damage is also reported which may be due to the reactive of nitric oxide with superoxide and form peroxy nitric anion, that decomposed to produced nitric oxide hydroxyl scavengers of nitric oxide compete with oxygen and reduce the production of nitrite ion (Dadashpour *et al.,* 2011).Figure 3 revealed that the nitric oxide free radical scavenging activities of fresh and dry leaves of *Pergularia deamia.*

Figure 3

Nitric oxide radical scavenging activity of *Pergularia deamia*

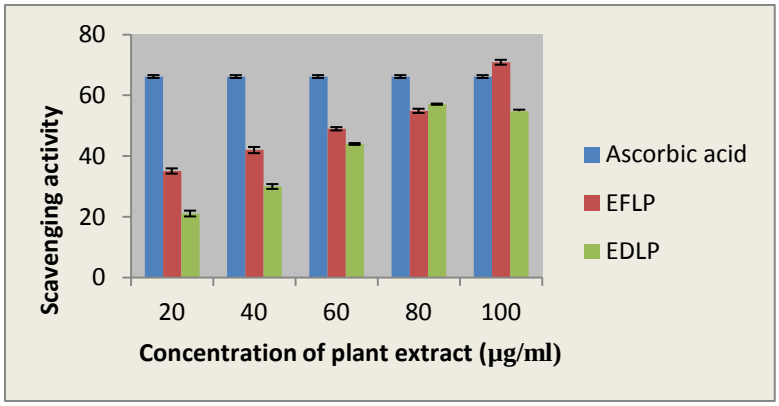


Figure 3 present the per cent inhibition of nitric oxide radical by the ethanolic extract of both fresh and dry leaves of *Pergularia deamia* .

In this study the ethanol extract of fresh leaves of *Pergularia deamia* showed appreciable inhibition of 70.9 ± 0.8against nitric oxide radical than that of ethanol extract of dry leaves of *Pergularia deamia* 21.1 ± 0.96 .

Sharef *et al*. (2014) reported the nitric oxide radical scavenging activity of *Zingiber officinale* for 50% inhibition was found to be 83.38 µg/ml.

Kamboj *et al.* (2014) explained that the ethanol extract of leaves, stem and roots of *X.strumarium* showed better activity when compared with other solvent extracts.

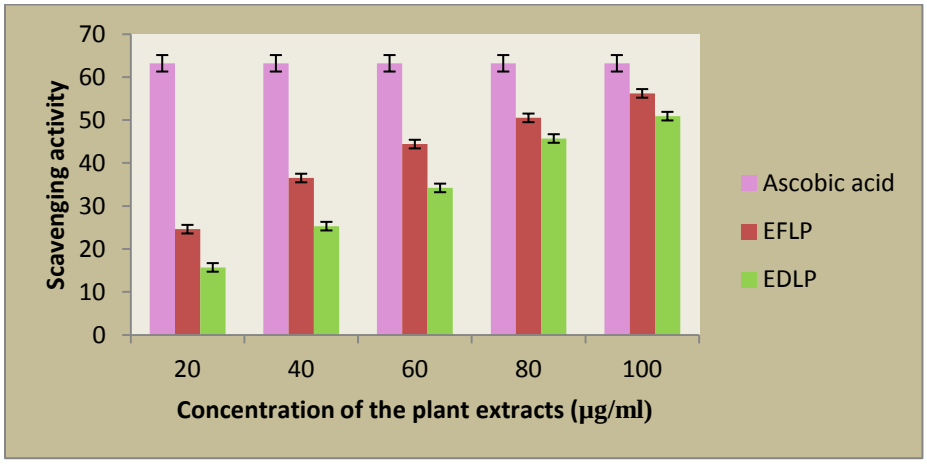
Hence from the above reports, it is clearly demonstrated that the extracts of *Pergularia deamia* effectively scavenged nitric oxide generation indicative of its antioxidant potential.

4.2.3 Hydrogen peroxide radical scavenging activity

Hydrogen peroxide occurs naturally at low concentration levels in the air, water, human body, plants, microorganisms, food and beverages. It is widely used as a bleaching agent in the textile, paper and pulp industries. In the body, H_2O_2 is rapidly decomposed into oxygen, water and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate lipid peroxidation and cause DNA damage (Chanda and Dave, 2009). The per cent inhibition of hydrogen peroxide scavenging activities of *Pergularia deamia* was induced and the results are indicated in Figure 4.

Figure 4

Hydrogen peroxide radical scavenging activity of *Pergularia deamia*



Both the ethanol extract of fresh leaves and dry leaves, exhibited a very good scavenging activies of 56± 2.61and 50 ±0.04 at 100 µg/ml concentration.

Muthu and Durairaj (2008) reported that the *Annona muricata* extract was found to be dose dependant with mximum inhibition of 86.4 percent 100 µg/ml concentration.

Harza *et al.* (2008) reported that the *Spondias pinata* showed the 23% of hydrogen peroxide activity.

(Dogra *et al.*, 2011) reported that the plant leaves of *Camellia sinenis* to exhibited higher amount of scavenging activity of 67.8%. Therefore the present study exhibits that the extract of

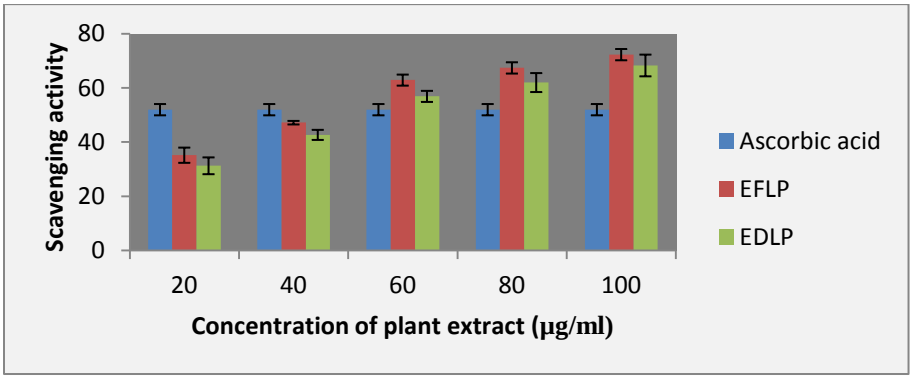
Pergularia deamia exhibits good antioxidant activity and could serve as a combat against the diseases caused by free radicals.

4.2.4 Superoxide radical scavenging activity

Superoxide radical can be converted into hydrogen peroxide by the action of superoxide dismutase and the H_2O_2 can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions (e.g., iron and copper), or by UV photolysis (Shyur *et al*., 2005). The scavenging activity of different extracts of *Pergularia deamia* against superoxide radical was evaluated and is presented in Figure 5.

Figure 5

Superoxide radical scavenging activity of *Pergularia deamia*



It was found that the ethanol extract of fresh leaves of *Pergularia deamia* showed highest inhibitory activity when compared with ethanol extracts of dry leaves of *Pergularia deamia*. The superoxide radicals scavenging of all the extract was found to be concentration dependent that is as the concentration of the extract increased the radical scavenging activity was also found to be increased.

Similar dose dependent study was observed by Sathisha *et al.*(2011) that *Curcuna* and *Coffee bean* showed significant superoxide radical scavenging activity with the IC 50 value of 0.4±0.14 and 0.52 ± 0.18µg/ml.

The superoxide radical scavenging activities of the extract of *Spodias pinnata* were found to have marked increased with increase in concentration. At a concentration 200 µg/ml, ethanol extract exhibited the highest inhibition of superoxide radical (Jain *et al.*, 2014).

Superoxide radicals are less reactive but they can give rise to toxic hydroxyl radicals thereby damaging cellular macromolecules directly or indirectly with severe consequences. The superoxide radicals have been proved to play crucial role in ischemia-reperfusion, injury. These radicals are also involved in many pathological process. Thus scavenging of superoxide radicals would be a promising remedy for this disease (Pal *et al.*, 2015).

Since *Pergularia deamia* was found to be exhibits significant *in vitro* free radical scavenging activity, it could be suggested to be used to reduce the risk of disease caused by free radicals.

4.2.5 Hydroxyl radical scavenging activity

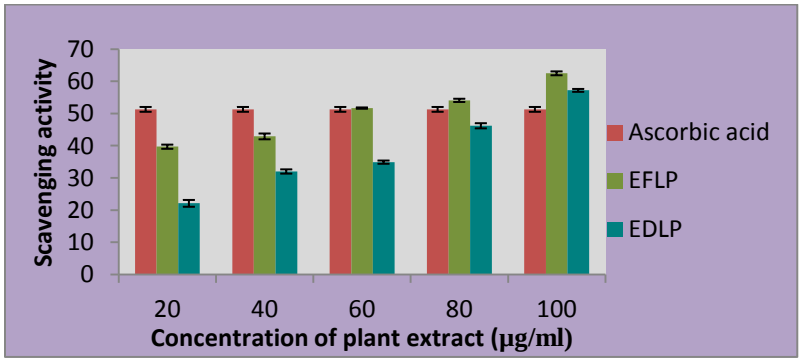
Hydroxyl radicals are the most reactive and predominant radicals generated endogenously during aerobic metabolism. The radicals is extremely reactive oxygen species capable of modifying almost every molecule in the living cells. It can cause strand damages in DNA leading to carcinogenesis, mutagenesis and cytotoxicity. They are capable of quench initiation of lipid peroxidation process by abstracting hydrogen atom from unsaturated fatty acid.

Hydroxyl radical scavenging activity was quantified by measuring the inhibition of the degradation of 2- deoxyribose by the free radicals generated by the Fenton reaction (Saeed *et al.*, 2012).

The percent inhibition of hydroxyl radical scavenging activity of leaves of *Pergularia deamia* was studied and the results are indicated in Figure 6

Figure 6

Hydroxyl radical scavenging activity of *Pergularia deamia*



The percent inhibition of hydroxyl radical by ethanolic extract of fresh and dry leaves of *Pergularia deamia* were concentration dependent and the fresh leaves (EFLP) should highest scavenging activity of 62% as compared with compared with standard ascorbic acid, which was found to be 57% concentration of 100 µg/ml.

Similar results were repeated by Pavithra and Vadivukarasi, (2015) who reported that the extract of *Kedrostis foetidissima* exhibited higher hydroxyl radical scavenging activity.

Saumaya and Basha (2010) narrated that the *Ginseng* and *Langerstroemia speciosa* extracts also exhibited higher value for hydroxyl radical scavenging activity.

Hence our reported clearly demonstrate that the ethanolic extract of leaves of *Pergularia deamia* (EFLP and EDLP).scavenged hydroxyl radicals and it was propotional to the concentration of extract. The antioxidative property the *Pergularia deamia* might be due to the presence of active phyto constituents.

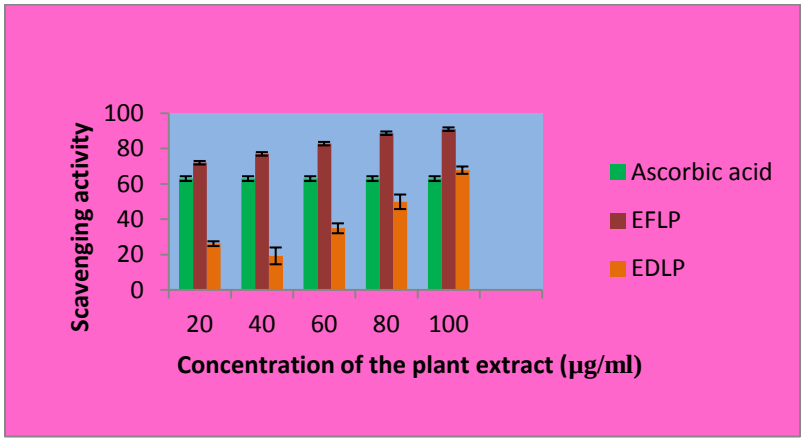
ABTS radical scavenging activity

ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain-breaking antioxidants.

ABTS in a blue chromophore produced by a reaction between ABTS and potassium persulphate and in the presence of the plant extract the cation radical gets reduced and the remaining reduced cation concentration after reaction with antioxidant compound was then quantified.

Figure 7

ABTS radical scavenging activity of *Pergularia deamia*



The *Pergularia deamia* extract efficiently scavenged ABTS radicals generated by the reaction between 2,2'-azinobis (3- ethylbenzothiazolin-6-sulphonic acid) (ABTS).

Kazazic, (2016) have reported that the *Papaver rhoeas* tested against ABTS radical scavenging activity of ethanolic extract possessed excellent antioxidant capacity by increased with the increasing concentration of the extract as compared to aqueous extract.

In the present study the ethanolic extract of both fresh and dry leaves of *Pergularia deamia* was found to be effective scavenger of ABTS reduced and it can be explored as novel and potential antioxidant.

5.0 SUMMARY AND CONCLUSION

Since ancient times, plants have been an exemplary source of medicine. Indian has about 45000 plant species and among them several thousand have been claimed to possess medicinal properties. Plant based substances have been used extensively because of its efficacy, safety having nearly no side effects.

Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value that have yet to be discovered, though large number of plants is constantly being screened for their antioxidant and antimicrobial effects. For this reason, studies of natural medicinal plants are attractive to investigators due to their potent antioxidant and free radical scavenging activity.

Therefore there is an increasing interest in the antioxidant effects of compounds derived from the herbs which could be relevant in relation to their nutritional incidence and their nutritional incidence and their role in health and diseases.

Pergularia daemia (L) a medicinal plant belonging to the family *Asclepiadaceae*, which is a slender twinning perennial herb, was selected for the present study. The study was formulated with the objective to assess the free radical scavenging and antioxidant potential of *Pergularia daemia*.

Enzymic antioxidants (catalase, peroxidase, superoxide dismutase, polyphenol oxidase, and glutathione peroxide) and non enzymatic antioxidants (ascorbic acid, α – tocopherol, vitamin A, polyphenol and glutathione) were analyzed in the leaves of *Pergularia daemia*. The leaves of *Pergularia daemia* was used to ascertain antioxidants studies. The leaves of *Pergularia daemia* were extracted with solvent namely ethanol and fresh leaves were used for the determination of free radical scavenging and antioxidant activity.

Pergularia daemia was found to be good sources of enzymic antioxidants. The activity of glutathione peroxidase was higher in the leaves (59.56 ± 0.57 U/g) of *Pergularia daemia* among all the enzymes assessed. The leaves of *Pergularia daemia* were found to contain all the non – enzymic antioxidants analyzed. Our results in the present report suggest that the *Pergularia daemia* exhibited potent antioxidant effect.

Fresh leaves of *Pergularia daemia* was found to possess the free radicals scavenging activity. The ethanol extract of the leaves (91 per cent) of *Pergularia daemia* showed strong inhibition against ABTS radicals tested. *Pergularia daemia* extract were found to have a potent superoxide, nitric oxide and hydrogen peroxidase activity.

The present results clearly indicate the extracts from *Pergularia daemia* may serve as a potent free radical scavengers acting possibly as primary antioxidants. However, further investigation *in vivo* experiments are still necessary to further shed light on their efficacy in disease risk reduction.

Due to their antioxidant and free radicals scavenging activities, *Pergularia daemia* has promising potential as a source of natural antioxidant and free radicals agents. Further work is needed to isolate the secondary metabolites from the extract studied inorder to test specific antioxidant and free radical scavenging activity and the underlying mechanisms.

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APPENDIX I

ESTIMATION OF CATALASE ACTIVITY

(Luck, 1974)

PRINCIPLE

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease.

REAGENTS

1. Phosphate buffer (0.067M PH 7.0)

   Dissolved 3.522g of $KH_2PO_4$and 7.268g of $KHPO_4.2H_2O$ in distilled water and made up the volume to one litre.

2. Hydrogen peroxide – Phosphate buffer

   Dissolved 0.16 ml of H_2O_2 (10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1 cm light path.

PROCEDURE

ENZYME EXTRACT

The sample was homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Used fresh extract for assay.

ASSAY

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01-0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If ‘t’ was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

CALCULATION

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036µ mole/ml.

APPENDIX II

ESTIMATION OF PEROXIDASE ACTIVITY

(Reddy *et al.*, 1995)

PRINCIPLE

In the presence of the hydrogen donor pyrogallol, peroxidase converts H2O2 to water and oxygen. The oxidation of pyrogallol to the coloured product purpurogalli can be quantified spectrophotometrically at 430nm. The formation of the product is proportional to the activity of the enzyme and can be used as a measure of the same.

$$\text{Pyrogallol} + H_2O_2 \longrightarrow \text{Oxidized pyrogallol} + H_2O$$

REAGENTS

1. Pyrogallol(0.05M); Phoshphate buffer (pH 6.5): 630 mg of pyrogallol in 100ml of 0.1M Phosphate buffer.
2. Hydrogen peroxide (1%)

PROCEDURE

Macerated one gram of the sample with 5 ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300 g for 15 min. Used the supernatant as the enzyme source. All procedure were carried out at 0-5° C.

Pipetted out 3ml of 0.05 M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400 nm. Added 0.5 ml of 1% hydrogen peroxide in the test cuvette. Recorded the change in the absorbance every 30 seconds upto 3 minutes.

CALCULATION

Change in absorbance / min	= X
Weight of the plant material taken	= 300 mg
Volume of the extract taken for the assay	= 0.02 ml
Change in absorbance for 1.5 ml extract	= (X / 0.02) x 1.5 – Y
(i.e) Peroxidase activity in 300 mg plant tissue	= Y
Peroxidase activity / g plant tissue	= Yx (1000/300) Units

APPENDIX III

ESTIMATION OF SUPEROXIDE DISMUTASE

(Misra and Fridovich, 1972)

PRINCIPLE

The assay of SOD is based on the inhibition of formation of NADHphenazine methosulphate-nitroblue tetrazolium formazon, the extent of which can be assayed spectrophotometrically at 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT)) (300µM)
4. NADH (700µM)
5. Glacial acetic acid
6. n-butanol

PROCEDURE

PREPARATION OF ENZYME EXTRACT

Gmelina arborea leaves (0.5g) were ground with 3.0ml of sodium pyrophosphate buffer, centrifuged at 2000g for 10 minutes and the supernatant was used for the assay.

ASSAY

The assay mixture contained in a total volume of 3.0ml, 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of enzyme preparations and 1.0ml of water. NADH (0.2ml) was added to start the reaction.

The assay mixture was incubated at 30ºC for 90 seconds and the reaction was stopped by the addition of 1.0ml of glacial acetic acid. n-butanol (4ml) was added to the above mixture, allowed to stand for 10 minutes and then centrifuged at 2000g for 5 minutes. The intensity of the chromogen in the butanol layer was measured at 560nm against butanol as blank. The system devoid of enzyme served as control. One unit of enzyme activity is defined as the amount of enzyme causing a 50% reduction in NBT oxidation/minute.

APPENDIX IV

ASSAY OF POLYPHENOL OXIDASE

Esterbauer *et al*., (1977)

PRINCIPLE

Phenol oxidases are copper proteins, which catalyze the aerobic oxidation of certain phenolic substrates to quinines, which are auto-oxidized to dark brown pigments generally known as melanins, which can be estimated spectrophotometrically at 495nm.

REAGENTS

1. Reaction medium - Tris-HCl (50mM, pH 7.2), sorbitol (0.4M), NaCl (10mM)
2. Catechol (0.01M)
3. Phosphate buffer (0.1M, pH 6.5)

PROCEDURE

PREPARATION OF ENZYME EXTRACT

The enzyme extract was prepared by macerating 5g of leaf tissue in 20ml reaction medium containing tris-HCl. The homogenate was centrifuged at 2000g for 10 minutes at 4ºC, the supernatant was used for the assay.

ASSAY

Both phosphate buffer (2.5ml) and catechol solution (0.3ml) was pipette out into the experimental cuvette and the spectrophotometer was set at 495nm. The sample (0.2ml) was added to the same cuvette and the changes in absorbance were monitored for every 30 seconds up to 5 minutes. One unit of either catechol oxidase/laccase is defined as the amount of enzyme that transforms one µmole of dihydrophenol to one µmole of quinine/minute. The activity of PPO can be calculated using the formula,

$$\text{Enzyme unit} = K \times (\Delta A/\text{minute})$$

where,

K for catechol oxidase = 0.272

K for laccase = 0.242

APPENDIX V

ESTIMATION OD GLUTATHIONE PEROXIDASE ACTIVITY

(Rotruck *et al.*, 1977)

Principle

A known amount of enzyme preparation was used to react with hydrogen peroxide in the presence of GSH for a specified time pereiod when the screening was measured by the method of Ellman

$$2GSH + H_2O_2 \xrightarrow{Se - GPx} GSSH + 2H_2O$$

Reagents

1. 0.4M Tris Buffer
2. 10µM sodium azide
3. 10% TCA
4. 0.4µM EDTA
5. 19µM hydrogen peroxide

Procedure

To 2ml of Tris buffer, 0.2ml of EDTA, 0.1ml of sodium azide and 0.5ml of plant extract were added following by 0.1ml hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with the tube containing all the reagent expect sample. After 10 min the reaction was arrested by the addition of 0.5ml of 10% TCA centrifuged and supernatant was assayed for glutathione by the method of Ellman

The activities are expressed as µg GSH consumed/min/mg protein

APPENDIX VI

ESTIMATION OF ASCORBIC ACID

(Roe and Kuether, 1953)

PRINCIPLE

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophotomerically at 540nm.

REAGENTS

1. 4%TCA
2. 9N H_2SO_4
3. 2% 2, 4-dinitrophenyl hydrazine: Dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. Stock standard solution: Dissolved 100mg of ascorbic acid in 100ml of 4%TCA
7. Working standard: Diluted 10ml of the stock solution to 100ml with 4%TCA

PROCEDURE

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay.

The assay volumes were made up 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours.

The osazones formed were dissolved in 2.5ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX VII

ESTIMATION OF α-TOCOPHEROL

(Emmerie-Engel method, 1938 as described by Rosenberg, 1992)

PRINCIPLE

Tocopherol can be estimated using Emmerie – Engel reaction which based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction read at

460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

REAGENTS

1. Absolute alcohol
2. Xylene
3. 2, 2'- dipyridyl
4. Standard solution:

Dissolved 10mg/ 10ml of α-tocopherol in absolute alcohol 91mg of œ- tocopherol is equivalent to 100mg of tocopherol acetate.

EXTRACTION OF PLANT TISSUE

The sample was homogenized with water in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask. Added 50ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatman No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

PROCEDURE

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetteed out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard,added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution.

The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520nm} - \text{Reading at 460nm}}{\text{Reading of standard at 520nm} \times 0.24 \times 15}$$

APPEND IX

ESTIMATION OF POLY PHENOLS

(Mallick and Singh, 1980)

PRINCIPLE

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650nm.

REAGENTS

1. 80% ethanol
2. Diluted Folin - Ciocalteau reagent
3. 20% Sodium carbonate
4. Stock solution:

   100mg of catechol was made up with 100ml distilled water
5. Working standard:

   10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

PROCEDURE

PREPARATION OF PLANT EXTRACT

Pre-weighed leaf sample (0.5g) was ground in 5ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was collected and the residue was re-extracted with 2.5ml of 80% ethanol. After repeated centrifugation, the supernatants were collected and pooled. The ethanol was evaporated and the residue was dissolved in a known volume of distilled water and used for the estimation of phenol.

ESTIMATION

Aliquots (0.2 to 2.0ml) of the standard catechol solution were made up to 3ml with distilled water. Folin-Ciocalteau (0.5ml) reagent was added to each test tube. After 3 minutes, 2.0ml of 20% sodium carbonate was added to each tubes. After mixing the tubes thoroughly, all the tubes were heated in a boiling water bath for exactly one minute and allowed to cool at room temperature. The blue colour was recorded at 650nm against a reagent blank. The concentration of phenols in the sample was calculated from the standard curve constructed on an electronic calculator set to the linear regression mode and expressed as mg phenols/g leaf.

APPENDIX X

ESTIMATION OF REDUCED GLUTATHIONE

Moron *et al*., (1979)

PRINCIPLE

Reduced glutathione (GSH) was measured by its reaction with 5,5'- dithiobis-2-nitrobenzoic acid (DTNB) (Ellman's reaction) to give a yellow coloured compound that absorbs at 412nm.

REAGENTS

1. TCA (5%)
2. TCA (25%)
3. Sodium phosphate buffer (0.2M, pH 8.0)
4. DTNB (0.6M in 0.2M sodium phosphate buffer)

PROCEDURE

PREPARATION OF PLANT EXTRACT

The leaves of *Gmelina arborea* (0.5g) were ground with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and the supernatant (0.1ml) was taken for the estimation of GSH.

ESTIMATION

The volume of different aliquots (0.2 to 1.0ml) was made up to 1ml with phosphate buffer. Freshly prepared DTNB (2ml) was added to the tubes and the intensity of the yellow colour was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentrations ranging from 2 to 10nmoles of GSH. The concentration of GSH in the samples was calculated from this and the result were expressed as nmoles GSH/g leaf.

APPENDIX XI
DPPH RADICAL SCAVENGING ACTIVITY

(Mensor *et al*., 2001)

PRINCIPLE

Antioxidants react with DPPH and convert it to diphenyl-picryl hydrazine by donating its OH group. The degree of discoloration from purple to yellow colour can be measured at 519nm, which is a measure of the radical scavenging potential of the extracts.

REAGENTS

1. DPPH (0.3mM in methanol)
2. Methanol

PROCEDURE

The different solvent extracts and crude aqueous extract (5µl) was added with 0.5ml of methanolic solution of DPPH and 0.495ml of methanol. The mixture was then allowed to stand at room temperature for 30 minutes. DPPH methanol solution was used as positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was read at 518nm in a spectrophotometer. The per cent inhibition was calculated using the following formula;

$$\text{Scavenging activity (\%)} = 100 - \frac{\text{A (Control)}}{\text{A(Control) - A(Sample)}} \text{ X } 100$$

APPENDIX XII

NITRIC OXIDE SCAVENGING ACTIVITY

(Green and Hill, 1984)

PRINCIPLE

An aqueous solution of sodium nitroprusside spontaneously generates nitric oxide at physiological pH, which interacts with oxygen to produce nitrite ions, which is measured at 546nm.

REAGENTS

1. Sodium nitroprusside (100mM)
2. Phosphate buffered saline (PBS) (pH 7.4)
3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride)

PROCEDURE

Sodium nitroprusside (2.0ml), phosphate buffered saline (0.5ml) and each of the six different plant extracts (0.5ml) were mixed and incubated at 25ºC for 30 minutes. Griess reagent (0.5ml) was added and allowed to stand for 30 minutes. The control tube was prepared without leaf extracts. The absorbance of the pink coloured chromogen was read at 546nm against a reagent blank.

$$\text{Nitric oxide scavenging activity (\%)} = \frac{\text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX XIII

HYDROGEN PEROXIDE SCAVENGING EFFECTS

(Ruch *et al*., 1989)

PRINCIPLE

H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance at 230nm spectrophotometrically.

REAGENTS

1. H_2O_2 (40mM in 0.1M phosphate buffer)
2. Phosphate buffer (0.1M, pH 7.4)

PROCEDURE

The plant extract was diluted to a concentration of 10mg in 10µl. This extract (10µl corresponding to 10mg) was added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without H2O2 for each sample. The percent inhibition was calculated using the formula,

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX XIV
SUPEROXIDE SCAVENGING ACTIVITY

(Winterbourn *et al*., 1975)

PRINCIPLE

The extent of superoxide generation was studied on the basis of inhibition of the production of nitroblue tetrazolium formazon of the superoxide ion by the plant extracts and is measured spectrophotometrically at 560nm.

REAGENTS

1. EDTA (0.1M containing 1.5mg of sodium cyanide/10 ml)
2. Nitroblue tetrazolium (NBT) (1.5mM)
3. Riboflavin (0.12mM)
4. Phosphate buffer (0.067M, pH 7.6)
5. Dimethyl sulfoxide (DMSO)

PROCEDURE

The assay mixture contained 0.02ml of plant (solvent and crude aqueous) extracts with 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.63ml of phosphate buffer. DMSO, instead of plant extract, was considered as control. All tubes were vortexed and the initial absorbance was read at 560nm. The tubes were illuminated uniformly using a fluorescent lamp for 30 minutes. The absorbance was read again at 560nm. The difference in optical density before and after illumination is the measure of superoxide generation and the percentage inhibition was calculated using the formula,

$$\% \text{ Superoxide Scavenging} = \frac{A \text{ (After illumination)} - A \text{ (Reference)}}{A \text{ (Control)}} \times 100$$

APPENDIX XV

HYDROXYL RADICAL SCAVENGING EFFECTS

(Elizabeth and Rao 1990).

PRINCIPLE

Hydroxyl radicals are generated from a Fe_2+/ascorbate/EDTA/H_2O_2 system, which attacks deoxyribose and eventually produces thiobarbituric acid reactive substances (TBARS). The ability of the plant extracts to inhibit TBARS formation is measured spectrophotometrically at 532nm.

REAGENT

1. Deoxyribose (28mM)
2. $FeCl_3$ (1mM)
3. EDTA (1mM)

4. H_2O_2 (10mM)

5. Ascorbate (1mM)

6. KH_2PO_4-KOH buffer (20mM, pH 7.4)

7. Thiobarbituric acid (1%)

8. HCl (25%)

PROCEDURE

The reaction mixture contained deoxyribose (0.1ml), $FeCl_3$ (0.1ml), H_2O_2 (0.1ml), ascorbate (0.1ml), buffer (0.1ml) and 20µl of leaf extracts which corresponded to 10mg concentration. The total volume was made up to 1ml with water. The tubes were capped tightly and incubated in a water bath at 37ºC for one hour. The reaction was terminated by the addition of TBA (0.5ml) and HCl (0.5ml). The tubes were heated in a boiling water bath for 20 minutes for colour development. The intensity of the pink colour formed, as the indication of TBARS formation, was measured at 532nm. The per cent TBARS produced for positive control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the extract treated groups.

$$\text{Hydroxyl radical scavenging activity (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \times 100$$

APPENDIX XVI

ABTS RADICAL SCAVENGING EFFECTS

(Shirwaikar *et al*., 2006)

PRINCIPLE

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation de-colourisation assay was employed to assess the radical-scavenging effect of the leaf extracts of the candidate plant. ABTS is a chromogen, which changes into a coloured mono-cation radical form (ABTS+) in the presence of oxidative agent and the ABTS+ has an absorption peak at 750nm. Antioxidants will reduce ABTS+ into its colourless form and the extent of decolourisation corresponds to the per cent reduction of ABTS+.

REAGENTS

1. Ethanol

2. ABTS solution (7mM ABTS with 2.45mM ammonium persulfate).

The solution was incubated at room temperature for 12-16 hours before use.

PROCEDURE

The six different extracts (100µl each) were added to ABTS solution (300µl) and the final volume of each was made up to 1ml with ethanol. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula,

$$\text{Inhibition (\%)} = \frac{\text{A (Control)} - \text{A (Sample)}}{\text{A (Control)}} \text{ X } 100$$