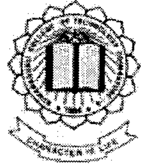
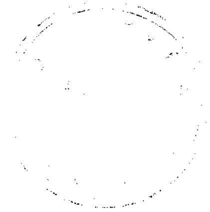


EVALUATION OF ANTIOXIDANT POTENTIAL IN SELECTED GREEN LEAFY VEGETABLES

P-2183

A PROJECT REPORT

Submitted by



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SUBHASREE. B.

In partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY, COIMBATORE

ANNA UNIVERSITY: CHENNAI 600 025

APRIL 2008

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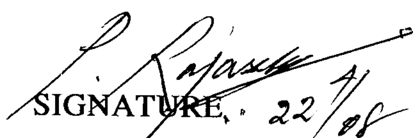
BONAFIDE CERTIFICATE

Certified that this project report titled **"Evaluation of antioxidant potential in selected green leafy vegetables"** is the bonafide work of "**Ms. R. Laxmi Keerthana, Ms. Lijina Susan Rajan and Ms. Subhasree. B.**" who carried out the project work under my supervision.



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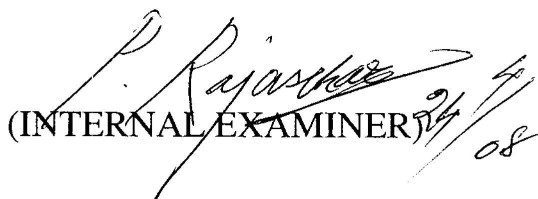

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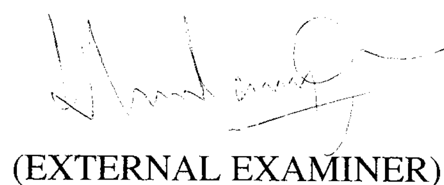

(EXTERNAL EXAMINER)

DEDICATED TO OUR
BELOVED PARENTS

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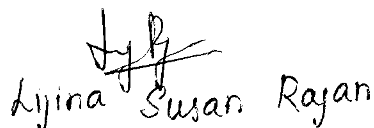
R. Laxmi Keerthana



Lijina Susan Rajan

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ABSTRACT

ABSTRACT

Antioxidants play a very important role in the defense against the damage caused by free radicals in the body. Free radicals are produced in the body either naturally or on the exposure of radiation, cigarette smoke, etc. and can be implicated in many diseases like cancer, atherosclerosis, arthritis, Parkinson's disease, Alzheimer's disease and can also be held responsible for aging and other age-related diseases. The body can produce antioxidants to aid in its defense against free radical damage. But in most cases dietary supplement of antioxidants is recommended. Plants are a very good source of antioxidants and other nutrients. Some green leafy vegetables remain under-exploited for scientific study. This project aims to screen four such green leafy vegetables for the presence of antioxidants. The plants chosen were *Centella asiatica, Trigonella foenum-graecum, Sauropus androgynus and Pisonia alba.* The plant constituents were extracted using methanol and acetone. These extracts were subjected to *in vitro* free radical scavenging assays like DPPH scavenging assay, ABTS cation radical scavenging assay, nitric oxide scavenging assay, total antioxidant capacity assay and hydroxyl radical scavenging assay. Lipid peroxidation assay using goat liver was also carried out. Assays to determine the phytochemicals responsible for antioxidant activity were also carried out. These include assays for the determination of phytochemical antioxidants like flavonoids, total phenols, total carotenoids, lycopene, Vitamins A and C. The results were analyzed statistically and all the plants were found to possess significant antioxidant potential.

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ABBERVIATIONS

µg	microgram
µl	microliter
µm	microgram
ABTS	2, 2,-azinobis (3-ethylbenzoline-6- sulfonic acid)
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
DMRT	Duncun's Multiple Range Test
DNA	Deoxyribose Nucleic Acid
DNPH	2,4-dinitro phenyl hydrazine
DPPH	1, 1-diphenyl -2-picryl hydrazyl
EDTA	Ethylene Diamine Tetra Acetic acid
FDA	Food and Drug Administration
$FeCl_3$	Ferric Chloride
g	gram
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulphuric acid
HOCl	Hypochlorous acid
LDL	Low Density Lipoprotein
LPO	Lipid peroxidation
MDA	Malondialdehyde
mg	milligram
min.	Minute
ml	milliliter
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium hydroxide
NBT	Nitro Blue Tetrazolium
NO	Nitric oxide
O_2	Oxygen
OFR	Oxygen-Free Radicals
$^{\cdot}OH$	Hydroxyl radical
PG	Propyl Gallate
PUFA	Polyunsaturated fatty acid

RBC	Red blood cells
ROM	Reactive Oxygen Metabolites
ROOH	Organic hydroperoxide
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBHQ	Butylated hydroxy toluene, Terbutyl hydroquinone
TCA	Trichloro Acetic acid
XDH	Xanthine dehydrogenase

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INTRODUCTION

1. INTRODUCTION

The human body is composed of many different types of cells. Cells are composed of many different types of molecules. Molecules consist of one or more atoms of one or more elements joined by chemical bonds. The atoms consist of a nucleus, protons, neutrons and electrons. The number of protons in the atom's nucleus determines the number of electrons surrounding the atom. Electrons are involved in chemical reactions and are the substances that bond atoms together to form molecules. Electrons surround or "orbit" an atom in one or more shells. The most important structural feature of an atom for determining its chemical behavior is the number of electrons in its outer shell. A substance that has a full outer shell tends not to enter in chemical reactions. Because atoms seek to reach a state of maximum stability, an atom will try to fill its outer shell by:

- Gaining or losing electrons to fill or empty its outer shell
- Sharing its electrons by bonding together with other atoms to complete its outer shell

Normally bonds don't split in a way that leaves a molecule with an unpaired electron. The formation of radicals may involve the breaking of covalent bonds in a process that requires a significant amount of energy, which is known as the bond dissociation energy.

Free radicals and other reactive oxygen species are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals. Free radicals are atomic or molecular species with unpaired electrons. These unpaired electrons are highly reactive. They are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability. The attacked molecules now become free radicals, thus initiating the chain reaction. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions

Reactive oxygen species (ROS) are an entire class of highly reactive molecules derived from the metabolism of oxygen. ROS, including superoxide radicals, hydroxyl radicals, and hydrogen peroxide, are often generated as byproducts of biological reactions or from exogenous factors. *In vivo*, some of these ROS play positive roles in cell physiology; however, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations

leading to cancer, degenerative disorders, and other diseases (Cerutti, 1991; Finkel and Holbrook 2000).

Pro-oxidants are chemicals that induce the production of free radicals or inhibit the antioxidant system (Puglia and Powell, 1984). The overdose of these chemicals can damage cells and tissues. For example, the overdose of the analgesic paracetamol can cause fatal damage to the liver (Jaeschke *et al.*, 2002), partly through the production of reactive oxygen species (James *et al*, 2003).

Oxygen, an essential element for life, can also be a reason for the destruction of tissue and/or impair its ability to function normally (Kehrer 1993). Oxidants or free radicals or oxygen-free radicals (OFR) or more generally called as reactive oxygen species (ROS) are formed due to various exogenous and endogenous factors. A free radical contains one or more unpaired electrons and is capable of independent existence. The formation of oxygen radicals could be the reason for the damaging effects of O_2. A class of enzymes called superoxide dismutases (SOD) is responsible for the catalytic removal of superoxide free radical, (Lee *et al.*, 2001). An average person has around 10,000–20,000 free radicals attacking each body cell every day. In some cases, ROS are produced specifically to serve essential biological functions, whereas in other cases, they are the byproducts of metabolic processes (Shigenaga *et al.*, 1994).

Generation of oxygen radicals, such as superoxide radical, hydroxyl radical and non-free radical species, such as H_2O_2 and singlet oxygen is associated with cellular and metabolic injury, and accelerating aging, cancer, cardiovascular diseases, neurodegenerative disorders, and inflammation (Stadtman., 1992; Sun., 1990). Previous epidemiological studies have consistently shown that consumption of fruits and vegetables has been associated with reduced risk of chronic diseases, such as cardiovascular diseases and cancers (Gerber *et al.*, 2002; Kris-Etherton *et al.*, 2002; Serafini *et al.*, 2002) and neurodegenerative disorders, including Parkinson's and Alzheimer's diseases (Di Matteo and Esposito, 2003) as well as inflammation and problems caused by cell and cutaneous aging. Fruits and vegetables contain different antioxidant compounds, such as Vitamin C, Vitamin E and carotenoids, whose activities have been established in recent years.

Antioxidants are defined as the substance that when present in low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that

substance (Halliwell and Gutteridge, 1989). For the *in vivo* situation, the concept of antioxidants includes antioxidant enzymes, iron binding and transport proteins and other compounds affecting signal transduction and gene expression (Gutteridge, 1989). In case of foods and beverages, antioxidants are related to the protection of specific oxidation substrates or the formation of specific oxidation. Synergism, antagonism, co-antioxidants and oxidation retarders are the other useful concepts related to antioxidants. Synergism can be defined as the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than if they were alone. Antagonism can be defined likewise by substituting "more" with "less", whereas co antioxidants may be defined by substituting "more" with "same". The compounds that reduce the rate of oxidation without showing a distinct lag phase of oxidation are retarders of oxidation. Antioxidant action is measured as a decrease in over-all rate of oxidation and as the length of the lag phase.

Antioxidants are divided into two classes: preventive antioxidants and chain breaking antioxidants. Preventive antioxidants inhibit oxidation by reducing the rate of chain initiation. Preventive antioxidants convert the hydroperoxides to molecular products that are not potential sources of free radicals (Burton *et al.*, 1985). Most biological preventive antioxidants are also peroxide decomposers. Antioxidants can also be manufactured synthetically. These belong to the class of synthetic antioxidants. The main disadvantage with these antioxidants is their side effect when taken *in vivo* (Chen *et al.*, 1992). Most of the natural antioxidants are found to have higher antioxidant activity when compared with that of the synthetic ones. Several arguments suggest that the antioxidant components of fruits and vegetables contribute in the defense effect. Epidemiological studies and intervention trials on prevention of diseases such as cancer and cardiovascular disease in people have shown the positive effects of taking antioxidant supplements (Enstrom *et al.*, 1992; Rimm *et al.*, 1993).

Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate (Hollman, 2001). Common antioxidants include Vitamin A, Vitamin C, Vitamin E, and certain compounds called carotenoids (like lutein and beta-carotene) (Hayek, 2000). These plant-based dietary antioxidants are believed to have an important role in the maintenance of human health because our endogenous antioxidants provide insufficient protection against the constant and unavoidable challenge of reactive oxygen species (Fridovich, 1998).

Green leafy vegetables occupy an important place among the food crops as these provide adequate amounts of many vitamins and minerals for humans. They are rich source of carotene, ascorbic acid, riboflavin, folic acid and minerals like calcium, iron and phosphorous. In nature, there are many underutilized greens of promising nutritive value, which can nourish the ever-increasing human population. Many of them are resilient, adaptive and tolerant to adverse climatic conditions. Although, they can be raised comparatively at lower management costs even on poor marginal lands, they have remained underutilized due to lack of awareness and popularization of technologies for utilization. Now-a-days, underutilized foods are gaining importance as a means to increase the per capita availability of foods (Sheela *et al.*, 2004).

The nutritional value of green leafy vegetables and the lack of awareness of the antioxidant potential of some unexploited green leafy varieties has provoked us to evaluate and compare the antioxidant potential of four under-exploited green leafy varieties- ***Trigonella foenum-graecum*, *Centella asiatica*, *Sauropus androgynous* and *Pisonia alba/grandis*.**

The main objective of our study are categorized into two phases –

1. To evaluate and compare the *in vitro* antioxidant activities of the four plant varieties by different *in vitro* free radical scavenging assays.
2. To determine the levels of phytochemical antioxidants and vitamins A and C in the plant species and to correlate their presence and the free radical scavenging activity of the plant extracts.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 Free radicals

Free radicals can be defined as chemical species possessing an unpaired electron, which is formed by homolytic cleavage of a covalent bond of a molecule by the loss of a single electron from a normal molecule or by the addition of a single electron to a normal molecule. Free radicals are a group of active molecules with unpaired electrons that react with biological systems, resulting in cell damage. Antioxidants are capable of taking up these free radicals. In doing so, they protect healthy cells from damage and abnormal growth. A number of antioxidant activity assays have been published, and its antioxidant activity is often expressed as Trolox Equivalent.

Most of the molecular oxygen consumed by aerobic cells during metabolism is reduced to water by using cytochrome oxidases in mitochondria. However, when oxygen is partially reduced, it becomes activated and reacts readily with a variety of biomolecules. This partial reduction occurs in one step, by addition of one, two and four electrons to oxygen, which leads to successive formation of reactive oxygen metabolites (ROMs). These ROMs include superoxide anion, hydroperoxy radical, peroxide ion, hydrogen peroxide and hydroxyl radical (Green and Hill, 1984).

The oxygen and hydrogen peroxide so formed may lead to the formation of the most reactive $OH^{\cdot}$. This hydroxyl radical oxidizes lipids giving rise to lipid peroxidation. Hydrogen peroxide is known to cause DNA breaks in intact cells. Malondialdehyde (MDA) is the major reactive aldehyde resulting from the peroxidation of biological membranes of polyunsaturated fatty acid (PUFA) (Vaca *et al.,* 1998). MDA is the secondary product of LPO and used as an indicator of tissue damage (Ohkawa *et al.,* 1979). MDA can modify Xanthine oxidoreductase activity through interaction with Xanthine oxide and for Xanthine dehydrogenase (XDH). Lipid hydroperoxides may directly induce DNA chain breaking, and lipid peroxyl and alkoxyl radicals may cause base oxidation in DNA (Park, 1992).

The reactive oxygen metabolites produced during cell metabolism are believed to be involved in various disease processes including cancer. Their main targets are membrane lipids and enzymatic proteins, where they cause cell injury and death (Nadkarni *et al.,* 1991). The damage induced by activated oxygen species leads to fluid loss, protein modification; ion leaks and eventually cell lysis (Paller et *al.,* 1991). However, these problems are

overruled by administering the antioxidant compounds.

2.1.1 Examples of free radicals

	DESCRIPTION
Superoxide radical ($^{\cdot}O_2^-$)	One-electron reduction state of O_2. Rather unreactive, but can release Fe^{2+} from iron-sulfur proteins and ferritin. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor of metal catalyzed $^{\cdot}OH$ formation.
Hydrogen peroxide (H_2O_2)	Two-electron reduction state of O_2. Formed by dismutation of $^{\cdot}O_2^-$ or by the direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
Hydroxyl radical ($^{\cdot}OH$)	Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive and will attack most cellular components.
Organic hydroperoxide (ROOH)	Formed by radical reactions with cellular components such as lipids and nucleobases.
Alkoxy ($RO^{\cdot}$) and Peroxy ($ROO^{\cdot}$) radicals	Oxygen centered organic radicals. Lipid forms precipitate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
Hypochlorous acid (HOCl)	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
Peroxynitrite ($OONO^-$)	Formed in a rapid reaction between $^{\cdot}O_2^-$ and $NO^{\cdot}$. Lipid soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo cleavage to form hydroxyl radical and nitrogen dioxide.

Table 2.1.1. Examples of free radicals.

2.1.2. Reactive Oxygen Species

Reactive oxygen species (ROS) include oxygen ions, free radicals and peroxides both organic and inorganic. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural byproduct of the normal metabolism and have important roles in cell signaling. The two of the most important oxygen centered free radicals are the superoxide and the hydroxyl radical.

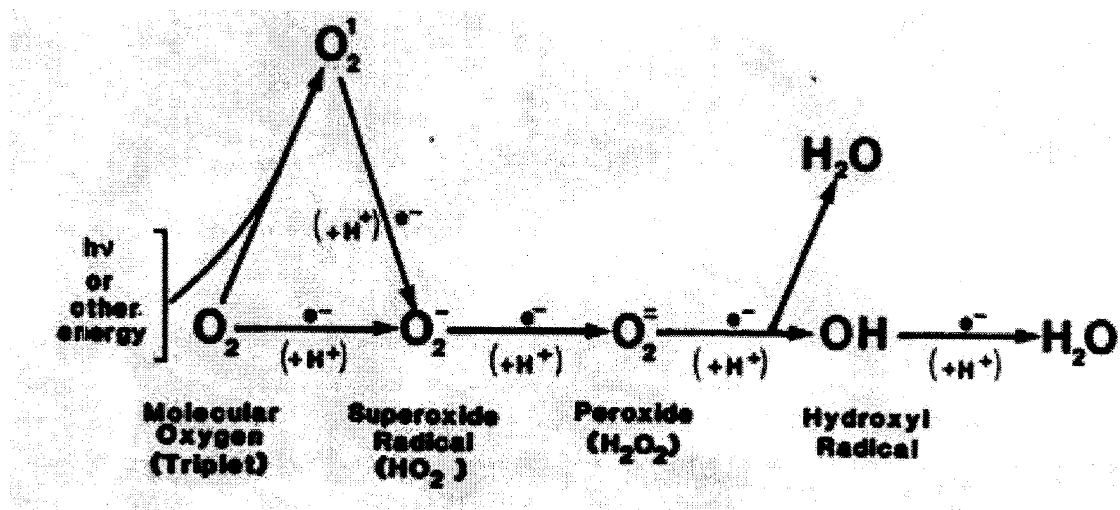


Figure 2.1.2. The active oxygen system

The molecular oxygen is reduced to water in four single-electron steps. Reduction of non-radical forms of oxygen is a "forbidden" process and thus usually involves spin-orbit coupling by a heavy metal or a halide or excitation to singlet state. An example is the Fenton's reaction, the reduction of peroxide to water and hydroxyl radical by ferrous ion. Hydroxyl radical is one of the most powerful oxidizing agents known. Simply put, reducing agents act as pro-oxidants by reducing non-radical forms of oxygen to radical forms, usually with heavy atom involvement. Similarly, they can act as antioxidants by reducing the radical forms of oxygen, by terminating the radical chain reactions. This dual property can be of great significance (Peter Proctor, 1989).

2.2. Formation of free radicals

Free radicals are produced due to various reasons. Free radicals are also produced inside organelles, such as the mitochondrion. Mitochondria convert energy for the cell into a usable form, adenosine triphosphate (ATP). The process, in which ATP is produced, called

oxidative phosphorylation, involves the transport of protons (hydrogen ions) across the inner mitochondrial membrane by means of the electron transport chain. In the electron transport chain, electrons are passed through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the last. The last destination of the electron along this chain is an oxygen molecule. Normally this oxygen is reduced to form water; however, in about 0.1-2% of the electrons passing through the chain, oxygen is prematurely and incompletely reduced to give the superoxide radical. Superoxide is not particularly reactive, but can inactivate specific enzymes or initiate lipid peroxidation. If too much damage is caused, a cell undergoes programmed cell death or apoptosis.

Thus the most important source of reactive oxygen under normal conditions in aerobic organisms is probably the leakage of activated oxygen from mitochondria during normal oxidative respiration.

Other enzymes capable of producing superoxide are xanthine oxidase, NADPH oxidases and cytochromes P450. Hydrogen peroxide is produced by a wide variety of enzymes including several oxidases.

Metals such as iron, copper, chromium, vanadium and cobalt are capable of redox cycling in which a single electron may be accepted or donated by the metal. This action catalyzes reactions that produce reactive oxygen species. The most important reactions are probably Fenton's reaction and Haber-Weiss reaction, in which hydroxyl radical is produced from reduced iron and hydrogen peroxide.

Certain organic compounds can also produce reactive oxygen species. One of the most important classes of these are the quinones. Quinones can redox cycle with their conjugate semiquinones and hydroquinones, in some cases catalyzing the production of superoxide from dioxygen or hydrogen peroxide from superoxide.

The immune system uses the lethal effects of oxidants by making the production of oxidizing species a central mechanism of killing pathogens; with activated phagocytes producing both ROS and reactive nitrogen species. These include superoxide, nitric oxide and their particularly reactive product, peroxynitrite (Nathan&Shiloh,2000). Although the use of these highly reactive compounds in the cytotoxic response of phagocytes causes damage to the host tissues, the non-specificity of the oxidants is an advantage since they will damage

almost every part of the target cell (Rice-Evans and Gopinath, 1995). This prevents a pathogen from escaping this part of the immune response by mutation of a single molecular target.

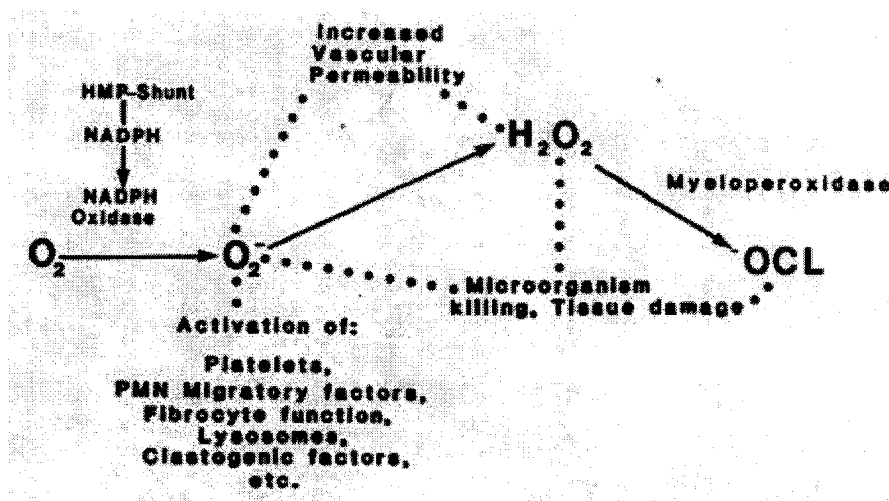


Figure 2.2. The role of active oxygen species in inflammation

The granulocytes and other phagocytes possess membrane NADPH oxidase, which takes reducing equivalents from the hexose monophosphate shunt and transfers these to molecular oxygen to produce superoxide and other active oxygen species. A further myeloperoxidase converts the peroxide produced in this system to microbicidal products, probably including hypochlorite. Production of activated products by this system probably plays a key role in cell-mediated immunity and microbicidal activity. There is evidence for similar systems in T-lymphocytes, platelets and mucus. (Peter Proctor, 1989).

Another major mechanism for the endogenous production of activated species is by autoxidation- catalyzing charge-transfer agents such as copper, iron, and manganese.

Free radicals are produced in the biological system due to the influence of several external factors as well. Free radicals are produced on exposure to environmental stress, exposure to cigarette smoke, radiation, anti-cancer drugs, etc.

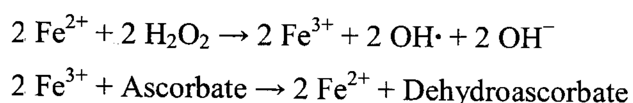
2.2.1. Role of Pro-oxidants in free radical formation

Pro-oxidants are chemicals that induce the production of free radicals or inhibit the antioxidant system (Puglia and Powell, 1984). The overdose of these chemicals can damage cells and tissues. For example, the overdose of the analgesic paracetamol can cause fatal damage

to the liver (Jaeschke *et al.*, 2002), partly through the production of reactive oxygen species (James *et al.*, 2003).

Some substances can act as either antioxidants or pro-oxidants, depending on the specific set of conditions (Herbert, 1996). Some of the conditions that are important include the concentration of the chemical and if oxygen or transition metals are present. While thermodynamically very favored, reduction of molecular oxygen or peroxide to superoxide or hydroxyl radical is fortunately spin forbidden. This greatly reduces the rate of these reactions, thus allowing aerobic organisms to live. As a result, the reduction of molecular oxygen typically involves the initial formation of singlet oxygen or the spin-orbit coupling through the reduction of a transition state metal such as manganese, copper, iron, etc. This reduced metal then transfers the single electron to molecular oxygen or peroxide.

Transition metals can act as pro-oxidants. Vitamins that are reducing agents can be pro-oxidants. Vitamin C has antioxidant activity when it reduces oxidizing substances such as hydrogen peroxide (Duarte and Lunec, 2005). However it can also reduce metal ions, which leads to the generation of free radicals through the Fenton reaction (Carr and Frei, 1999).



$$2\ Fe^{2+} + 2\ H_2O_2 \rightarrow 2\ Fe^{3+} + 2\ OH\cdot + 2\ OH^-$$
$$2\ Fe^{3+} + \text{Ascorbate} \rightarrow 2\ Fe^{2+} + \text{Dehydroascorbate}$$

The pro-oxidant properties of reductants can also have important clinical consequences. For example, in humans, uric acid accounts for roughly half the antioxidant ability of plasma. In fact, uric acid may have substituted for ascorbate in human evolution (Jaeschke *et al.*, 2002). Like ascorbate, uric acid can also mediate the production of reactive oxygen species and thus act as a pro-oxidant.

In humans, elevated homocysteine levels are associated with production of free radicals. Several important anticancer drugs both bind DNA and generate reactive oxygen species. These include adriamycin, anthracyclines, bleomycin, cisplatin, etc.

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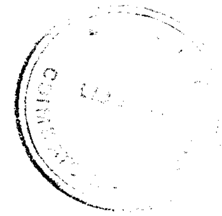

2.3. Biological significance of Free radicals

2.3.1. Positive roles of free radicals

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

Thus reactive oxygen species play an indispensable role in the normal functioning of biological system.

2.3.2. Role of oxidative stress

But free radicals can also cause extensive damage to the cells and tissues. The major mechanism of damage is due to the induction of oxidative stress. Oxidative stress is caused by the imbalance between the production of ROS and a biological system's inability to readily detoxify the reactive species or easily repair the resulting damage. All forms of life maintain a reducing environment within their cells. The enzymes through a constant input of metabolic energy preserve this reducing environment. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all the components of the cell, including proteins, lipids and DNA.

In chemical terms, oxidative stress is a large increase in the cellular reduction potential or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione (Schafer & [illegible] 2001). The effects of oxidative stress depend on the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stress can cause necrosis (Lennon *et al.*, 1991).

A particularly destructive aspect of oxidative stress is the production of ROS, which include free radicals and peroxides. Some of the less reactive of these species can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds including quinones into more aggressive radical species that can cause extensive cellular damage (Valko *et al.*, 2005). Under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli *et al.*, 1998; Lee and Shacter 1999).

2.3.3. Free radicals and Human Disease

Free radicals are highly reactive they participate in a number of reactions that can result in cell damage. Many forms of cancer are thought to be the result of reactions between free radicals and DNA, resulting in mutations that can adversely affect the cell cycle and potentially lead to malignancy. Some of the symptoms of aging such as atherosclerosis are also attributed to free radical induced oxidation of many chemicals making up the human body. In addition free radicals contribute to the alcohol-induced liver damage, perhaps more than alcohol itself. Radicals in cigarette smoke have been implicated in the inactivation of alpha-1- antitrypsin in the lungs. This process leads to the development of emphysema.

Free radicals may also be involved in Parkinson's disease, senile and drug-induced deafness, schizophrenia, and Alzheimer's disease. The classic free radical syndrome, the iron storage disease hemochromatosis, is typically associated with a constellation of free radical related symptoms including movement disorder, psychosis, skin pigmentary melanin abnormalities, deafness, arthritis and diabetes mellitus. (Ames , 1983; Ames , 1995).

Generally the harmful effects of reactive oxygen species on the cell are most often:

- Damage to DNA
- Oxidations of polyunsaturated fatty acids in lipids
- Oxidations of amino acids in proteins
- Oxidatively inactivate specific enzymes by oxidation of co-factors

Oxidative stress due to free radicals contributes to tissue injury following irradiation and hyperoxia. It is linked to cardiovascular disease, since oxidation of LDL in the vascular endothelium is a precursor to plaque formation. Oxidative stress also plays a very important

role in ischemic cascade due to oxygen reperfusion injury following hypoxia. This cascade includes roles both in stroke and heart attacks.

Transition metals also produce free radicals and are hence responsible for many diseases. For example, chronic manganism is a classic pro-oxidant disease (Puglia and Powell, 1984). Another disease associated with the chronic presence of a pro-oxidant transition-series metal is hemochromatosis associated with elevated iron levels. Similarly, Wilson's disease is associated with elevated tissue levels of copper. Such syndromes tend to be associated with a common symptomology. This typically includes the various combinations of psychosis, dyskinesia, pigmentary abnormalities, fibrosis, deafness, diabetes and arthritis (James *et al.*, 2003). Wilson's disease and striatal iron have similarly been linked to human Parkinsonism.

Fibrosis is another pro-oxidant free radical symptom. A variety of oxygen-generating agents can produce fibrotic changes. Examples include oxygen itself, nitrofurantoins, and bleomycin, which produce pulmonary fibrosis. Radical generating agents such as iron and copper are also associated with liver fibrosis and fibrotic changes in other organs such as the heart. The induction of vitreous scarring by interocular iron or copper is also well known, as is the association of homocystinuria with fibrotic lesions of the arteries.

Free radicals can also mediate disorders of the purine metabolism. Uric acid and other purines can mediate a Fenton-type reaction with peroxide. Purines also catalyze the autoxidation of epinephrine under certain conditions. The latter may involve a Fenton-type reaction with peroxide produced by adrenaline autoxidation. Uric acid can be involved in the neurological symptoms of Lesch-Nyhan Syndrome. It may also be involved in the gouty inflammatory disease. High uric acid levels are also involved in atherosclerosis, metabolic syndrome and in stroke.

Excess bilirubin, a product of RBC breakdown can lead to jaundice, which could eventually damage the central nervous system. Elevated levels of homocysteine are also associated with increased incidence of atherosclerosis and may play a role in Alzheimer's. Homocysteine is a powerful reducing agent and can induce oxidative stress. (Valko *et al.*, 2005; Halliwell, 2007; Hao and Marek, 2005; Schneider, 2005).

2.4. Defense mechanism and Antioxidants

Because free radicals are essential for life, the body has a number of defense mechanisms to minimize free radical induced damage and to repair the damage, which does occur. Antioxidants are substances with free radical chain reaction breaking properties. The antioxidants are this any substances that delay, inhibit or prevent the oxidation of molecules due to free radicals.

2.4.1. Role of apoptosis in defense against free radical damage

Apoptosis or programmed cell death is one of the major protective mechanisms to remove the damaged tissues and cells from the body and thus enables the body to repair or remove the damage caused by free radicals. A brief mechanism of apoptosis for the removal of cells containing damaged mitochondria is described here. Bcl-2 proteins are layered on the surface of mitochondria, detect damage and activate a class of proteins called Bax, which punch holes in the mitochondrial membrane causing cytochrome C to leak out. The Cytochrome C binds to Apaf-1, or apoptotic protease activating factor-1, which is free floating in the cell's cytoplasm. Using energy from the ATPs in the mitochondrion, the Apaf-1 and cytochrome C bind together to form apoptosomes. These apoptosomes bind to and activate caspase-9, another free floating protein. The caspase-9 then cleaves the proteins of the mitochondrial membrane, causing it to break down and start a chain reaction of protein denaturation and eventually phagocytosis of the cell.

2.4.2 Antioxidant as Scavengers

To deal with the free radicals or so called ROS the body is equipped with an effective defense system which includes various enzymes and high and low molecular weight antioxidants. Antioxidants neutralize free radicals by donating one of their own electrons, ending the electron stealing reaction. The antioxidants do not themselves become free radical by donating electrons because they are stable in other form. These act as scavenger and play the housekeeper's role by mopping up free radicals before they get a chance to create havoc in a body. Thus they may be well defined as the substances that are capable of quenching or stabilizing free radicals.

Antioxidants have also been suggested to have well defined role as preservatives. These have been defined by the US Food and Drug Administration (FDA) as substance used

to preserve food by retarding deterioration, rancidity or discoloration caused by oxidation. Lipid peroxidation is an important deteriorative reaction of foods during processing and storage. Toxic substance formed by lipid peroxidation may lead to adverse effects such as carcinogenesis, cell DNA mutagenesis and aging. Antioxidants therefore, according to their mode of action, have also been classified as the compounds that terminate the free radical chain in lipid peroxidation by donating electrons or hydrogen to fat containing a free radical and to the formation of a complex between the chain and a free radical. Antioxidants stop the reactions by contributing hydrogen from the phenolic hydroxyl hydrogen from the phenolic hydroxyl groups, themselves forming stable free radicals that do not initiate or propagate further oxidation of lipids (free radical terminators). Some of the important synthetic antioxidants of this class are butylated hydroxyanisole (BHA), butylated hydroxy toluene, terbutyl hydroquinone (TBHQ), propyl gallate (PG) and tocopherols.

Antioxidants also referred to as chelators which bind metal ions such as copper and iron that catalyze lipid peroxidation; oxygen scavengers are those compounds that react with oxygen in closed systems and secondary antioxidants which function by breading down the hydroperoxides.

In recent years, the use of some synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects. This concern has resulted in an increased interest in the investigation of the effectiveness of naturally occurring compounds with antioxidant properties. A number of plant and medicinal mushrooms constituents have been recognized to have positive effects when tested against the oxygen reactive compounds. Foods rich in antioxidants have been shown to play an essential role in the prevention of cardiovascular diseases; cancers, neurodegenerative diseases, the most well known of which are Parkinson's and Alzheimer's diseases, inflammation and problem caused by cell and cutaneous aging (Shahidi and Wanasundara, 1992).

2.4.3. Types of antioxidants

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

2.4.4. Enzymatic antioxidants

The enzymes responsible for the defense against the free radical damage include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. SOD is present in two places naturally in the cell. SOD that is present in the mitochondria contains manganese (MnSOD). This SOD is transcribed in the nucleus and has the mitochondrial targeting sequence, thereby localizing it to the mitochondrial matrix. SOD that is present in the cytoplasm of the cell contains copper and zinc (CuZn SOD). The genes that control SOD are located in the chromosomes 21, 6 and 4. When SOD comes into contact with superoxide, it reacts with it to form of hydrogen peroxide. The stoichiometry of this reaction is that for each 2 superoxide radicals encountered by this enzyme, 1 hydrogen peroxide is produced. This is really dangerous to the cell because it can readily transform hydrogen peroxide into a hydroxyl radical, one of the most destructive free radicals. Catalase, which is concentrated in the peroxisomes located next to the mitochiondria reacts with hydrogen peroxide to form water and oxygen. Glutathione peroxidase reduces hydrogen peroxide by transferring the energy to a very small sulfur containing protein called glutathione. The selenium contained in these enzymes acts as a reactive center, carrying reactive electrons from peroxide to the glutathione. Peroxiredoxins degrade hydrogen peroxide both within the mitochondria, cytosol and nucleus.

2.4.5. Natural non-enzymatic antioxidants

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

The Vitamins C and E are thought to protect the body against the destructive effects of free radicals. Antioxidants neutralize free radicals by donating an electron ending the electron "stealing" reactions. The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form. They act as scavengers helping to prevent cell and tissue damage that could lead to cellular damage and disease.

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

lipid-soluble antioxidants protect cell membranes from lipid peroxidation.(Sies , 1997). These compounds may be synthesized in the body or obtained from the diet (Vertuani *et al.*, 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquione mostly present within cells, while others such as uric acid are more evenly distributed throughout the body (Table 2.4.5).

The relative importance and interactions between these different antioxidants is a complex area, with the various metabolites and enzyme systems having synergistic and interdependent effects on one another (Chaudiere-Ferrari,1995; Sies, 1993). The action of one antioxidant may depend on the proper function of other members of the antioxidant system (Vertuani *et al.*, 2004). The amount of protection provided by any one antioxidant therefore depends on its concentration, its reactivity towards the particular reactive oxygen species being considered, and the status of the antioxidants with which it interacts (Vertuani *et al.*, 2004).

Some compounds contribute to antioxidant defense by chelating transition metals and preventing them from catalyzing the production of free radicals in the cell. Particularly important is the ability to sequester iron, which is the function of iron-binding proteins such as transferrin and ferritin (Imlay, 2000). Selenium and zinc are commonly referred to as antioxidant nutrients, but these chemical elements have no antioxidant action themselves and are instead required for the activity of some antioxidant enzymes, as is discussed below.

ANTIOXIDANT METABOLITE	SOLUBILITY	CONCENTRATION IN HUMAN SERUM (μM)	CONCENTRATION IN LIVER TISSUE (μmol/kg)
Ascorbic acid (Vitamin C)	Water	50-60	260 (Human)
Glutathione	Water	325-650	6400 (Human)
Lipoic acid	Water	0.1-0.7	4-5 (Rat)
Uric acid	Water	200-400	1600 (Human)
Carotenes	Lipid	β-carotene: 0.5-1.0 Vitamin A: 1-3	5 (Human, total carotenoids)
α-Tocopherol (Vitamin E)	Lipid	10-40	50 (Human)
Ubiquinol (Coenzyme Q)	Lipid	5	200 (Human)

Table 2.4.5. Non-Enzymatic Antioxidants

2.4.5.1. Ascorbic acid

Ascorbic acid or "vitamin C" is a monosaccharide antioxidant found in both animals and plants. As it cannot be synthesised in humans and must be obtained from the diet, it is a vitamin (Smirnoff, 2001). Most other animals are able to produce this compound in their bodies and do not require it in their diets (Linster and Van, 2007). In cells, it is maintained in its reduced form by reaction with glutathione, which can be catalysed by protein disulfide isomerase and glutaredoxins (Meister, 1994; Wells *et al.*, 1990). Ascorbic acid is a reducing agent and can reduce and thereby neutralize reactive oxygen species such as hydrogen peroxide (Padayatti *et al.*, 2003). In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the antioxidant enzyme ascorbate peroxidase, a function that is particularly important in stress resistance in plants (Shigeoka *et al.*, 2002). Vitamin C is the most abundant water-soluble antioxidant in the body. It acts primarily in the cellular fluid. It also helps return Vitamin E to its active form. Vitamin C is also needed for the production of serotonin.

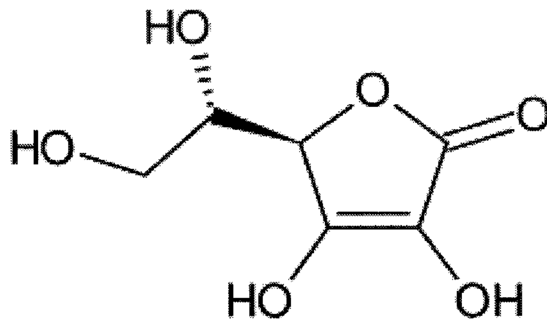

Figure 2.4.5.1. Ascorbic acid

2.4.5.2. Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life (Meister and Anderson, 1994). It is not required in the diet and is instead synthesized in cells from its constituent amino acids (Meister, 1988). 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 and enzyme systems as well as reacting directly with oxidants (Meister&Anderson, 1994). Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Meister&Anderson, 1994).

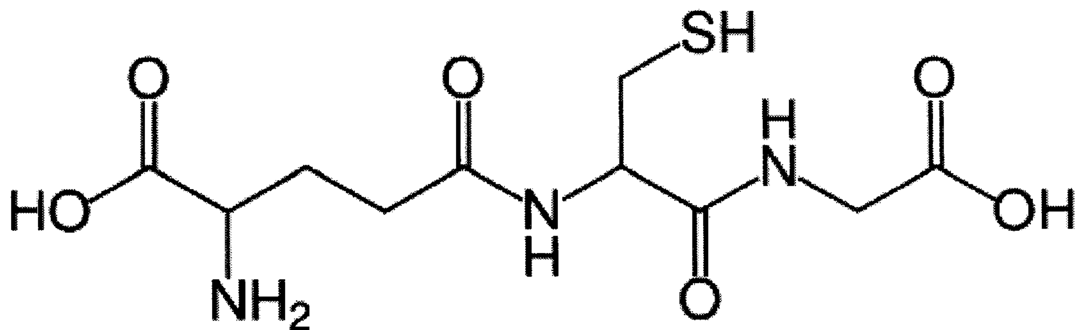

Figure 2.4.5.2. Glutathione

2.4.5.3. Melatonin

Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood-brain barrier (Reiter *et al.*, 1997). Unlike other antioxidants, melatonin does not undergo redox cycling, which is the ability of a molecule to undergo repeated reduction and oxidation. Redox cycling may allow other antioxidants (such as vitamin C) to act as pro-oxidants and promote free radical formation. Melatonin, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan *et al.*, 2000).

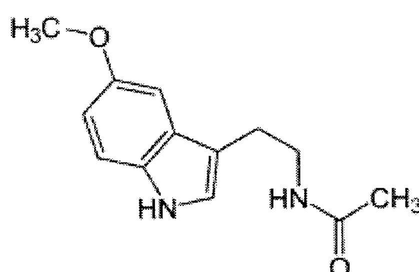

Figure 2.4.5.3. Melatonin

2.4.5.4. Tocopherols and tocotrienols (vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble antioxidant vitamins (Herrera&Barbas,2001). Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form (Brigelius&Traber,1999). The α-tocopherol form is the most important lipid-soluble antioxidant and protects cell membranes against oxidation by reacting with the lipid radicals produced in the lipid peroxidation chain reaction (Herrera& Barbas, 2001).This removes the free radical intermediates and prevents the propagation reaction from continuing. The oxidised α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by ascorbate, retinol or ubiquinol (Wang& Quinn,1999). The functions of the other forms of vitamin E are less well-studied, although γ-tocopherol is a nucleophile that may react with electrophilic mutagens, (Brigelius& Barbas,1999) and tocotrienols may have a specialised role in neuroprotection (Sen *et al.*, 2006).

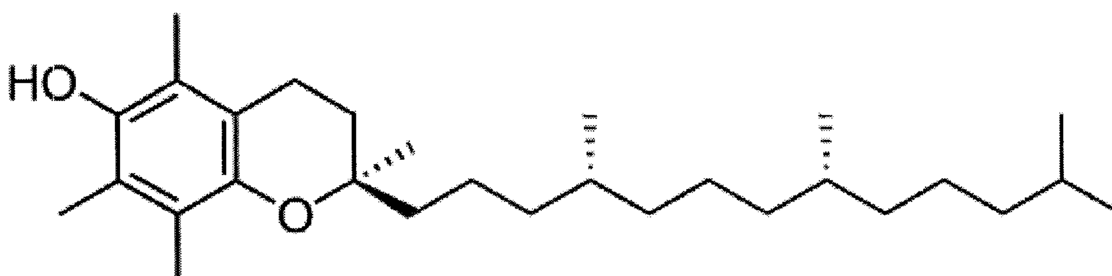

Figure 2.4.5.4. α-Tocopherol

Vitamin E is the most abundant fat-soluble antioxidant in the body. It is one of the most efficient chain breaking antioxidants available. It is also the primary defender against lipid peroxidation. It also optimizes immune system's T-cell activation. Vitamin E works synergistically with the mineral selenium to form other antioxidants. Vitamin E is the most important antioxidant for the health of the eyes.

2.4.5.5. Carotenoids

Carotenoids also play a major role. Lutein optimizes the immune system's B-cell activation and helps in vaccine recognition. Beta-carotene optimizes the types of cells present in the blood and increases the antibody count in the blood. It also helps in vaccine recognition

2.4.5.6. Ubiquinol

Coenzyme Q10 is very important to restore damaged skin and prevent further skin damage due to free radicals.

2.4.6 Antioxidant capacity assays

2.4.6.1 DPPH radical scavenging activity

The 1, 1-diphenyl -2-picryl hydroxyl (DPPH) radical was widely used as the model system to investigate the scavenging activities of several natural compounds such as phenolic and anthocyanins or crude mixtures such as the ethanol extract of plants. DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. When DPPH is placed in an assay system containing free radical scavengers such as flavonoids, the color vanishes. The color changes from purple to yellow after reduction, which can be

quantified by its decrease of absorbance at wavelength 517nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition.

2.4.6.2 Trolox Equivalent Antioxidant capacity Assay

In this improved version, $ABTS^{\cdot}$, the oxidant is generated by persulfate oxidation of 2, 2-azinobis (3-ethylbenzoline-6- sulfonic acid) - ($ABTS^{2-}$). Specifically, 7 mM of ABTS ammonium was dissolved in water and treated with 2.45 mM of ammonium persulphate and the mixture was then allowed to stand at room temperature for 12-16 h to give a dark blue solution. This solution was diluted with ethanol or buffer (pH 7.4) until the absorbance reached 0.7 at 745 nm. One milliliter of the resulting solution was mixed with 10 μl of sample. The absorbance was read at 30°C for every minute for 6 minutes after mixing at 30°C. The difference of the absorbance reading is plotted versus the antioxidant concentrations to give a straight line.

2.4.6.3 Hydroxyl radical and Nitric oxide scavenging assay

The hydroxyl radical scavenging activity was measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton's reaction mixture. Hydroxyl radical scavenging activity was measured by studying the competition between deoxy ribose and the extract for hydroxyl radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system. The hydroxyl radicals attack deoxyribose, which eventually results in TBARS formation.

Nitric oxide is a potent phototropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity.

2.4.6.4 Lipid peroxidation inhibition assay

Initiation of lipid peroxidation by ferrous sulphate takes place either through hydroxy radical by Fenton's reaction. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the hydroxy radical or the superoxide radicals or by changing the Fe^{3+}/Fe^{2+} or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself.

Iron catalyzes the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. The hydroxyl radical is highly reactive and can damage biological

molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane. Lipid hydroperoxides can be decomposed to produce apoxy and peroxy radical they eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damages, generation of cancer and aging related disease (Okhawa *et al.,* 1979). Thus, decrease in MDA level with increase in the concentration of the extracts indicates the role of the extracts as an antioxidant.

2.4.6.5 Ferric Reducing Antioxidant Potential Assay

The reducing capacity was investigated by measuring Fe^{3+}-Fe^{2+} conversion. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activities of putative antioxidants have been attributed to various mechanisms such as prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued proton abstraction and radical scavenging.

2.4.7. Antioxidants and peroxidation

Antioxidants are seemingly magical nutrients that can repair cell damage that happens in all our bodies over time. Although antioxidants are produced naturally, our body needs a supply of antioxidants from dietary sources.

The process of peroxidation due to free radicals continues in a chain reaction and cells are damaged. Peroxidation is important because it helps the body destroy cells that have outlived their usefulness and kills germs and parasites. However, peroxidation, when left unchecked, also destroys or damages healthy cells.

Antioxidants help prevent widespread cellular destruction by donating components to stabilize free radicals. More important, antioxidants return to the surface of the cell to stabilize, rather than damage, other cellular components.

When there are not enough antioxidants to hold peroxidation in check, free radicals begin damaging healthy cells, which can lead to problems. For example, free radical damage to immune cells can lead to an increased risk of infections.

2.5. Antioxidants from dietary sources

2.5.1. Importance of Antioxidant rich dietary sources

The body naturally creates some antioxidants, but it relies heavily on a proper diet to get the rest of its natural antioxidants. Spinach as well as blueberries, apples and several other plant foods are rich in antioxidants. Flaxseed contains lignans, which may have antioxidant effects. Flaxseeds are said to fight cancer due to the fact that the omega-3 fatty acids found within it act as antioxidants in the body. The antioxidants in dark chocolate have shown to make a difference in cardiovascular health. Research shows that anti aging creams that do not have antioxidants do not work as effectively as those that contain it. Free radicals are the forerunners of devastating diseases like cancer, osteoporosis and heart disease: antioxidants can help get rid of these free radicals from our body. Garlic also serves as an antioxidant and some studies even indicate that it can help to protect against cancer. Thus fruits and vegetables contain essential antioxidants for the body. The effect of *Aloe vera* is interesting because it makes Vitamin C, Vitamin E and other antioxidants work well.

2.5.2. Natural Sources of antioxidants

Many people these days take antioxidants to counter the detrimental effect of free radicals. In fact, artichokes are among the best sources of antioxidants. Berries in general are among the foods, which are the most antioxidant-dense. Caretonoids are antioxidants that are in carrots, apricots and all major citrus fruits including oranges. Beta-carotene is one popular natural antioxidant and is present in various natural food products. Strawberries are high in antioxidants. Apart from natural sources supplements can go a long way in increasing the antioxidant content in the body.

The other food sources rich in antioxidants and their antioxidant potential have been tabulated in Table 2.5.2. (Cao *et al.*, 1996)

Total Antioxidant Capacity of Tea and Common Vegetables[a]

Item	$ORAC_{ROO^{\cdot}}$[b] WM basis	$ORAC_{OH^{\cdot}}$[b] WM basis	$ORAC_{Cu}$[c] WM basis	Antioxidant score[d]
kale	17.7	6.2	0.2	24.1
garlic	19.4	1.1	2.7	23.2
spinach	12.6	2.8	1.6	17.0
Brussels sprouts	9.8	5.4	0.6	15.8
alfalfa sprouts	9.3	4.6	0.6	14.5
broccoli flowers	8.9	2.4	1.6	12.9
beets	8.4	3.1	0.2	11.7
red bell pepper	7.1	0.6	0.4	8.1
corn	4.0	2.2	1.0	7.2
onion	4.5	0.5	0.6	5.6
eggplant	3.9	1.1	0.1	5.1
cauliflower	3.8	1.1	0.2	5.1
cabbage	3.0	1.5	0.3	4.8
potato	3.1	1.0	0.5	4.6
sweet potato	3.0	1.0	0.3	4.3
leaf lettuce	2.6	1.4	0.1	4.1
string bean	2.0	1.7	0.2	3.9
carrot	2.1	0.8	0.5	3.4
yellow squash	1.5	1.1	0.2	2.8
iceberg lettuce	1.2	0.7	0.4	2.3
celery	0.6	0.3	0.2	1.1
cucumber	0.5	0.3	0.3	1.1

[a] Data expressed as means of three samples purchased and analyzed independently

[b] Data expressed as µmol of Trolox equiv/g of wet matter (WM) or dry matter (DM).

[C] Data expressed as $x10^3$ units/g of wet matter (WM) or dry matter (DM).

[d] Antioxidant score = $ORAC_{ROO^{\cdot}}$ + $ORAC_{OH^{\cdot}}$ + $ORAC_{Cu}$ (WM basis).

Table 2.5.2. Antioxidant activity of various plants

2.6. Evaluation of Antioxidant potential in selected green leafy vegetables

From the above table it is evident that research on antioxidant activity has been carried out in many of the commonly available edible plants and almost all these fruits and vegetables have shown significant antioxidant activity. However, some of the green leafy vegetables still remain under exploited. These green leafy vegetables have a great nutritive value and have been used in traditional medicine for the treatment or prevention of free radical induced diseases like arthritis, diabetes, cancer, etc. This project therefore aims to bring such plants into limelight and hence the objective of this project is to screen four commonly available edible plant varieties for the presence of antioxidant activity.

The four green leafy vegetables chosen are:

1) *Centella asiatica*
2) *Trigonella foenum-graecum*
3) *Pisonia grandis*
4) *Sauropus androgynus*

The features of the selected green leafy vegetables are explained in detail.

2.6.1. *Trigonella foenum-graecum*

Fenugreek (*Trigonella foenum-graecum*) or Menthya (Kannada) or Venthayam (Tamil) or Menthulu (Telugu) or Methi (Bangla, marathi) belongs to the family Fabaceae. Fenugreek is used both as a herd (the leaves) and as a spice (the seed).

2.6.1.1.Classification

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Genus : Trigonella
Species : *T.foenum - graecum*

2.6.1.2. Distribution

Fenugreek is cultivated worldwide as a semi-arid crop. Fenugreek seeds have been recovered from Tell Halal, Iraq (radiocarbon dating to 4000 BC). Fenugreek is native to the Mediterranean region, the Ukraine, India and China and it is widely cultivated in these regions. The fenugreek seed that is used pharmacologically comes exclusively from cultivated plants originating in India, Morocco, China and Turkey. The herd has been used for centuries as a cooking spice in Europe and remains a popular ingredient in prickles, curry powders and spice mixtures of India and Asia. The maple aroma and flavor of fenugreek has led to its use in imitation of maple syrup. In Egypt, fenugreek seeds are prepared, as tea by boiling and then sweetened. In other Middle East regions, fenugreek is used in a variety of sweet confectionaries. The seed is used in Ethiopia as a natural herbal medicine in the treatment of diabetes.

2.6.1.3. Morphology

A member of the bean family, fenugreek grows as an erect annual with long, slender stems reaching 30 to 60 cm in height. The plant bears grey-green, tripartite, toothed leaves. White or pale yellow flowers appear in summer and develop into long, slender, sword-shaped seedpods with a curved, beak-like tip. Each pod contains about 10 to 20 small, yellowish-brown, angular seeds.

2.6.1.4. Chemical components

The leaves contain at least seven saponins, known as graecunins. These compounds are glycosides of diosgenin. Seeds contain 0.1% to 0.9% diosgenin and are extracted in a commercial basis (Varchney & Jani, 1979). Plant tissue cultures from seeds grown under optimal conditions have been found to produce as much as 2% diosgenin with smaller amounts of gitongenin and trigogenin. The seeds also contain the saponin, fenugrin B (Sauvaire & Baccou, 1978; Elujoba & Hardman, 1987). Several coumarin compounds have been identified in fenugreek seeds (Gangrade *et al.*, 1979) as well as number of alkaloids (e.g., trigonelline, gentianine, carpaine). A large proportion of the trigonelline is degraded to nicotinic acid and related pyridines during roasting. These degradation products are, in part, responsible for the flavor of the seed. The seeds also yield as much as 8% of fixed, foul-smelling oil. The C-glycoside flavones vitexin, vitexin glycoside and the arabinoside isoorientin have been isolated from the plant. Three minor steroidal sapogenins also have

been found in the seeds. These include smilagenin, sarsapogenin, and yuccagenin (Adanska and Lutomski, 1971). Some medical constituents fenugreek contains are complex carbohydrates and fiber (25-45%) and protein (25-30% rich in lysine and tryptophan but in low in sulfur-containing amino acids).

2.6.1.5. Traditional uses and Medicinal properties

The leaves of the plant have been used traditionally for the treatment of several diseases. The development of many of these diseases involves free radicals and hence the antioxidants in these plants might play a crucial role in the treatment of these diseases.

For Hyperglycemia:

Fenugreek affects the gastrointestinal transit time, slowing glucose absorption (Jelilin ., 2000; Sauvaire , 1998).

Non-insulin dependent diabetes

Ingestion of the extracted seeds improves plasma glucose and insulin response.

Insulin dependent diabetes

Ingestion of the seed powder reduces plasma glucose, glycosuria, and the daily insulin requirement.

Kidney stones

Fenugreek may decrease calcium oxalate deposition in the kidneys, reducing the risk of kidney stone formation.

Lactation

Fenugreek has documented uterine stimulant effects. Thus should be avoided during pregnancy. It has been used to stimulate milk production in nursing mothers. Excretion into milk has not been studied.

Cholesterol - lowering effects

Fecal bile acid and cholesterol excretion are increased by fenugreek administration. This may be secondary to a reaction between bile acids and fenugreek-derived saponins causing the formation of micelles too large for the digestive tract to absorb. Another hypothesis attributes the cholesterol-lowering activities to the fiber-rich gum portion of the seed that reduces the rate of hepatic synthesis of cholesterol. It is likely that both mechanisms contribute to the overall effect (Madar & Stark, 2002).

Figure 2.6.1. *Trigonella foenum-graecum*

2.6.2. *Centella asiatica*

Centella asiatica has many common names such as Gotu Kola, Asiatic pennywort (English), Vallarai (Tamil), Mandookaparni (Sanskrit), Tholkari (Bengali), Luei Gong Gen, Antanan, Pegagan, Pegaga, Kula kud and Brahmi (Hindi)(although this last name is shared with *Bacopa monnieri* and other herbs). The botanical synonyms include *Hydrocotyle asiatica L.* and *Trisanthus Cochinchinensis* (Lour.).

2.6.2.1.Classification

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Apiales
Family	: Apiaceae
Genus	: Centella
Species	: *C.asiatica*

2.6.2.2. Distribution

Centella grows along ditches and in low wet areas. In India and Southeast Asia, the plant frequently suffers from high levels of bacterial contamination, possibly from having been harvested from sewage ditches. It is native to Northern Australia, Indonesia, Iran, Malaysia, Melanesia, New Guinea and other parts of Asia(Dutta and Basu, 1967).

2.6.2.3. Morphology

The plant consists of vertical rootstalk. Stem is prostrate and slender, creeping with long stolens and is nearly glabrous or hairy on younger parts. Leaves are cordate or hastate or orbicular or reniform or palmately lobed consisting of long petiole and small stipules. Crowded leaves can be seen at nodes, and they have long stalks and sheathing leaf bases. Leaf blades are dentate, crenate with thick radiate veins and dark green in color. Flowers are small, sessile and dark pink in color. They arise as simple umbels of 3-6 flowers at the ends of slender peduncles arising from the axils of leaves and much shorter thin petioles supported below by an involucre of 2 boat - shaped membranous persistent bracts. The fruits when

bruised have an aromatic pungent odour, nauseous and bitter taste. Seeds are solitary in each mericarp, and have a pendulous embryo, which is laterally compressed.

Figure 2.6.2. ***Centella asiatica***

2.6.2.4. Chemical Components

The plants have been found to possess the following chemical constituents some of which have an important role in the antioxidant activity of the plant.

- Vallarine
- Asiaticoside
- Hydrocotylin
- Pectic acids
- Steroids
- Hersaponin
- Bacogenin
- Monnierin
- Triterpene
- Tannin

The main two active constituents in Centella are Bacoside A and B. Bacoside A assists in release of nitric oxide that allows the relaxation of the aorta and veins, to allow the blood to flow more freely through the body. Bacoside B is a protein attributed to nourishing the brain cells.

2.6.2.5. Traditional uses and Medicinal properties

Centella asiatica is an all-healing drug of mighty potency and capable of rejuvenating the system thus enabling to live for ages. *Centella asiatica* can be taken as a panacea drug, and it is also said to have remarkable power in increasing intelligence.

For Syphilis

When internally given, the leaves have a effect on syphilitic skin diseases and in secondary and constitutional syphilis especially in those cases where subjacent cellular tissues are principally affected.

As a memory tonic

When the leaves are taken as a tonic, it is said to increase memory power. It is also taken as tablets for increasing intelligence quotient.

For Dysentery

The root of *Centella asiatica* when given with milk and liquorices arrests dysentery.

For all types of fever

The leaves of *Centella asiatica, Ocimum sanctum* and pepper are grinded and are made into tablets and are taken twice a day. This is thought to help in bringing down fever.

For Children's abdominal disorders

The ghee of *Centella asiatica* and the decoction made out of fenugreek and the leaves are reported to have good effect in the treatment of children's abdominal disorders.

Effect on enteric pathogens

The leaves of *Centella asiatica* are reported to consist of triterpenoid glycosides, free acids, volatile oils and flavonoids. It has been found that the activity of the plant extract against the microorganisms is mainly concentrated on the triterpene asiaticoside. The triterpenes weaken the membranous tissues, which results in dissolution of the cell walls of the microorganisms so that they can be more efficiently eliminated.

Role in preventing age-related depletion of antioxidant status

The leaves when supplemented to the regular diet significantly improve the antioxidant status of aging organisms.

For wound-healing

Asiaticoside derived from the leaves is known to possess good wound-healing activity. This can be possibly attributed to increased angiogenesis and collagen formation .

2.6.3. *Sauropus androgynous*

Sauropus androgynous, also known as katuk, star gooseberry, or sweet leaf, is a shrub grown in some tropical regions as a leaf vegetable.

The genus *Sauropus*, of the Phyllanthaceae family, comprises about 40 species of herbs, shrubs or subshrubs, sometimes with woody bases. These plants can be monoecious or dioecious.

2.6.3.1. Classification

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnliopsida
Order	: Malpighiales
Family	: Phyllanthaceae
Genus	: Sauropus
Species	: *S. androgynous*

2.6.3.2. Distribution

Generally *Sauropus* species are distributed in Southeast Asia, Malesia and Australia. *Sauropus androgynus* is one of the most popular leaf vegetables in South Asia and Southeast Asia.

2.6.3.3. Morphology

Sauropus species have alternate, entire leaves with short petioles and small stipules. Flowers appear at axiles and mainly form clusters. There are 6 perianth segments divided in 2 whorls, with female flowers often having bigger perianths. At male flowers, the perianth is tube-like, with 3 stamen. The fruit is berry-like, ovoid or globose, and fleshy.

Sauropus androgynus's multiple upright stems can reach 2.5 m high and bear dark green oval leaves 5–6 cm long. It is notable for high yields and palatability. The shoot tips have been sold as tropical asparagus.

2.6.3.4. Chemical components

The percentage of various components in *Sauropus androgynus* is as follows:

Protein 49%

fiber 14-18%

2.6.3.5. Traditional uses and Medicinal properties

This plant is known to be a good source of Vitamins A, B and C. In medical terms, it is also called a blood building element and a cell rejuvenator. It is also helps in detoxifying the blood.

As this species is not highly exploited, its medical uses are yet to be brought in to light.

Figure 2.6.3. Sauropus androgynus

2.6.4. *Pisonia alba/grandis*

Pisonia grandis is a tree species. One of its common name in Malayalam is Sandi. Some species, for example *Pisonia brunoniana* of New Zealand, Norfolk Island, Lord Howe Island and Hawaii, *Pisonia umbellifera*, and possibly *Pisonia grandis* widespread in the tropical Indo-Pacific region, are referred to as Birdcatcher or Catchbird trees because their sticky seeds reportedly trap small birds. Such sticky seeds are postulated to be an evolutionary feature of some island species for adherence of their seeds to birds; thereby facilitating distribution of seeds between islands.

2.6.4.1. Classification

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Caryophyllales
Family	: Nyctaginaceae
Genus	: Pisonia
Species	: *P.grandis*

2.6.4.2. Distribution

Its distribution is from the western Indian Ocean to eastern Polynesia. It is found mostly on coral islands like the Hawaiian Islands, and is indigenous to Lisianski, Maui. Large shrubs or trees up to 10-30 m tall are with grayish cream or dull pale brown, thick, smooth trunks. The wood is very soft and brittle. The distal internodes are usually short, but not condensed to an enlargement.

2.6.4.3. Morphology

Leaves are deciduous in very dry seasons. The arrangement is opposite to sub opposite; sometimes also alternate on same tree. Leaves are usually falcate, thin (or fleshy when exposed to salt spray), and broadly elliptic to oblong or ovate, 9-30 cm long, 6-18 cm wide. The lateral veins are distinct, with 6-10 pairs, arching slightly apically, inconspicuously anatomizing close to margins. The tertiary and higher order venation is very fine. The upper surface is glabrous, lower surface is also glabrous or appressed puberulent along the midrib

and has short pubescent domatia in lateral vein axils. The apex is acute to acuminate, base acute to rounded, truncate, or rarely subcordate, petioles 2-3 or more cm long.

Flowers are unisexual (the plants monoecious or dioecious), pedicellate, irregularly crowded at tips of branches in the form of terminal, corymbiform cymes. These are distally somewhat rounded, 5-8 cm long, enlarging and become more open in the fruit. The peduncles are less than 1/2 the length of cymes, and have alternate or irregular branching. Anthocarps are cylindrical to clavate, 1 cm long. The apical portion is fertile, does not gradually taper to a rostrum. The ribs are prominently glandular spinulose, and very sticky.

2.6.4.4. Traditional uses and Medicinal properties

The leaves pf the plant are used in several forms of ethnic treatment of diseases. The leaves are used to treat rheumatism or arthritis. Leaves are carminative (expels flatulence). Leaves coated with eau de cologne are used to rub against elephantoid swellings.

Figure 2.6.4. ***Pisonia grandis***

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Chemicals used

1,1-Diphenyl-2-Picryl hydrazyl (DPPH), methanol, Ammonium persulfate, 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), acetone, sodium nitroprusside, sulphanilamide, napthyl ethylene diamine dihydrochloride, o-phosphoric acid, deoxyribose, Ethylene diamine tetraacteic acid (EDTA), ferric chloride, trichloro acetic acid (TCA), thiobarbituric acid (TBA), ferrous sulphate, acetic acid, sulfuric acid, ammonium molybdate, disodium hydrogen phosphate, potassium ferricyanide, hydrogen peroxide, sodium nitrate, aluminium chloride, sodium hydroxide, petroleum ether, potassium hydroxide, calcium carbonate, oxalic acid, ascorbic acid, 2,4-dinitrophenyl hydrazine (DNPH), etc.

All reagents used were of the analytical grade.

3.2. Plant materials

The leaves of the plants *Centella asiatica, Trigonella foenum-graecum, Sauropus androgyus and Pisonia alba* were collected from various places in Coimbatore (Tamil Nadu, India) during the months of February-March 2008. The plant specimens were authenticated at the Botanical Survey of India, Coimbatore.

3.3. Methods

3.3.1. Phase I

The following assays were used to estimate the free radical scavenging activity of the various plant extracts by *in vitro* antioxidant capacity assays.

3.3.1.1. Preparation of the plant extracts

The leaves were shade-dried for around one week. The dried leaves were then crushed to make a coarse powder. The dried powder was weighed and solvent extraction at a 10% concentration was carried out. The solvents used were Methanol and Acetone. Exhaustive extraction was carried out for about 36 hours in a shaker at 37°C with gentle shaking. The extracts of the plants were evaporated at room temperature. The residues obtained were re-evaporated to remove impurities. The residues obtained after this step were

used for carrying out various radical scavenging assays. The excess residue of each plant extract was stored in a dessicator for further use.

3.3.1.2. Estimation of DPPH radical scavenging activity

The DPPH radical scavenging activity was measured spectrophotometrically as summarized in Appendix 1.

3.3.1.3. Estimation of ABTS cation radical scavenging activity

The ABTS cation radical scavenging activity of the plant extracts was assayed by a modified spectrophotometric method as described in Appendix 2.

3.3.1.4. Estimation of Nitric oxide scavenging activity

The nitric oxide scavenging activity of the various plant extracts was measured spectrophotometrically as elaborated in Appendix 3.

3.3.1.5. Estimation of Hydroxyl radical scavenging activity

A spectrophotometric method as described in Appendix 4 was used to assay the hydroxyl radical scavenging activity of the various plant extracts.

3.3.1.6. Estimation of Lipid peroxidation inhibition activity

The extent of inhibition of lipid peroxidation of the various plant extracts was determined spectrophotometrically by the method described in Appendix 5.

3.3.1.7. Estimation of Total antioxidant capacity

The total antioxidant capacity of the plant extracts was determined by the phosphomolybdenum method as described in Appendix 6.

3.3.1.8. Determination of Ferric Reducing Antioxidant Potential

The reducing power of the various plant extracts was measured using a spectrophotometric method as described in Appendix 7.

3.3.2. Phase II

The following assays were carried out to determine the various non-enzymatic antioxidants present in the plants.

3.3.2.1. Estimation of flavonoids

The flavonoids present in the various plants were estimated using the procedure described in Appendix 8.

3.3.2.2. Estimation of total phenols

The method described in Appendix 9 was used to determine the total phenolic content of the various selected green leafy vegetables.

3.3.2.3. Estimation of levels of β-carotene

Vitamin A present in each of the selected plants was determined using the spectrophotometric method described in Appendix 10.

3.3.2.4. Estimation of levels of Vitamin C (Ascorbic acid)

The levels of ascorbic acid were determined using the procedure described in Appendix 11.

3.3.2.5. Estimation of levels of total carotenoids and lycopene

The pigments present in the plants were determined using the protocol as given in Appendix 12.

3.4. Statistical analysis

3.4.1. Statistical analysis of free radical scavenging assays

The statistical analysis of the data obtained was carried out using two-way ANOVA from which the significance of interaction between the various plant species and the concentration of the plant extracts used in carrying out the assays was obtained. The mean differences between the four species within each concentration were analyzed using DMRT analysis. The coefficient of variance, which indicates the extent of variation in the

observations made during the assays, was also determined. The standard error of each assay was determined, which estimates of the precision of the experiment.

Statistical analysis of determination of non-enzymatic antioxidants

The statistical analysis of the data obtained was carried out to determine the plant species that contained the maximum quantity of the various non-enzymatic antioxidants. The DMRT analysis was carried out. One-way ANOVA was also carried out. Correlation and regression analysis was also carried out.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Oxidative stress has been implicated in the pathology of many diseases, inflammatory conditions, cancer and aging (Marx, 1987). And the best way to eliminate free radicals is with the help of antioxidants like Vitamins A, E and C. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation, etc. and thus prevent the onset of deadly diseases. (Youdim & Joseph, 2001).

Literature suggests that antioxidant activity is high on herbal plants and vegetables (Asmah Rahmat *et al.*, 2003). Fruits and vegetables are rich in many nutrients. Green leafy vegetables are highly rich in nutrients like Vitamin A and Vitamin C, which are potent non-enzymatic antioxidants. Studies carried out by researchers have however shown that low consumption of vegetables is associated with an increased risk of cancer (Tavani & Vecchia, 1995). This clearly indicates that apart from the antioxidants synthesized naturally, the body requires a supplement of dietary antioxidants. This can be obtained only by the consumption of an antioxidant rich diet.

Some of the green leafy vegetables are under exploited, but are potent sources of natural antioxidants like vitamins, carotenoids, flavonoids, phenols, etc. The selected varieties include *Centella asiatica, Trigonella foenum-graecum, Pisonia grandis* and *Sauropus androgynus*. Each plant species has a potent antioxidant activity and its beneficial effects have been proved by experiments both *in vivo* and *in vitro*.

Some of these selected green leafy vegetables have been traditionally used in medicine to treat several free radical mediated diseases. *Centella asiatica* has been one of the most important herbs used since time immemorial in traditional medications, as in Ayurveda. For example *Centella asiatica* has been used in wound healing. The plant has been used in ethnic formulations for this purpose. *In vivo* studies have been carried out in *Centella asiatica* to find out its potential capability to reduce age-related changes in antioxidant defense system, lipid peroxidation and protein carbonyl contents in the rat brain. Supplementation with *Centella asiatica* proved to be effective in lowering the regional lipid peroxidation and protein cabonyl levels in the brain and in increasing the antioxidant status of aged rats. It has been traditionally used in the treatment of leprosy and tuberculosis. It has also been used to rejuvenate the system. It has a potential to increase the memory capacity of

an individual and thereby enhance intelligence. Thus the plant has quite a remarkable medicinal value and most of this is attributed to its antioxidant capacity.

Trigonella foenum-graecum is another plant that has a tremendous potential of treating many free radical related diseases. The plant has been well studied in the laboratories around the world, with the main objectives of the study being to understand the chemical constituents of the plants that are responsible for its healing effects. The plant has been widely used to treat diabetes mellitus. The novel amino acid 4-hydroxy isoleucine is the main component responsible for the effect. It appears to directly stimulate the secretion of insulin by the pancreas in a glucose-dependent manner (Sauvaire., 1998; Broca , 1999; Broca, 2000). Fenugreek has been proved to stimulate appetite and act as a poultice for local inflammation (Jelilin ., 2000). Steroid saponins have been proved to be responsible for the stimulation of appetite (Petit ., 1995). They also help in lowering of serum cholesterol by inhibiting the absorption of bile acids in a dose –dependent manner (Stark and Madar, 1993). Fenugreek has also been shown to improve the blood levels of glutathione and beta-carotene in normal and diabetic rats (Ravikumar & Anuradha, 1999). Conversely, fenugreek has been shown to decrease the levels of vitamin E and has been shown to have no effect on ascorbic acid. However, it still remains a potential candidate to improve the overall antioxidant status of the body.

Pisonia grandis is a commonly available plant that has been traditionally used in the treatment of the free radical induced disease- arthritis. The leaves expel flatulence and they can be applied externally on elephantiod swellings. Not much information is available about the plant. But its usage in the treatment of arthritis indicates that it might have potential antioxidant activity.

Sauropus androgynus is another under exploited plant, which has been found to possess antioxidant activity (Suhaila *et al.*, 1999). This might have some role in preventing cancer. An in vitro study was carried out to determine the plant's potential to inhibit the proliferation of cell lines for breast cancer. The plant has been proved to have a good anticancer effect. The vegetable also showed a higher antioxidant activity when compared to vitamin E (Asmah Rahmat *et al.*, 2003).

The potential role of antioxidants like Vitamin C, Vitamin E and beta-carotene, of minerals like zinc and selenium, and of antioxidant enzymes like catalase, glutathione peroxidase, have been extensively studied in the prevention of degenerative diseases, including tumor growth and carcinogenesis (Halliwell *et al.*, 1992).

So natural antioxidants play a very important role in the protection of the body against various diseases. Although the selected green leafy vegetables and their derivatives have not yet been subjected to extensive clinical trials and are not pharmaceuticals, animal trials and in vitro tests provide a solid evidence to support their supplemental usage in the prevention of free radical induced and age-related diseases. But, extensive standardization in the isolation, characterization and dosages are necessary to actually assure its rational usage and to confirm their efficiency. This project is just an attempt to scrape the tip of the iceberg. There is still plenty of room to carry extensive research work on these plants.

Phase I

4.1. Free radical scavenging assays

4.1.1. DPPH radical scavenging activity

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

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

Species	Mean percentage inhibition				
	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
T.f	1.68	5.62	12.97	21.62	27.11
C.a	7.79	23.68	37.07	52.34	61.99
P.a.	35.39	41.08	47.05	54.65	56.86
S.a	46.45	49.22	57.42	62.85	63.66

Table 4.1.1.1. Mean percentage inhibition of DPPH radicals by methanol extracts of plant species

SE of cell values = 0.725

Coefficient of variation = 3.28%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
Sa vs Tf	44.76 *	43.06 *	44.45 *	41.24 *	36.55 *
Sa vs C.a	38.66 *	25.543 *	20.35 *	10.52 *	1.67 *
Sa vs Pa	11.05 *	8.14 *	10.37 *	8.20 *	6.80 *
Pa. vs Tf	33.71 *	35.457 *	34.08 *	33.03 *	29.75 *
Pa vs Ca	27.60 *	17.40 *	9.98 *	2.31 *	5.13 *
Ca vs Tf	6.11 *	18.06 *	24.10 *	30.72 *	34.88 *

Table 4.1.1.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

*****: Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

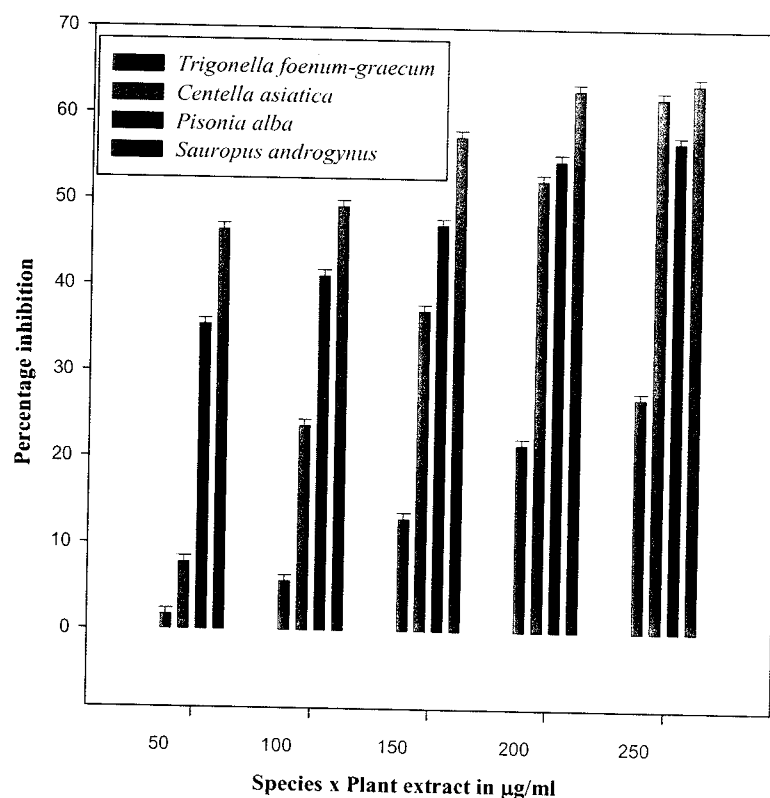

Figure 4.1.1. 1. Percentage inhibition of DPPH radicals by methanol extracts of plants species

Species	Mean percentage inhibition				
	50 µg/ml #	**100 µg/ml**	**150 µg/ml**	**200 µg/ml**	**250 µg/ml**
T.f	20.92	21.36	23.31	25.98	51.23
C.a	6.85	16.51	26.48	54.83	74.46
P.a.	13.88	17.43	22.69	24.96	27.03
S.a	44.02	48.12	50.67	52.05	54.59

Table 4.1.1.3. Mean percentage inhibition of DPPH radicals by acetone extracts of plant species

SE of cell values = 0.711

Coefficient of Variance = 3.63%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*;
S.a: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	23.10 *	26.76 *	27.36 *	26.07 *	3.36 *
Sa vs C.a	37.17 *	31.61 *	24.19 *	2.78 *	19.86 *
Sa vs Pa	30.14 *	30.69 *	27.97 *	27.10 *	27.56 *
Pa. vs Tf	7.04 *	3.93 *	0.61 ns	1.03 ns	24.20 *
Pa vs Ca	7.03 *	0.92 ns	3.79 *	29.87 *	47.42 *
Ca vs Tf	14.07 *	4.85 *	3.17 *	28.85 *	23.23 *

Table 4.1.1.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

*****: Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

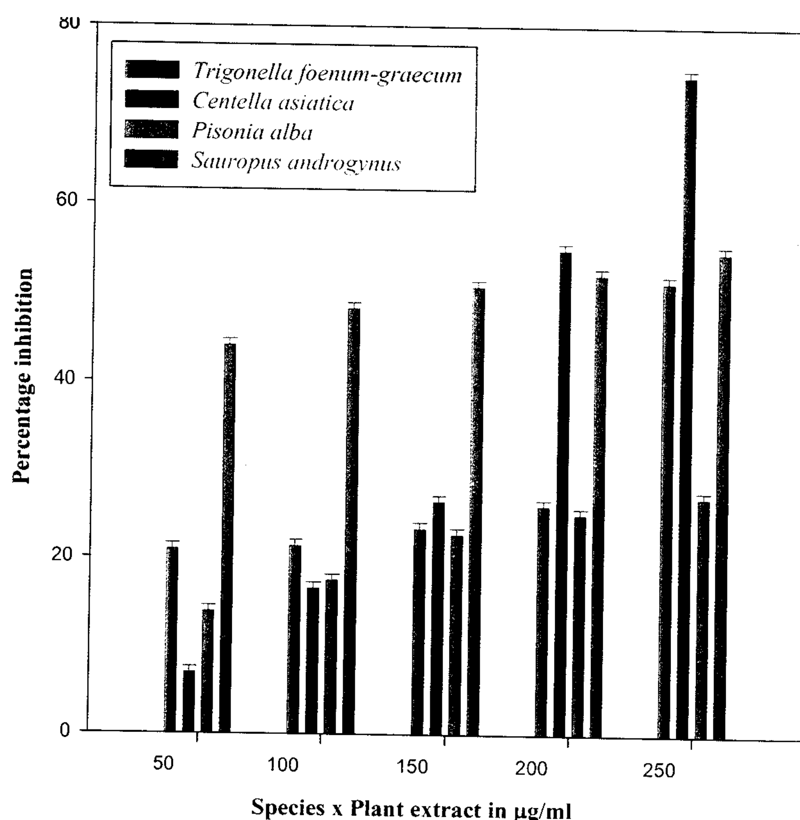

Figure 4.1.1.2. Percentage inhibition of DPPH radicals by acetone extracts of plants species

Species	Mean % inhibition across concentrations	
	Methanol extract	Acetone extract
T.f	13.80 ± 0.324	28.56 ± 0.318
C.a	36.57 ± 0.324	35.83 ± 0.318
P.a.	47.01 ± 0.324	21.20 ± 0.318
S.a	55.92 ± 0.324	49.89 ± 0.318

Table 4.1.1.5. Mean percentage inhibition of DPPH radicals across the concentration range by methanol and acetone extracts of plant species

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

The mean values across the concentrations indicate that the acetone and methanol extracts of the plant ***Sauropus androgynus*** are more potent in neutralizing DPPH free radical. Of these the methanol extract shows a slightly better scavenging activity. The methanol extract shows an inhibition of 46-64 % in the concentration range of 50-250 μg/ml. The acetone extract on the other hand shows an inhibition of 44-55% inhibition in the same concentration range.

An IC_{50} value is the concentration of sample required to scavenge 50% free radical or to prevent lipid peroxidation by 50%. These values were calculated for the in vitro assays and the antioxidant activity is observed for each of the eight extracts and the standard. The extracts were found to have different levels of antioxidant activity in different systems tested.

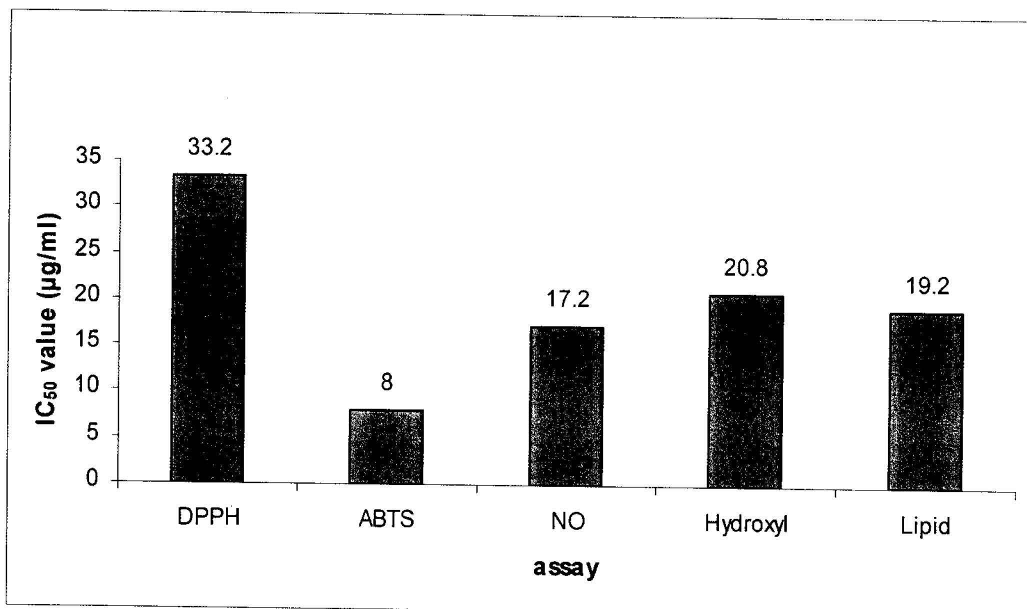

Figure 4.1.1.3 Comparison of IC_{50} values of ascorbic acid in different assays

The IC_{50} values of the standard Ascorbic acid were obtained and are depicted in the above graph. From the figure, it is seen that ascorbic acid is the most potent in scavenging ABTS radical. And it shows moderate potency in scavenging other radicals. It has the weakest scavenging potential for DPPH.

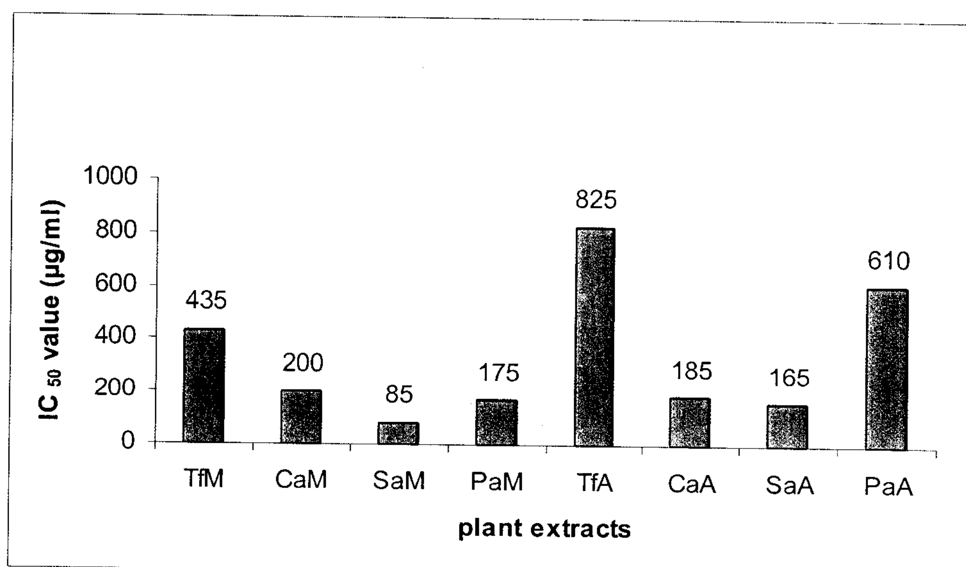

Figure 4.1.1.4 Comparison of IC_{50} values of DPPH radical scavenging assay

TfM- Methanolic extract of *Trigonella foenum-graecum*

CaM- Methanolic extract of *Centella asiatica*

SaM- Methanolic extract of *Sauropus androgynus*

PaM- Methanolic extract of *Pisonia alba*

TfA – Acetone extract of *Trigonella foenum-graecum*

CaA- Acetone extract of *Centella asiatica*

SaA- Acetone extract of *Sauropus androgynus*

PaA- Acetone extract of *Pisonia alba*

Among the methanolic extracts of the plant species *Sauropus androgynus* exhibits the highest potential for DPPH radical scavenging as it has the least IC_{50} value of 85 μg/ml. For the acetone extracts the least IC_{50} value is again for *Sauropus androgynus*. The value for methanolic extract of *Trigonella foenum-graecum* has been previously recorded as 444.1 μg/ml (Nooman *et al.*, 2007). This is consistent with our observation, which is 435 μg/ml. The value of ascorbic acid is 33.2 μg/ml for DPPH radical scavenging activity. This however indicates that the chosen plant extracts are not as potent as the standard. The methanol extract of *Sauropus androgynus* however is almost as good as ascorbic acid.

4.1.2. ABTS cation radical scavenging assay

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$, which has a characteristic long wavelength absorption spectrum (Sanchez- Moreno, 2002).

Species	Mean percentage inhibition				
	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
T.f	25.30	29.34	35.31	40.00	45.31
C.a	17.57	19.70	21.64	30.06	49.62
P.a.	33.10	55.74	71.32	92.07	99.63
S.a	18.25	23.46	32.21	34.55	50.70

Table 4.1.2.1. Mean percentage inhibition of ABTS cation radicals by methanol extracts of plant species

SE of cell values = 0.383

Coefficient of variation = 1.61%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. ($P = <0.001$)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	7.06 *	5.88 *	3.09 *	5.45 *	5.39 *
Sa vs C.a	0.68 ns	3.76 *	10.58 *	4.48 *	1.08 ns
Sa vs Pa	14.86 *	32.28 *	39.11 *	57.52 *	48.94 *
Pa. Vs Tf	7.80 *	26.40 *	36.02 *	52.07 *	54.33 *
Pa vs Ca	15.54 *	36.04 *	49.69 *	62.01 *	50.01 *
Ca vs Tf	7.74 *	9.65 *	13.67 *	9.94 *	4.31 *

Table 4.1.2.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

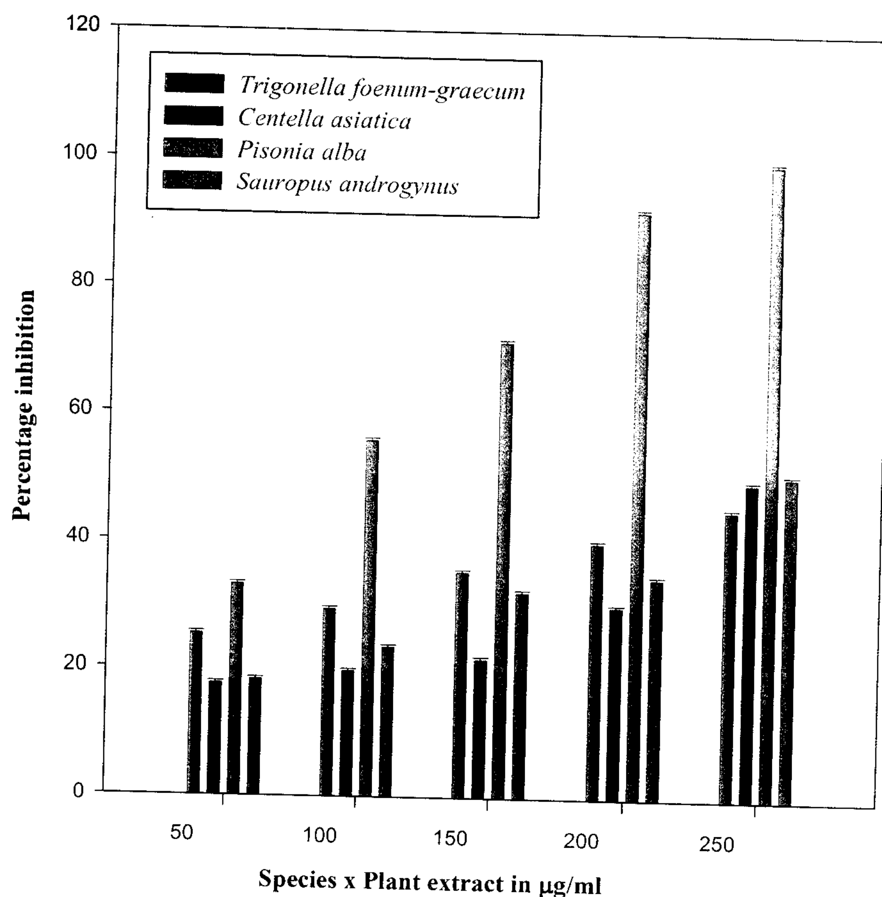

Figure 4.1.2.1. Percentage inhibition of ABTS cation radicals by methanol extracts of plants species

Species	Mean percentage inhibition				
	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
T.f	23.66	25.92	31.55	42.25	49.44
C.a	20.26	30.87	37.08	42.47	52.79
P.a.	19.32	22.78	26.14	28.17	30.34
S.a	15.61	17.77	22.98	28.02	29.48

Table 4.1.2.3. Mean percentage inhibition of ABTS cation radicals by acetone extracts of plant species

SE of cell values = 0.225

Coefficient of Variance = 1.31%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs. Tf	23.10 *	26.76 *	27.36 *	26.07 *	3.36 *
Sa vs. C.a	37.17 *	31.61 *	24.19 *	2.78 *	19.86 *
Sa vs. Pa	30.14 *	30.69 *	27.97 *	27.10 *	27.56 *
Pa. vs. Tf	7.04 *	3.93 *	0.61 ns	1.03 ns	24.20 *
Pa vs. Ca	7.03 *	0.92 ns	3.79 *	29.87 *	47.42 *
Ca vs. Tf	14.07 *	4.85 *	3.17 *	28.85 *	23.23 *

Table 4.1.2.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

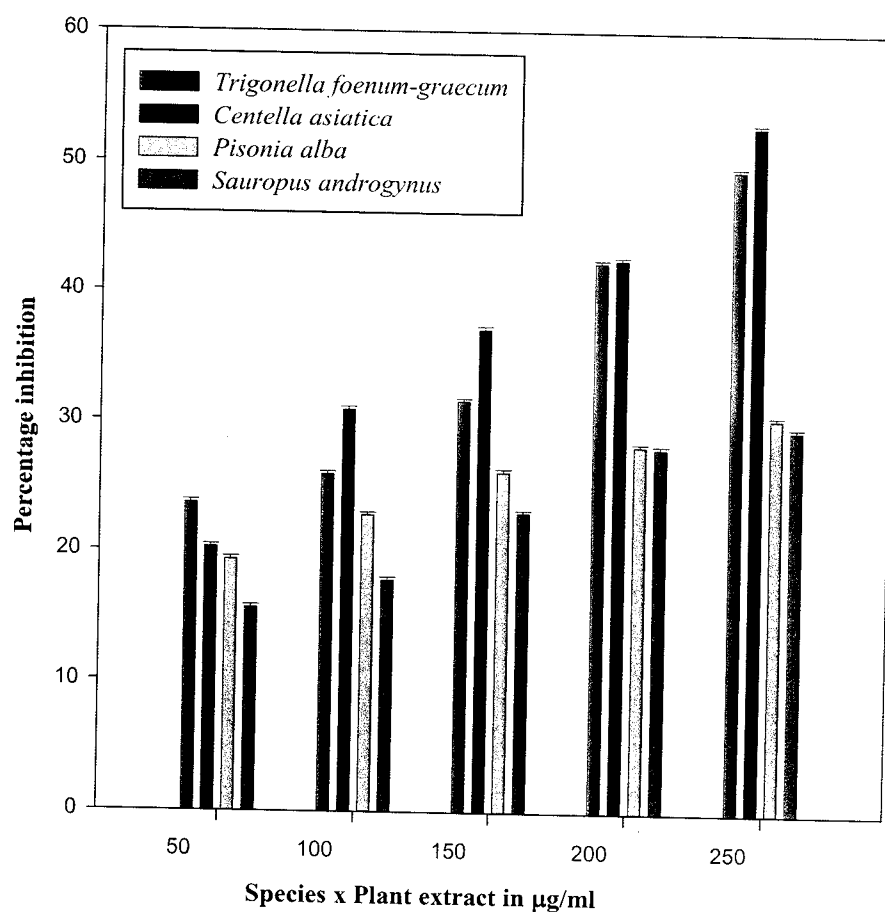

Figure 4.1.2.2. Percentage inhibition of ABTS cation radicals by acetone extracts of plants species

Species	Mean % inhibition across concentrations	
	Methanol extract	Acetone extract
T.f	35.05 ± 0.171	34.56 ± 0.101
C.a	27.72 ± 0.171	36.69 ± 0.101
P.a.	70.37 ± 0.171	25.35 ± 0.101
S.a	31.83 ± 0.171	22.77 ± 0.101

Table 4.1.2.5. Mean percentage inhibition of ABTS Cation radicals across the concentration range by methanol and acetone extracts of plant species

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol extract of ***Pisonia alba*** and the acetone extract of ***Centella asiatica*** are more potent in neutralizing ABTS cation free radical. Of these the methanol extracts show a slightly better scavenging activity. The methanol extract of *Pisonia alba* shows an inhibition of 33-99% in the concentration range of 50-100 µg/ml. The acetone extract of *Centella asiatica* shows an inhibition of 20-53 % in the same concentration range.

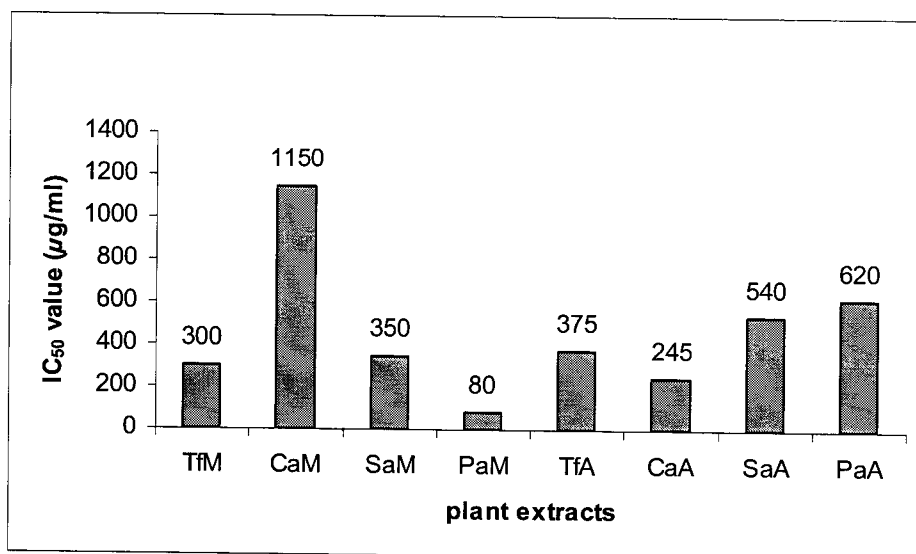

Figure 4.1.2.3 Comparison of IC_{50} values of ABTS cation radical scavenging activity

Among the methanolic extracts of the plant species *Pisonia alba* exhibits the highest potential for ABTS cation radical scavenging as it has the least IC_{50} value of 80 µg/ml. For the acetone extracts the least IC_{50} value is 245 µg/ml for *Centella asiatica*. The value of ascorbic acid is 8 µg/ml for ABTS cation radical scavenging activity. This however indicates that the chosen plant extracts are not as potent as the standard.

4.1.3. Nitric oxide scavenging assay

Nitric oxide is a free radical produced in mammalian cells, and is involved in the regulation of various physiological processes. However, excess production of NO is associated with several diseases (Ross, 1993; Ialenti *et al.*, 1993). Oxygen reacts with excess NO to generate nitrite and peroxynitrite anions, which act as free radicals (Cotran *et al.*, 1999).

Species	Mean percentage inhibition				
	50 µg/ml	**100 µg/ml**	**150 µg/ml**	**200 µg/ml**	**250 µg/ml**
T.f	28.44	20.98	35.04	61.91	75.41
C.a	22.63	33.34	35.09	36.26	38.54
P.a.	36.66	40.87	43.24	45.90	53.29
S.a	57.96	62.84	67.11	71.74	74.06

Table 4.1.3.1. Mean percentage inhibition of Nitric oxide radicals by methanol extracts of plant species

SE of cell values = 4.477; Coefficient of variation = 16.48%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	29.52 *	41.86 *	32.07 *	9.84 ns	1.35 ns
Sa vs C.a	35.33 *	29.51 *	32.02 *	35.49 *	35.52 *
Sa vs Pa	21.30 *	21.97 *	23.87 *	25.85 *	20.77 *
Pa. Vs Tf	8.22 ns	19.89 *	8.20 ns	16.01 *	22.12 *
Pa vs Ca	14.02 *	7.53 ns	8.15 ns	9.64 ns	14.75 *
Ca vs Tf	5.81 ns	12.35 ns	0.05 ns	25.65 *	36.87 *

Table 4.1.3.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

*****: Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

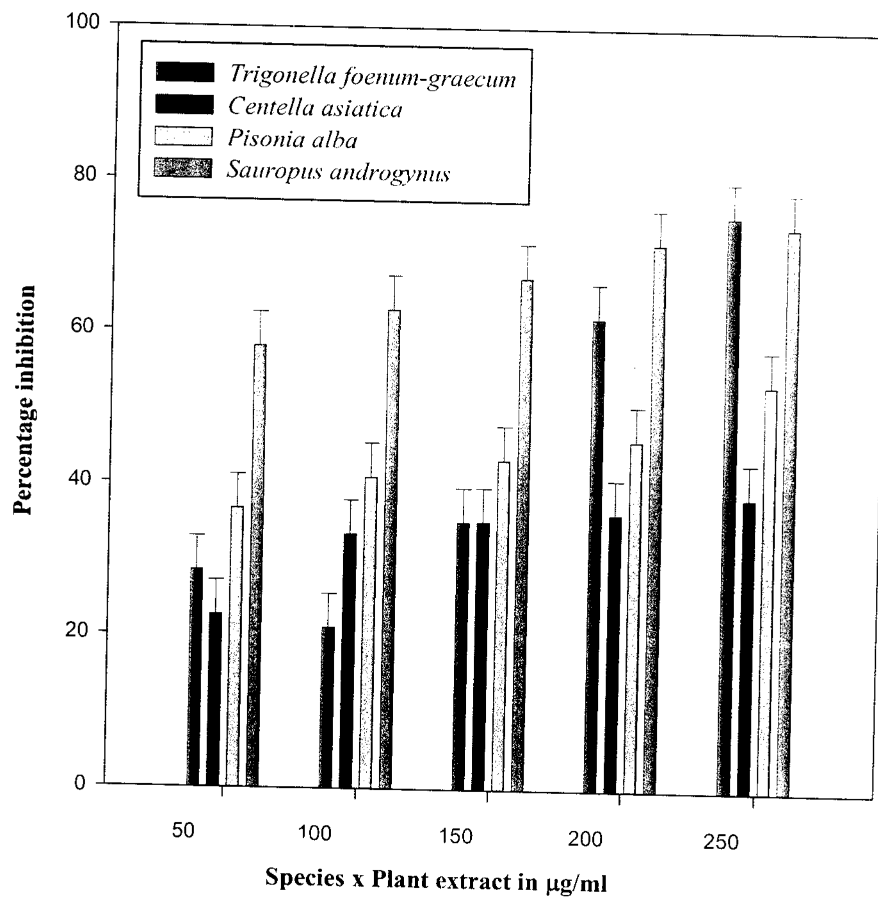

Figure 4.1.3.1. Percentage inhibition of Nitric oxide radicals by methanol extracts of plants species

Species	Mean percentage inhibition				
	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
T.f	8.95	20.47	40.08	64.75	73.85
C.a	46.37	51.05	57.84	61.58	65.20
P.a.	49.22	53.88	57.13	65.48	69.47
S.a	20.67	42.42	60.78	66.61	69.67

Table 4.1.3.3. Mean percentage inhibition of Nitric oxide radicals by acetone extracts of plant species

SE of cell values = 0.490; Coefficient of Variance = 1.63 %

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	11.72 *	21.95 *	20.70 *	1.86 *	4.17 *
Sa vs C.a	25.70 *	8.63 *	2.94 *	5.03 *	4.47 *
Sa vs Pa	28.55 *	11.46 *	3.65 *	1.12 ns	0.20 ns
Pa. vs Tf	40.27 *	33.41 *	17.05 *	0.73 ns	4.37 *
Pa vs Ca	2.85 *	2.83 *	0.71 ns	3.90 *	4.27 *
Ca vs Tf	37.42 *	30.58 *	17.75 *	3.17 *	8.64 *

Table 4.1.3.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)
ns: Differences not significant at P=0.05

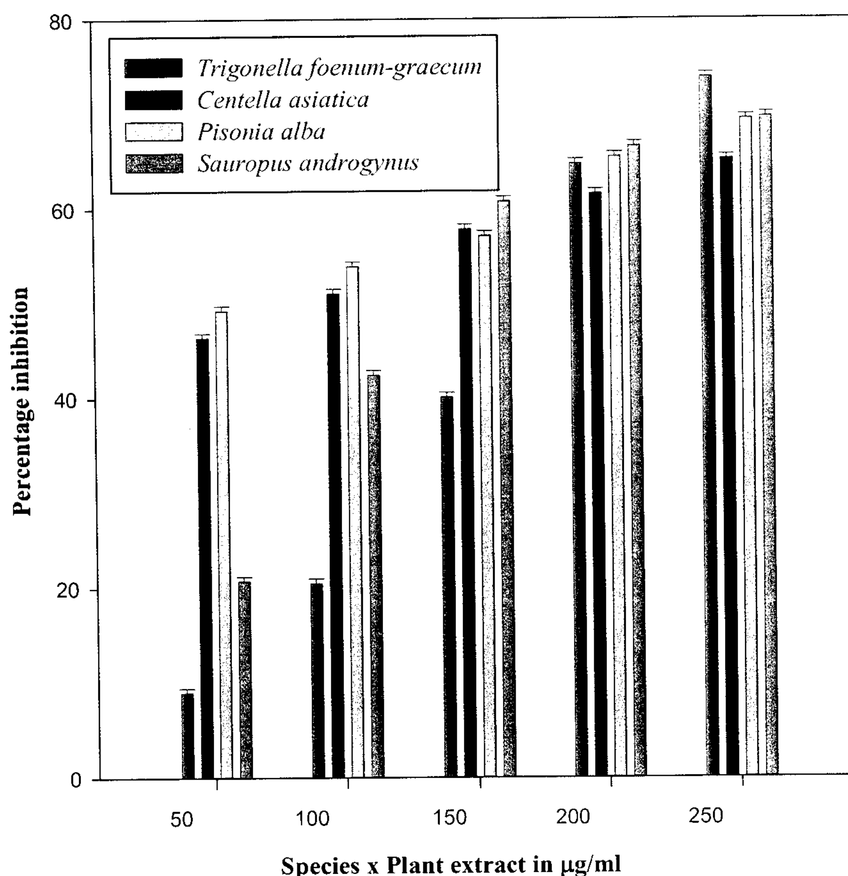

Figure 4.1.3.2. Percentage inhibition of Nitric oxide radicals by acetone extracts of plants species

Species	Mean % inhibition across concentrations	
	Methanol extract	Acetone extract
T.f	44.36 ± 2.002	41.62 ± 0.219
C.a	33.17 ± 2.002	56.41 ± 0.219
P.a.	43.99 ± 2.002	59.04 ± 0.219
S.a	66.74 ± 2.002	52.03 ± 0.219

Table 4.1.3.5. Mean percentage inhibition of Nitric oxide radicals across the concentration range by methanol and acetone extracts of plant species

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*;

S.a: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol extract of ***Sauropus androgynus***, which exhibited an inhibition of 57-74 % in the concentration range of 50-100 μg/ml and the acetone extract of ***Pisonia alba***, which exerted an inhibition of 49-70 % in the same concentration range are more potent in neutralizing Nitric oxide free radical.

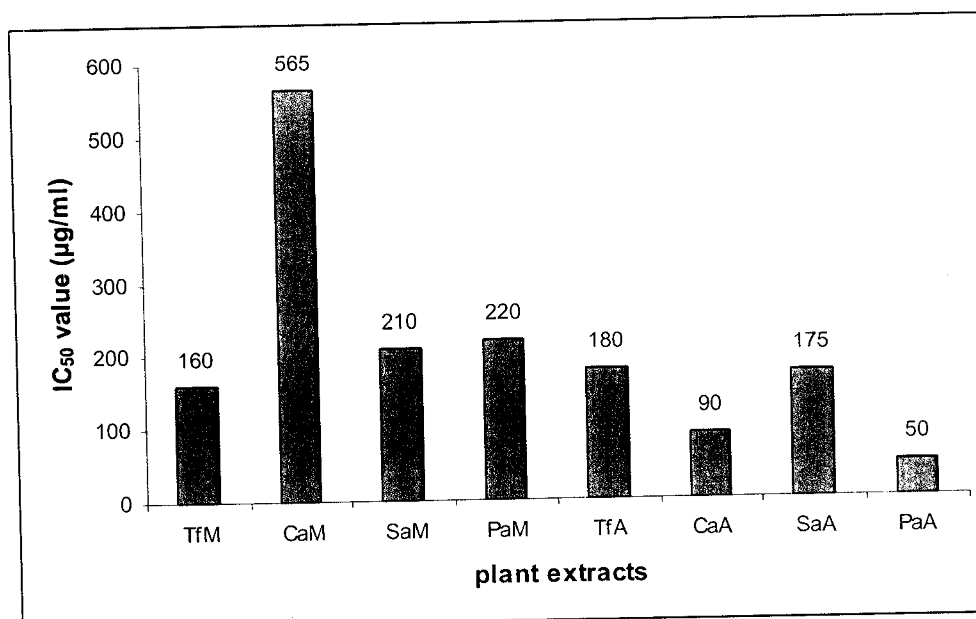

Figure 4.1.3.3 Comparision of IC_{50} values of Nitric oxide radical scavenging activity

Among the methanolic extracts of the plant species *Trigonella foenum-graecum* exhibits the highest potential for nitric oxide radical scavenging as it has the least IC_{50} value of 160 μg/ml. For the acetone extracts the least IC_{50} value is 50 μg/ml for *Pisonia alba*. The value of ascorbic acid is 17.2 μg/ml for Nitric oxide radical scavenging activity. This however indicates that the chosen plant extracts are not as potent as the standard

4.1.4. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay of the various plant extracts was measured as the percentage inhibition of hydroxyl radicals generated in the Fenton's reaction mixture. Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+} / Ascorbate/EDTA/H_2O_2 system. The hydroxyl radicals attack deoxyribose which eventually result in TBARS formation.

Species	Mean percentage inhibition				
	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
T.f	43.08	45.72	48.75	50.07	66.93
C.a	33.25	36.56	44.53	65.20	69.04
P.a.	11.65	13.38	17.99	32.18	38.06
S.a	11.19	21.22	28.26	33.10	35.99

Table 4.1.4.1. Mean percentage inhibition of Hydroxyl radicals by methanol extracts of plant species

SE of cell values = 0.905

Coefficient of variation = 4.20%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. ($P = <0.001$)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	20.49 *	24.49 *	31.89 *	16.97*	30.94 *
Sa vs C.a	16.27 *	15.33 *	22.06 *	32.10 *	33.05 *
Sa vs Pa	10.26 *	7.85 *	0.46 ns	0.90 ns	2.07 ns
Pa. Vs Tf	30.76 *	32.34 *	31.43 *	17.89 *	28.87 *
Pa vs Ca	26.53 *	23.18 *	21.60 *	33.02 *	30.98 *
Ca vs Tf	4.22 *	9.16 *	9.83 *	15.13 *	2.11 ns

Table 4.1.4.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

* : Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

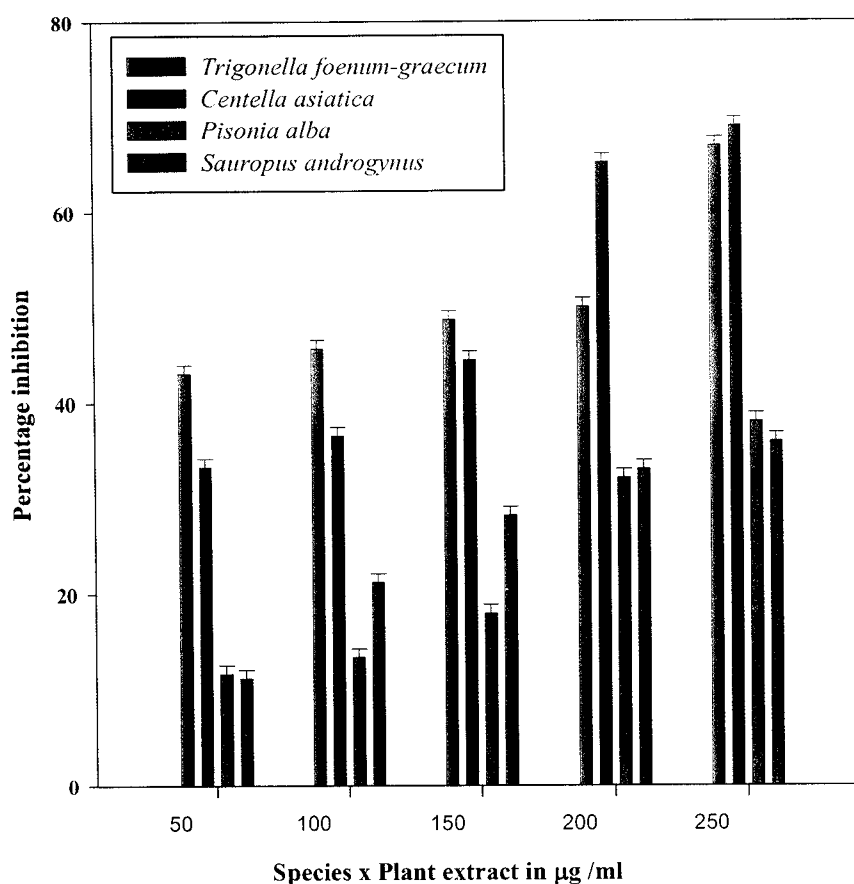

Figure 4.1.4.1. Percentage inhibition of Hydroxyl radicals by methanol extracts of plants species

Species	Mean percentage inhibition				
	50 µg/ml	**100 µg/ml**	**150 µg/ml**	**200 µg/ml**	**250 µg/ml**
T.f	44.40	46.25	50.72	51.78	53.49
C.a	43.83	49.71	52.61	54.12	56.21
P.a.	71.86	74.05	79.47	80.28	84.54
S.a	64.59	65.74	67.01	70.70	75.08

Table 4.1.4.3. Mean percentage inhibition of Hydroxyl radicals by acetone extracts of plant species

SE of cell values = 0.483; Coefficient of Variance = 1.35 %

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
Sa vs Tf	20.19 *	19.50 *	16.29 *	18.92 *	21.59 *
Sa vs C.a	20.76 *	16.03 *	14.40 *	16.58 *	18.87 *
Sa vs Pa	7.27 *	8.30 *	12.46 *	9.57 *	9.46 *
Pa. vs Tf	27.46 *	27.80 *	28.75 *	28.50 *	31.05 *
Pa vs Ca	28.03 *	24.33 *	26.86 *	26.15 *	28.33 *
Ca vs Tf	0.57 ns	3.47 *	1.89 *	2.34 *	2.72 *

Table 4.1.4.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

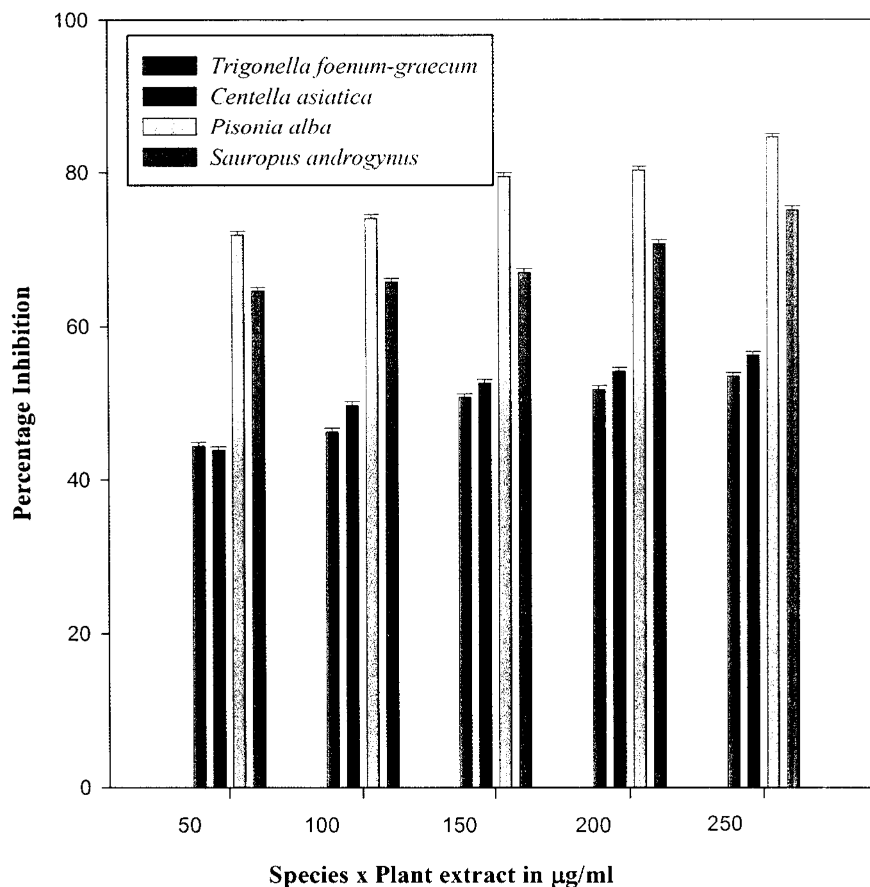

Figure 4.1.4.2. Percentage inhibition of Hydroxyl radicals by acetone extracts of plants species

Species	Mean % inhibition across concentrations	
	Methanol extract	Acetone extract
T.f	50.91 ± 0.405	49.33 ± 0.216
C.a	49.71 ± 0.405	51.30 ± 0.216
P.a.	22.65 ± 0.405	78.04 ± 0.216
S.a	25.95 ± 0.405	68.63 ± 0.216

Table 4.1.4.5. Mean percentage inhibition of Hydroxyl radicals across the concentration range by methanol and acetone extracts of plant species

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol extract of ***Trigonella foenum-graecum*** and the acetone extract of ***Pisonia alba*** are more potent in neutralizing Hydroxyl free radicals. The acetone extracts are more potent in this case. The methanol extract of *Trigonella foenum-graecum* exhibited an inhibition of 43-67 % in the concentration range of 50-100 μg/ml. The acetone extract of *Pisonia alba* on the other hand, showed an inhibition of 71-85 % in the same concentration range.

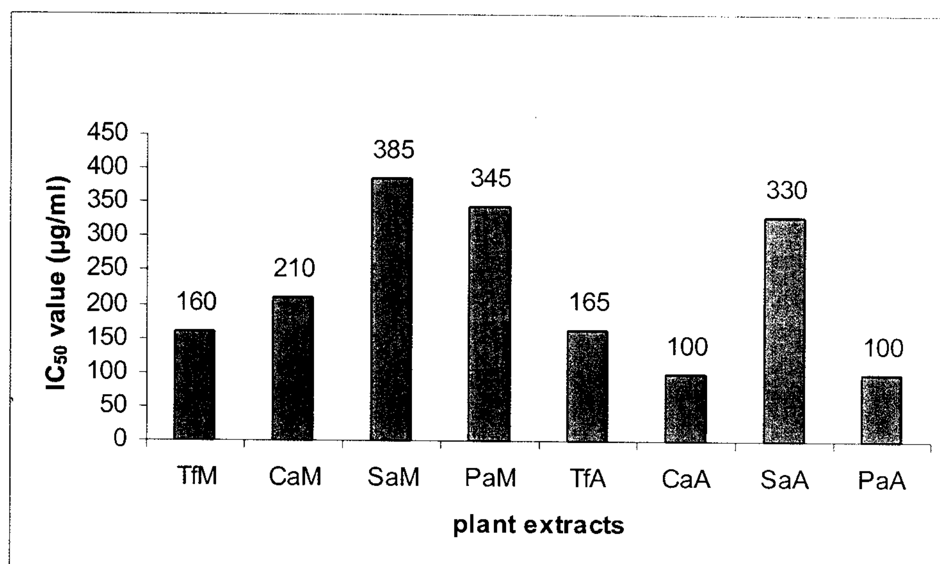

Figure 4.1.4.3 Comparision of IC_{50} values of Hydroxyl radical scavenging activity

Among the methanolic extracts of the plant species *Trigonella foenum-graecum* exhibits the highest potential for Hydroxyl radical scavenging as it has the least IC_{50} value of 160 μg/ml. For the acetone extracts the least IC_{50} value is 100 μg/ml for both *Pisonia alba* and *Centella asiatica*. The latter plant has already been established for its potency The value of ascorbic acid is 20.8 μg/ml for Hydroxyl radical scavenging activity. This however indicates that the chosen plant extracts are not as potent as the standard. But on the whole, leafy vegetables exhibit a good hydroxyl radical scavenging potential (Shyamala *et al.*, 2005).

4.1.5. Inhibition of lipid peroxidation assay

Lipid peroxidation (LPO) is the oxidative degradation of polyunsaturated fatty acids (PUFA) and involves lipid radicals. This is a basic membrane damage process and results in deleterious effects.

Species	Mean percentage inhibition				
	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
T.f	42.73	44.24	46.06	49.24	53.18
C.a	45.76	49.85	56.51	59.70	67.42
P.a.	20.38	22.98	26.90	34.67	48.37
S.a	32.58	36.67	37.93	40.35	45.86

Table 4.1.5.1. Mean percentage inhibition of Lipid peroxidation by methanol extracts of plant species

SE of cell values = 0.397; Coefficient of variation = 1.60%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	10.15 *	7.57 *	8.13 *	8.89 *	7.32 *
Sa vs C.a	13.18 *	13.18 *	18.58 *	19.35 *	21.56 *
Sa vs Pa	12.20 *	13.70 *	11.03 *	5.68 *	2.51 *
Pa. Vs Tf	22.35 *	21.27 *	19.16 *	14.57 *	4.81 *
Pa vs Ca	25.37 *	26.87 *	29.61 *	25.03 *	19.05 *
Ca vs Tf	3.03 *	5.61 *	10.45 *	10.46 *	14.24 *

Table 4.1.5.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

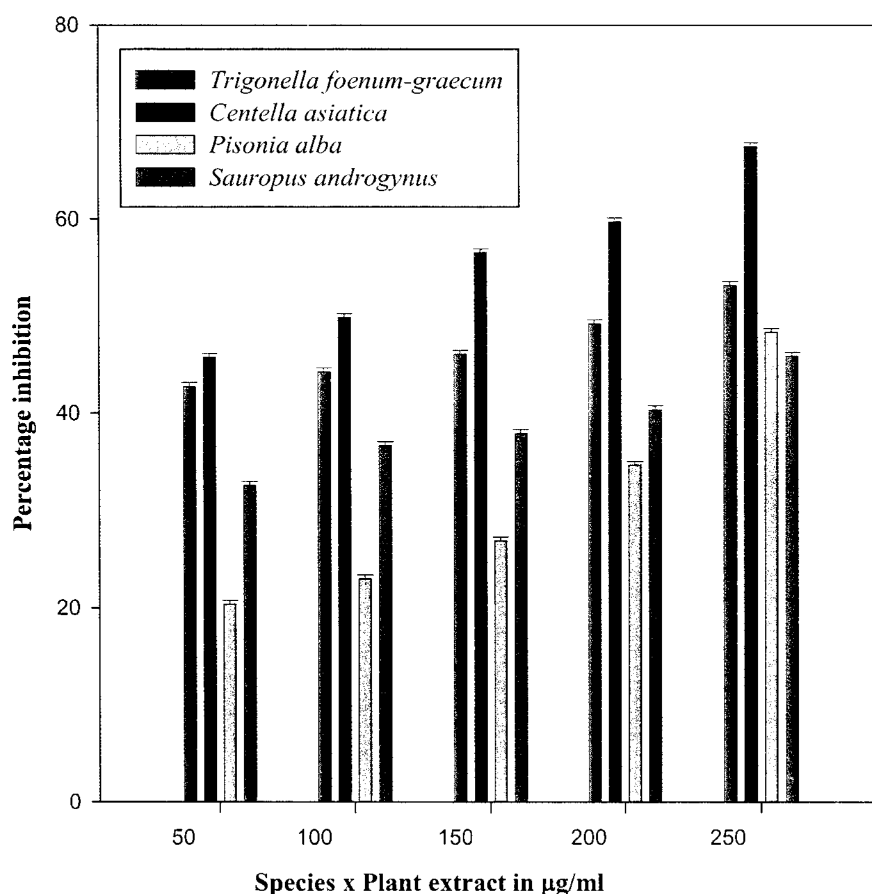

Figure 4.1.5.1. Percentage inhibition of Lipid peroxidation by methanol extracts of plants species

Species	Mean percentage inhibition				
	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
T.f	44.39	45.76	47.12	53.03	55.15
C.a	49.70	54.24	61.21	62.88	65.60
P.a.	17.88	20.55	25.56	30.66	38.35
S.a	33.25	32.83	42.94	51.63	57.31

Table 4.1.5.3. Mean percentage inhibition of Lipid peroxidation by acetone extracts of plant species

SE of cell values = 2.266; Coefficient of Variance = 8.82 %

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*;
S.a: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	11.15 *	12.92 *	4.18 ns	1.41 ns	2.16 ns
Sa vs C.a	16.45 *	21.41 *	18.27 *	11.25 *	8.29 *
Sa vs Pa	15.37 *	12.28 *	17.38 *	20.96 *	18.96 *
Pa. vs Tf	26.51 *	25.21 *	21.56 *	22.37 *	16.80 *
Pa vs Ca	31.82 *	33.69 *	35.65 *	32.22 *	27.25 *
Ca vs Tf	5.30 ns	8.49 *	14.09 *	9.85 *	10.45 *

Table 4.1.5.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)
ns: Differences not significant at P=0.05

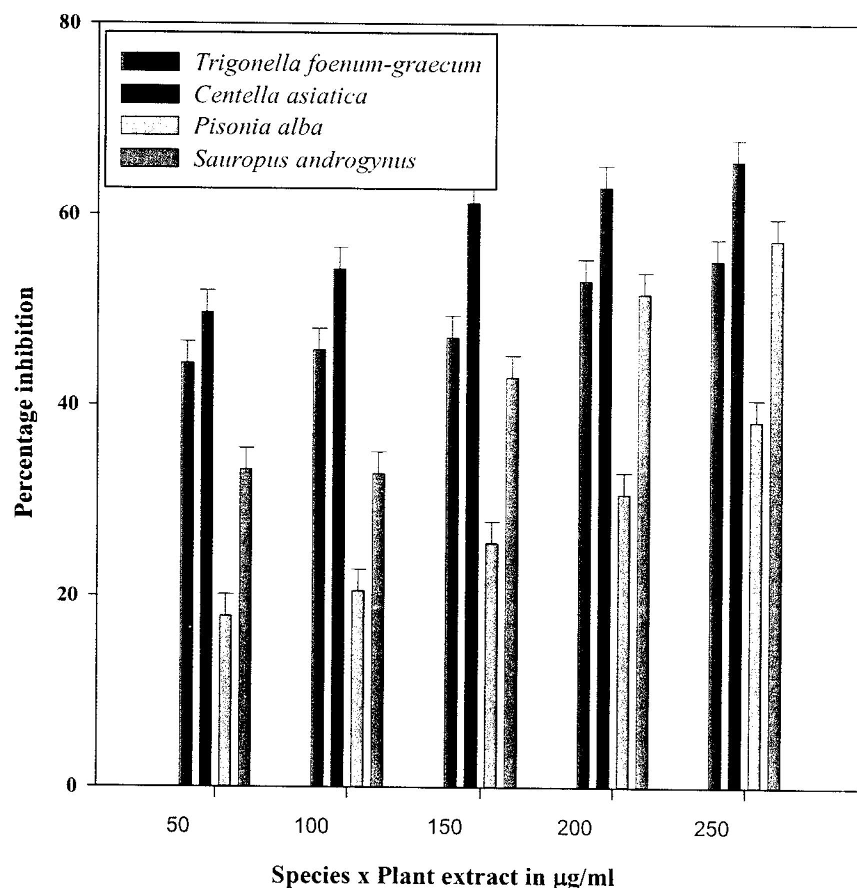

Figure 4.1.5.2. Percentage inhibition of Lipid peroxidation by acetone extracts of plants species

Species	Mean % inhibition across concentrations	
	Methanol extract	Acetone extract
T.f	47.09 ± 0.178	49.09 ± 1.013
C.a	55.85 ± 0.178	58.73 ± 1.013
P.a.	30.66 ± 0.178	26.60 ± 1.013
S.a	38.68 ± 0.178	43.59 ± 1.013

Table 4.1.5.5. Mean percentage inhibition of Lipid peroxidation across the concentration range by methanol and acetone extracts of plant species

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; P.a: *Pisonia alba*;

S.a: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol and acetone extracts of ***Centella asiatica*** are more potent in neutralizing Hydroxyl free radicals. The methanol extract exhibited an inhibition of 45-68 % in the concentration range of 50-100 μg/ml. The acetone extract showed an inhibition of 49-66 % in the same concentration range.

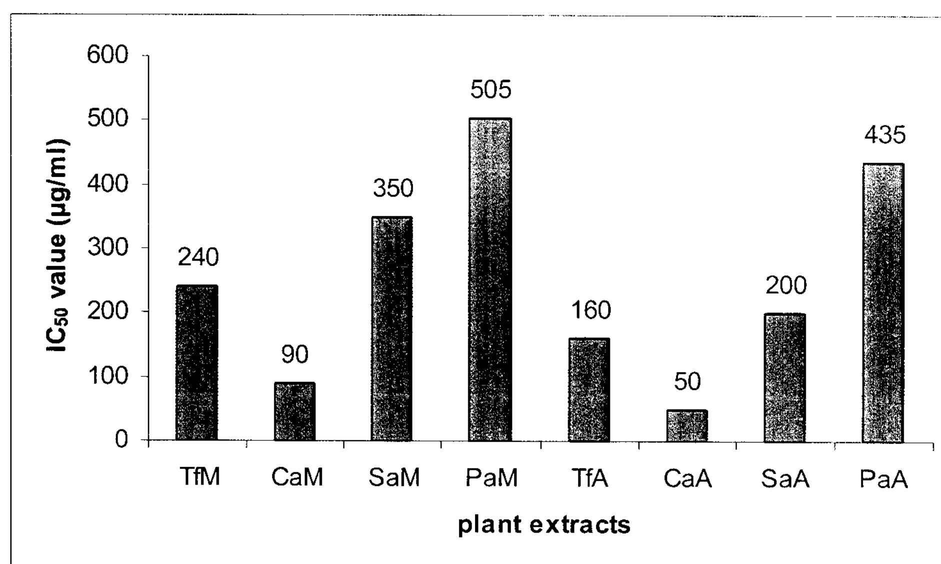

Figure 4.1.5.3 Comparision of IC_{50} values of lipid peroxidation inhibition activity

Among the methanolic extracts of the plant species *Centella asiatica* exhibits the highest potential for inhibition of Lipid peroxidation inhibition as it has the least IC_{50} value of 90 μg/ml. For the acetone extracts the least IC_{50} value is 50 μg/ml for *Centella asiatica.* The latter plant has already been established for its potency. The value of ascorbic acid is 19.2 μg/ml for inhibition of Lipid peroxidation. This however indicates that the chosen plant extracts are not as potent as the standard. Centella asiatica is potent in inhibition of lipid peroxidation as already established in previous research activities (Mahanom Hussin *et al.*, 2007).

4.1.6. Total antioxidant capacity assay

The total antioxidant assay gives an estimate of the overall antioxidant potential of the plant. There is a formation of phosphomolybdenum complex the intensity of which indicates the potential of the plant as a scavenger of free radicals.

Species	Mean concentration in ascorbic acid equivalents				
	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
T.f	0.810	1.033	1.530	1.647	1.818
C.a	0.233	0.483	0.643	0.757	1.693
P.a.	2.447	2.563	3.050	3.627	3.830
S.a	1.687	2.603	3.990	4.990	7.203

Table 4.1.6.1. Mean concentration in Ascorbic acid equivalents of methanol extracts of plant species

SE of cell values = 0.016;

Coefficient of variation = 1.18%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*;

S.a: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration in terms of equivalents of Ascorbic acid. ($P = <0.001$)

Species comparison	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
Sa vs Tf	0.877 *	1.570 *	2.460 *	3.343 *	5.385 *
Sa vs C.a	1.453 *	2.120 *	3.347 *	4.233 *	5.510 *
Sa vs Pa	0.760 *	0.040 ns	0.940 *	1.363 *	3.373 *
Pa. Vs Tf	1.637 *	1.530 *	1.520 *	1.980 *	2.012 *
Pa vs Ca	2.213 *	2.080 *	2.407 *	2.870 *	2.137 *
Ca vs Tf	0.577 *	0.550 *	0.887 *	0.890 *	0.125 *

Table 4.1.6.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

*****: Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

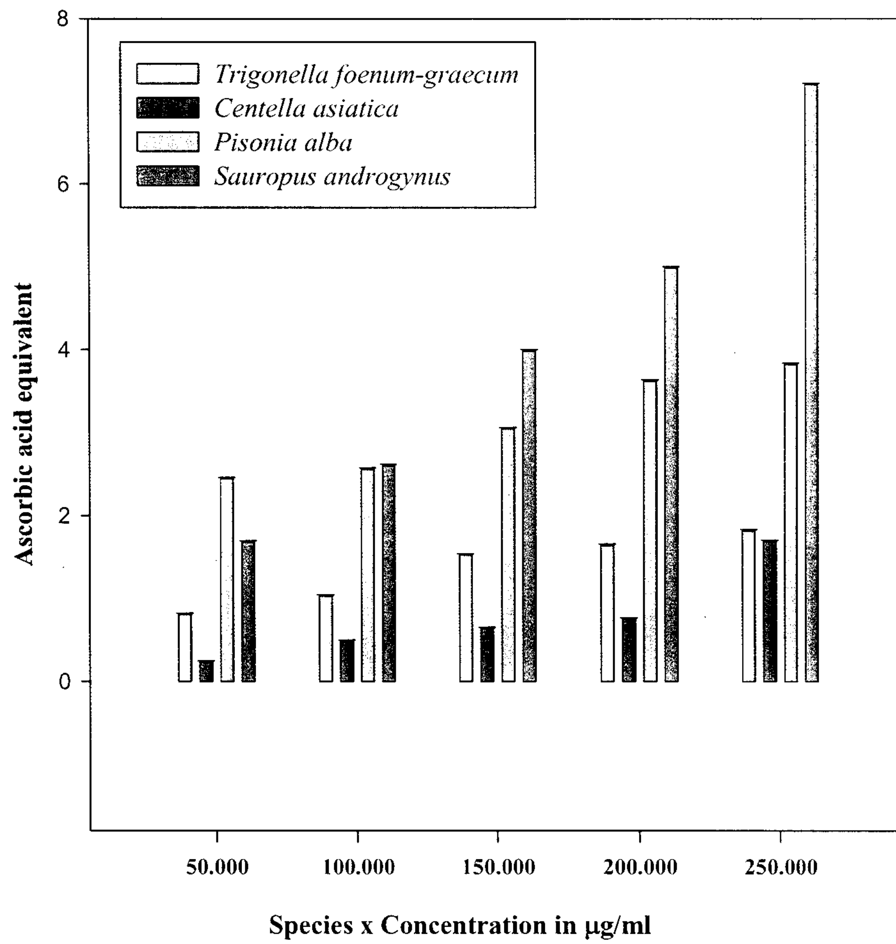

Figure 4.1.6.1. Concentration in terms of Ascorbic acid equivalents of methanol extracts of plants species

Species	Mean concentration in ascorbic acid equivalents				
	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
T.f	0.440	0.623	0.870	0.970	1.107
C.a	0.157	0.183	0.483	0.633	0.790
P.a.	0.740	1.390	1.497	1.840	2.043
S.a	1.577	1.670	1.713	1.753	1.880

Table 4.1.6.3. Mean concentration in Ascorbic acid equivalents of acetone extracts of plant species

SE of cell values = 0.036

Coefficient of Variance = 5.58 %

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	1.137 *	1.047 *	0.843 *	0.783 *	0.773 *
Sa vs C.a	1.420 *	1.487 *	1.230 *	1.120 *	1.090 *
Sa vs Pa	0.837 *	0.280 *	0.217 *	0.087 ns	0.163 *
Pa. vs Tf	0.300 *	0.767 *	0.627 *	0.870 *	0.937 *
Pa vs Ca	0.583 *	1.207 *	1.013 *	1.207 *	1.253 *
Ca vs Tf	0.283 *	0.440 *	0.387 *	0.337 *	0.317 *

Table 4.1.6.4. Comparison of Mean Differences among the species within each concentration of the acetone extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

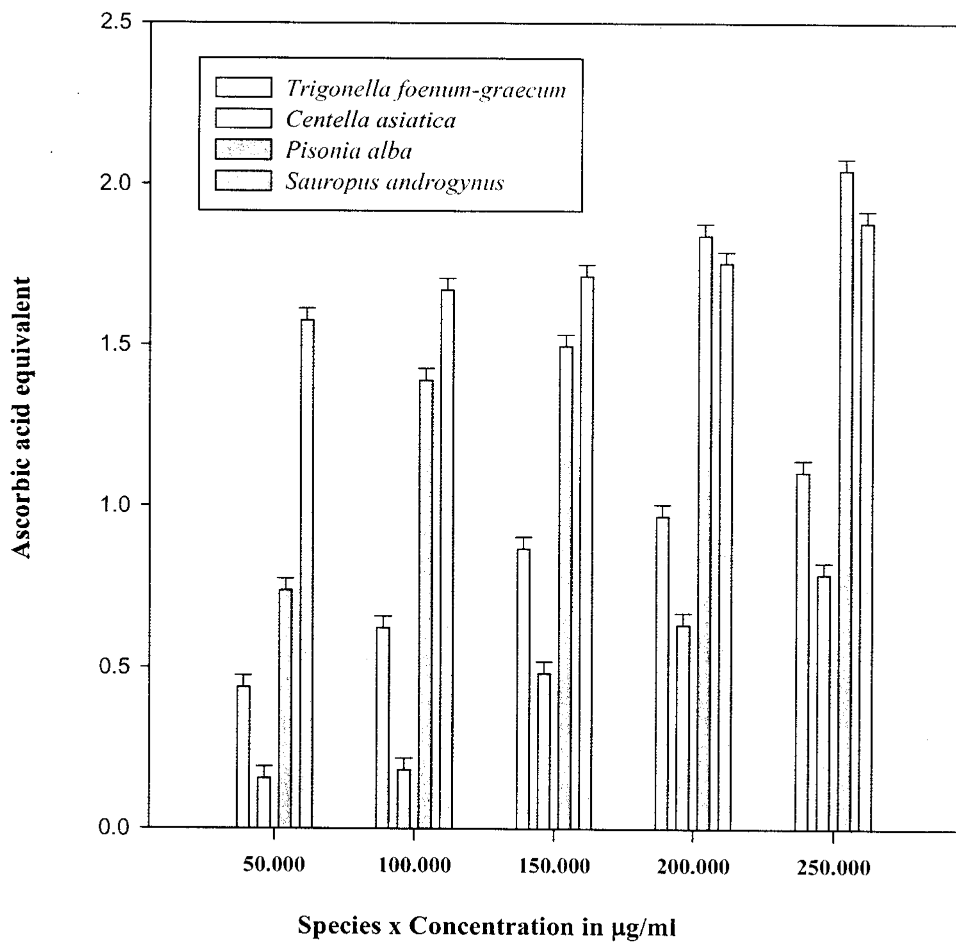

Figure 4.1.6.2. Concentration in terms of Ascorbic acid equivalents of methanol extracts of plants species

Species	Mean concentration in Ascorbic acid equivalents across concentrations (μg/ml)	
	Methanol extract	Acetone extract
T.f	1.368 ± 0.007	0.802 ±0.0161
C.a	0.762 ± 0.007	0.449 ±0.0161
P.a.	3.103 ± 0.007	1.502 ±0.0161
S.a	4.095 ± 0.007	1.719 ±0.0161

Table 4.1.6.5. Mean concentration in terms of Ascorbic acid equivalents across the concentration range for methanol and acetone extracts of plant species.

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*;
S.a: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol and acetone extracts of ***Sauropus androgynus*** exhibit the maximum total antioxidant capacity. The methanol extracts are more potent in this case.

4.1.7. Ferric Reducing Antioxidant Potential assay

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

Species	Mean absorbance of the extracts				
	50 μg/ml	100 μg/ml	150 μg/ml	200 μg/ml	250 μg/ml
T.f	0.831	0.846	0.877	0.935	1.012
C.a	0.371	0.781	1.341	1.758	2.203
P.a.	0.686	0.861	0.975	0.996	1.014
S.a	0.488	0.509	0.513	0.559	0.591

Table 4.1.7.1. Mean absorbance of methanol extracts of plant species

SE of cell values = 0.0025;Coefficient of variation = 0.4%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a**: *Centella asiatica*; **P.a**: *Pisonia alba*;
S.a: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration in terms of absorbance. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	0.343 *	0.338 *	0.363 *	0.376 *	0.421 *
Sa vs C.a	0.118 *	0.272 *	0.828 *	1.200 *	1.612 *
Sa vs Pa	0.197 *	0.353 *	0.461 *	0.438 *	0.423 *
Pa. Vs Tf	0.145 *	0.015 *	0.098 *	0.062 *	0.002 ns
Pa vs Ca	0.315 *	0.080 *	0.367 *	0.762 *	1.189 *
Ca vs Tf	0.460 *	0.065 *	0.465 *	0.824 *	1.191 *

Table 4.1.7.2. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

***:** Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)
ns: Differences not significant at P=0.05

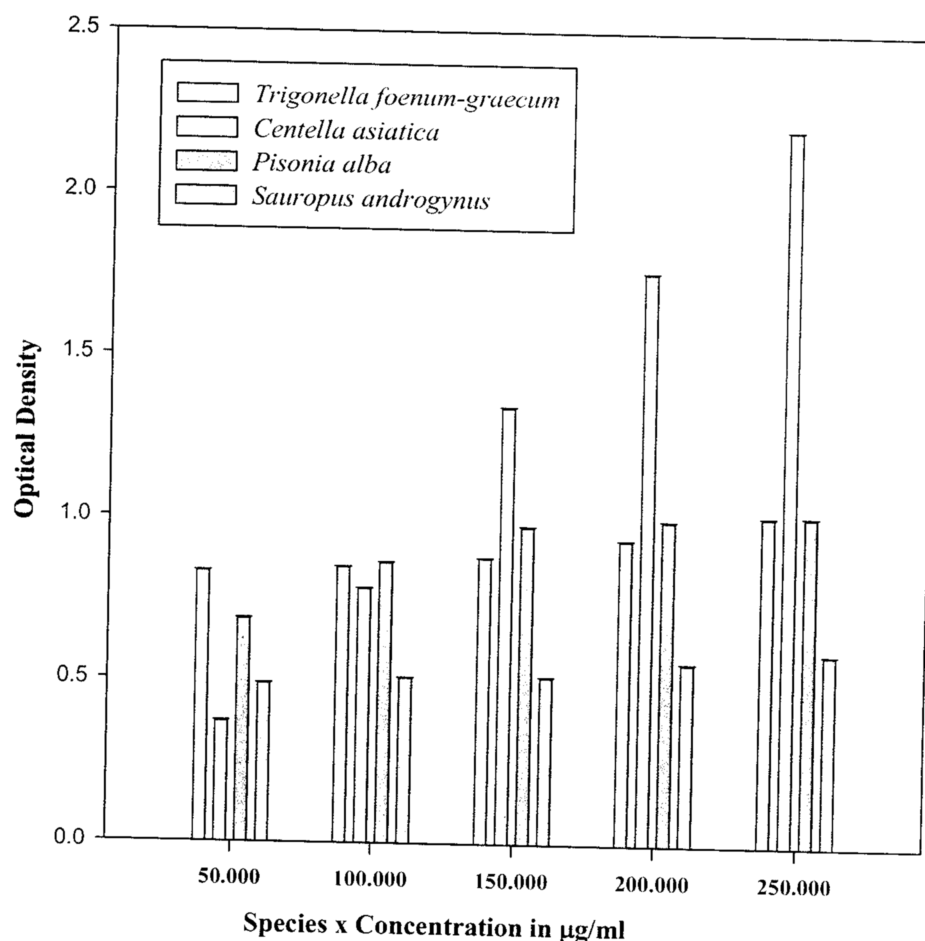

Figure 4.1.7.1. Mean absorbance of methanol extracts of plants species

Species	Mean absorbance of the extracts				
	50 µg/ml	**100 µg/ml**	**150 µg/ml**	**200 µg/ml**	**250 µg/ml**
T.f	0.138	0.143	0.170	0.180	0.190
C.a	0.700	1.405	1.739	2.203	2.543
P.a.	0.418	0.530	0.585	0.624	0.690
S.a	0.388	0.408	0.418	0.426	0.431

Table 4.1.7.3. Mean absorbance of methanol extracts of plant species

SE of cell values = 0.0018; Coefficient of variation = 2.82%

Values are expressed as mean of three replicates.

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

Statistical analysis:

The two-way ANOVA analysis was performed. It has been found that there is a statistically significant interaction between Species and Concentration in terms of absorbance. (P = <0.001)

Species comparison	50 µg/ml	100 µg/ml	150 µg/ml	200 µg/ml	250 µg/ml
Sa vs Tf	0.249 *	0.265 *	0.248 *	0.246 *	0.241 *
Sa vs C.a	0.312 *	0.997 *	1.320 *	1.777 *	2.112 *
Sa vs Pa	0.031 *	0.122 *	0.167 *	0.198 *	0.259 *
Pa. Vs Tf	0.280 *	0.387 *	0.415 *	0.444 *	0.500 *
Pa vs Ca	0.282 *	0.875 *	1.153 *	1.579 *	1.853 *
Ca vs Tf	0.562 *	1.262 *	1.568 *	2.023 *	2.353 *

Table 4.1.7.4. Comparison of Mean Differences among the species within each concentration of the methanol extracts by DMRT analysis

*: Differences significant at P=0.05 level by Duncun's Multiple Range Test (DMRT)

ns: Differences not significant at P=0.05

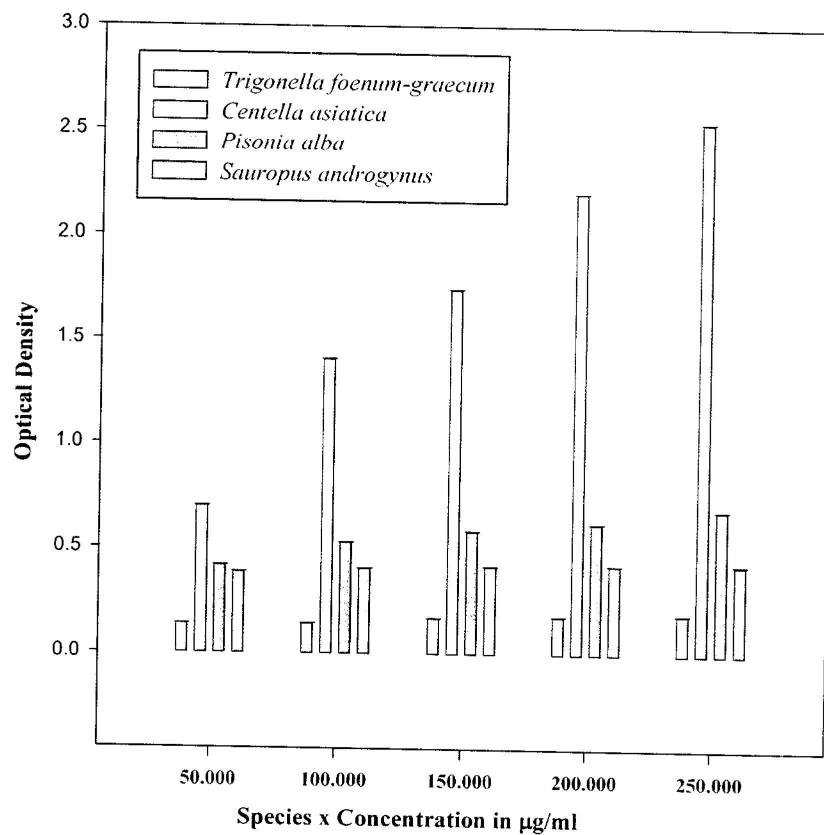

Figure 4.1.7.2. Mean absorbance of acetone extracts of plants species

Species	Mean absorbance of plant extracts across the concentration range	
	Methanol extract	Acetone extract
T.f	0.900 ± 0.001	0.164 ± 0.0008
C.a	1.291 ± 0.001	1.718 ± 0.0008
P.a.	0.906 ± 0.001	0.570 ± 0.0008
S.a	0.532 ± 0.001	0.414 ± 0.0008

Table 4.1.7.5. Mean absorbance across the concentration range for methanol and acetone extracts of plant species.

The values are expressed as the mean ± SE for three replicates

T.f: *Trigonella foenum-graecum*; **C.a:** *Centella asiatica*; **P.a**: *Pisonia alba*; **S.a**: *Sauropus androgynus*

The mean values across the concentrations indicate that the methanol and acetone extracts of ***Centella asiatica*** exhibit the maximum reducing power.

Phase II

4.2. Levels of non-enzymatic antioxidants

4.2.1. Levels of Flavonoids and Total phenols

Flavonoids are large compounds occurring in all plants. They occur as glycosides and contain several phenolic hydroxyl groups on their ring structures. Many flavonoids are found to be very good free radical scavengers and antioxidants (Scott & Slater, 1981; Robak & Gryglewski, 1988; Rafat *et al.*, 1987). Food-derived flavonoids such as the flavonols like Quercetin, Myrecetin have been reported to reduce the risk of cancer (Verma *et al.*, 1988; Yoshida *et al*, 1990). Flavonols are known to act as both free radical scavengers (Bors & Saran, 1987) and also as metal chelators (Afanaslejev *et al.*, 1989). Hence they can play a crucial role in the prevention of many deadly diseases.

Polyphenolic compounds have been reported to have multiple biological effects, including antioxidant activity (Kahkonen *et al.*, 1999). The antioxidant activity of the phenolics is mainly due to their redox properties, which enable them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators (Rice-Evans *et al.*, 1995).

Species	**Flavonoids* (mg/g tissue)**	**Total phenols # (mg/g tissue)**
Trigonella foenum-graecum	3.11 ± 0.112	16.28 ± 0.100
Centella asiatica	5.99 ± 0.068	9.91 ± 0.135
Pisonia alba	13.04 ± 0.165	6.20 ± 0.209
Sauropus androgynus	15.17 ± 0.066	19.21 ± 0.164

Table 4.2.1. Levels of Flavonoids and total phenols in plant species

The values represented are mean ± S.D. of three replicates

*Flavonoids are expressed as rutin equivalents.

#Total phenols are expressed as gallic acid equivalents.

Statistical analysis:

One-way ANOVA was carried out. A statistically significant difference ($P = <0.001$) was found among the mean values of plant species in the determination of flavonoids and total phenols.

The DMRT analysis was carried out and pair wise comparisons among the plant species and the following observations were made.

For flavonoids, all pair wise comparisons were found to be statistically significant.

Mean differences are statistically significant at $P=0.05$ level

For total phenols, all pair wise comparisons were found to be statistically significant.

Mean differences are statistically significant at $P=0.05$ level

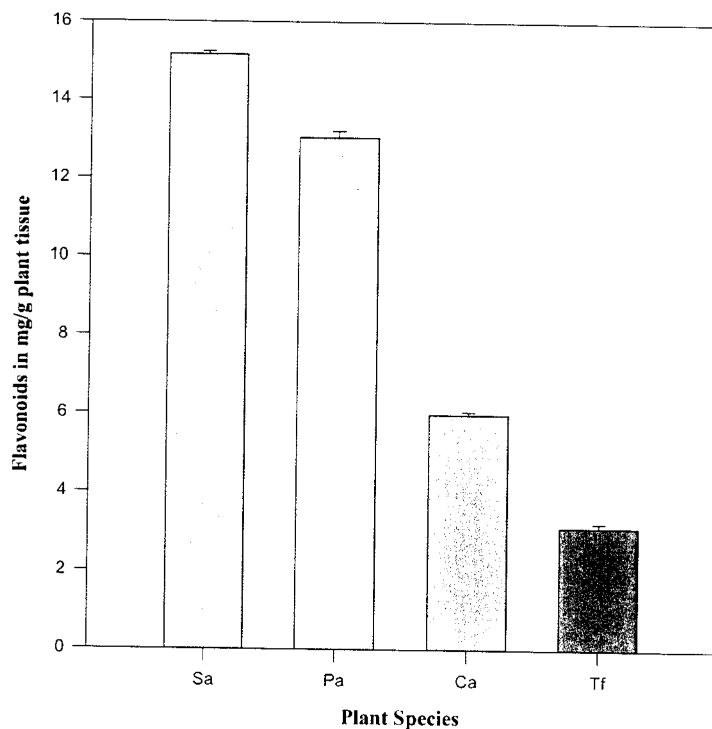

Figure 4.2.1.1. Levels of Flavonoids present in the various plant species

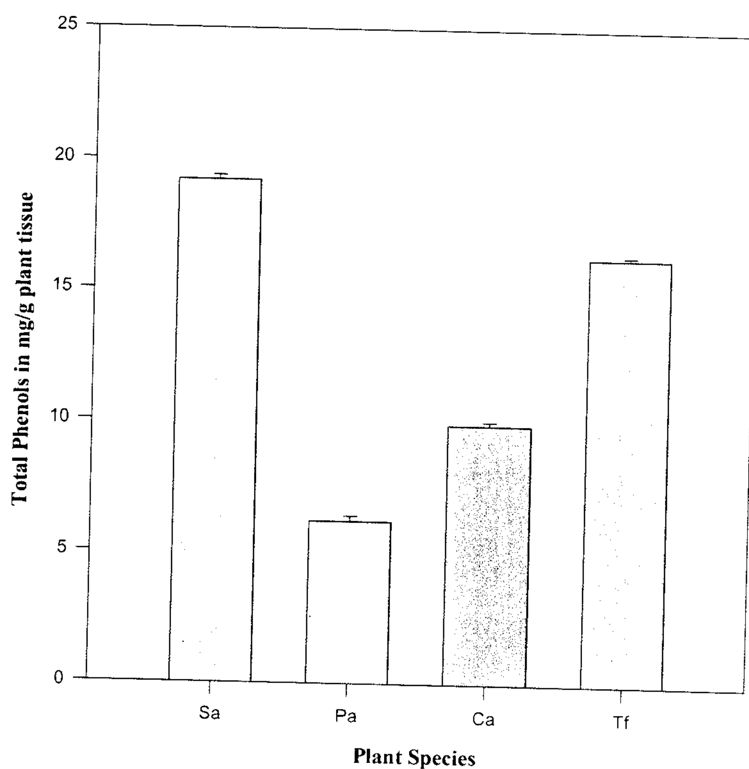

Figure 4.2.1.2. Levels of total phenols present in the various plant species

The results obtained indicate that the levels of Flavonoids and Total phenols are the highest in ***Sauropus androgynus.*** This suggests that both flavonoids and total phenols together may be responsible for the free radical scavenging activity of the plant. On the other hand, ***Trigonella foenum-graecum*** has the lowest levels of flavonoids, but has an equal content of total phenols as *Sauropus androgynus*. This may suggest that total phenols have a greater role to play in the free radical scavenging activity of this plant. ***Pisonia alba*** exhibits high levels of flavonoids and has low levels of total phenols. So, it might be inferred that the former plays a major role in the plant's antioxidant activity. ***Centella asiatica*** has moderately equal levels of both flavonoids and total phenols. (Zao Xin *et al.*, 2004).

4.2.2. Levels of Total Carotenoids and Lycopene

Green leafy vegetables are rich sources of carotenoids. Carotenoids, the tetraterpenoid compounds (C_{40}), are ubiquitous compounds in plants. These terpenoids existing as hydrocarbons (carotenes) or oxygenated derivatives, are accessory pigments in photosynthetic systems and give characteristic color to plants, particularly fruits and flowers.

Lycopene is a carotene. Certain amount of antioxidant activity may be attributed to these compounds in the plant leaves.

Species	Total Carotenoids (mg/ g sample)	Lycopene (mg/ 100 g sample)
Trigonella foenum-graecum	0.106± 0.239	0.287± 0.006
Centella asiatica	0.059 ± 0.051	0.343± 0.004
Pisonia alba	0.094 ± 0.046	0.151± 0.033
Sauropus androgynus	0.245± 0.324	0.293± 0.000

Table 4.2.2. Levels of total carotenoids and lycopene in plant species

The values represented are mean ± S.D. of three replicates

Statistical analysis:

One-way ANOVA was carried out. A statistically significant difference ($P = <0.001$) was found among the mean values of plant species in the determination of total carotenoids and lycopene.

The DMRT analysis was carried out and pair wise comparisons among the plant species and the following observations were made.

For total carotenoids, all pair wise comparisons except that of *T. foenum-graecum* and *Pisonia alba* were found to be statistically significant.

Mean differences are statistically significant at $P=0.05$ level

For lycopene, all pair wise comparisons except that of *T. foenum-graecum* and *Sauropus androgynus* were found to be statistically significant.

Mean differences are statistically significant at $P=0.05$ level

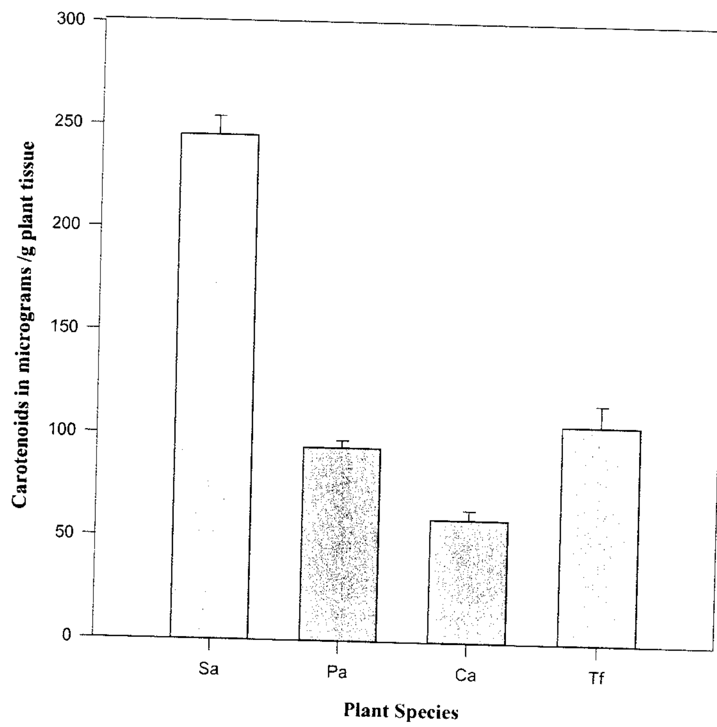

Figure 4.2.2.1. Levels of total carotenoids in the plant species

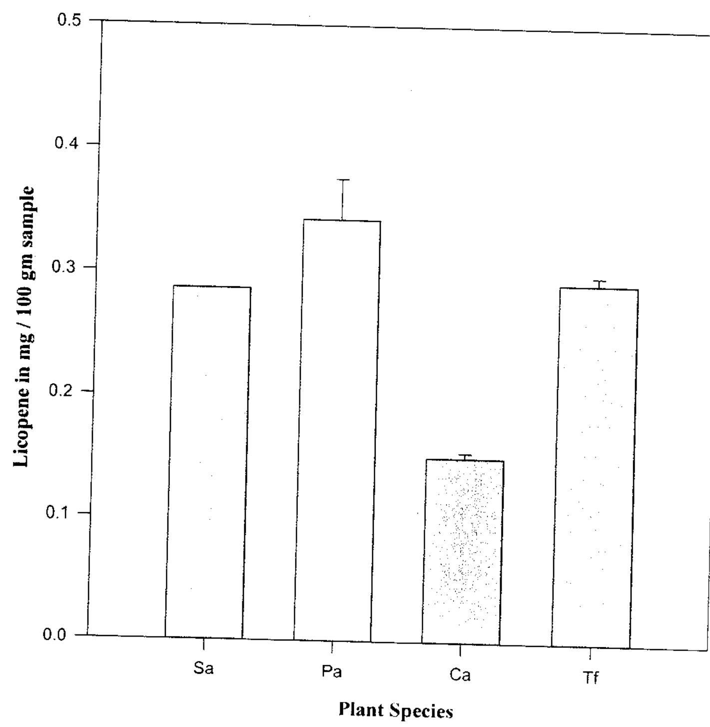

Figure 4.2.2.2. Levels of Lycopene in the plant species

The plant ***Sauropus androgynus*** has the maximum amount of total carotenoids. The plant with the highest levels of lycopene was found to be ***Pisonia alba.***

The levels of total carotenoids in most of the green leafy vegetables ranges from 10 to 35 mg/100 g plant tissue (Sheetal Gupta *et al.,* 2003). The values obtained in the experiment conform to this predetermined range of total carotenoid content. ***Sauropus androgynus*** has been found to possess about 24.5 mg /100 g plant tissue. Similarly the other plants also have been found to contain total carotenoid levels that fall within this range.

4.2.3. Levels of Vitamin C and β-carotene

The two are the major antioxidant components present in many plant species. They are major antioxidants and have crucial roles in the quenching of free radicals and thereby terminating the electron 'stealing' reactions. Thereby they play important roles in the defense against free radical damage.

Species	Levels of Vitamin C (mg/g tissue)	Levels of β-carotene* (mg/g tissue)
Trigonella foenum-graecum	1.009± 0.0318	0.0139± 0.0003
Centella asiatica	0.715± 0.0483	0.0235± 0.0006
Pisonia alba	0.928± 0.0234	0.0310± 0.0006
Sauropus androgynus	0.469± 0.0385	0.1560± 0.0002

Table 4.2.3. Levels of Vitamin C and β-carotene in plant species

The values represented are mean ± S.D. of three replicates

* β-carotene is expressed as equivalents of retinol.

Vitamin A (provitamin A carotenoids) = 6 μg β-carotene + 12 μg α-carotene

Statistical analysis:

One-way ANOVA was carried out. A statistically significant difference (P = <0.001) was found among the mean values of plant species in the determination of levels of Vitamin C and β-carotene.

The DMRT analysis was carried out and pair wise comparisons among the plant species and the following observations were made.

For Vitamin C, all pair wise comparisons were found to be statistically significant.

Mean differences are statistically significant at P=0.05 level

For β-carotene, all pair wise comparisons were found to be statistically significant.

Mean differences are statistically significant at P=0.05 level

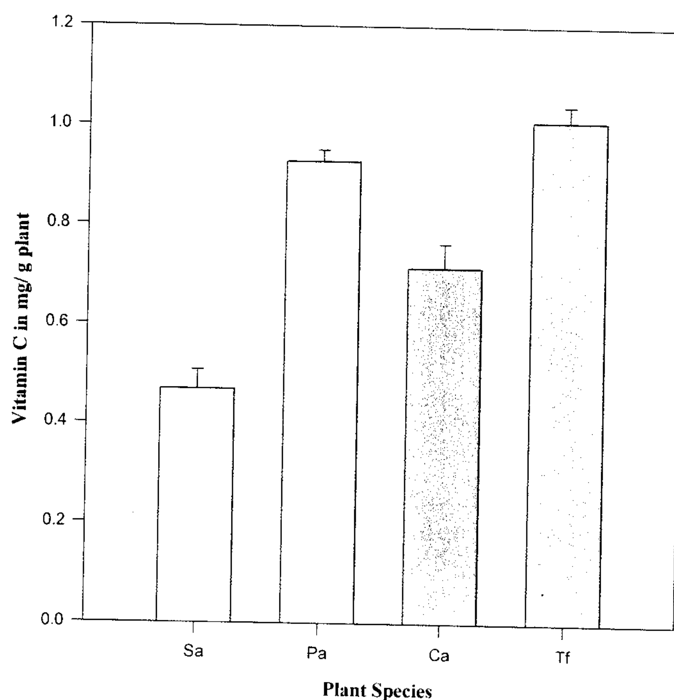

Figure 4.2.3.1. Levels of Vitamin C in the plant species

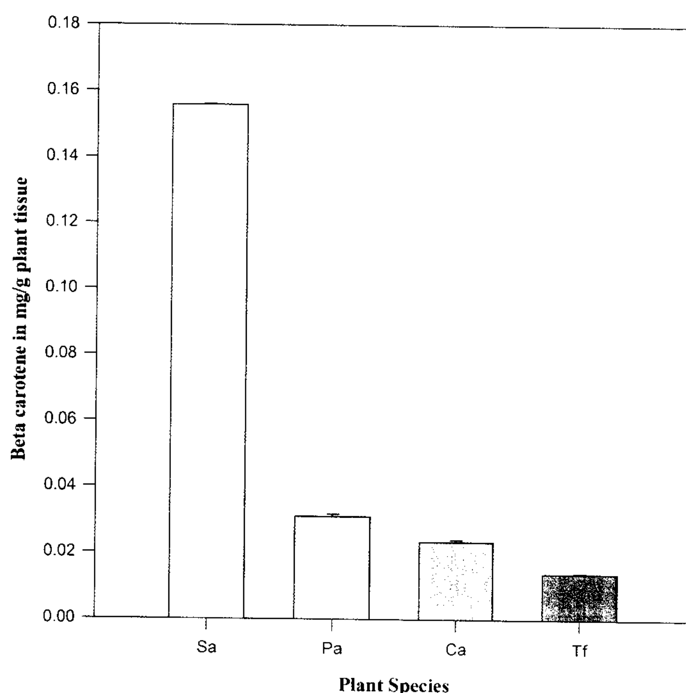

Figure 4.2.3.2. Levels of β-carotene in the plant species

High levels of Vitamin C have been found in all the four plant species. ***Trigonella foenum-graecum*** has been found to possess the highest levels of Vitamin C. ***Pisonia alba*** has been found to have almost equal levels of Vitamin C. All the plant species have however been observed to possess lower levels of β-carotene.

Sauropus androgynus has the maximum content of β-carotene. It has already been proved that the plant possesses really high values of Vitamin A. (Saskia de Pee *et al.*, 1998). The levels of β-carotene are expressed here as retinol equivalents. This implies that the result obtained in this section of the experiment is consistent with the previously recorded observations. The green leafy vegetables normally contain levels of β-carotene that are 5.21-14.05 % (Rajyalakshmi *et al.*, 2001) or 13-25 % (Sheetal Gupta *et al.*2003), of the total carotenoid levels in the plants. The values obtained for the levels of β-carotene fall approximately within the specified range.

The Ascorbic acid values range from 8.7-88.3 mg /100 g in fresh greens (Mamatha Kumari *et al.*, 2003). The results obtained for the selected plant species show that the Ascorbic acid levels range from 46-100 mg /100 g plant tissue, which conforms to the previously made assessments.

4.2.4. Correlation and regression analysis

Correlation and regression analysis was performed to determine the interrelationship between the non-enzymatic antioxidants present in the plants and the free radical scavenging potential displayed by the plant species in the phase I of the project. There are several non-enzymatic antioxidants in the plants. The main compounds analysed were Flavonoids, total phenols, total carotenoids, lycopene, Vitamin C and β-carotene. The statistical analysis between the non-enzymatic antioxidants and the total antioxidant capacity of the plant extracts in terms of ascorbic acid equivalents enables the determination of the major components among these antioxidants actually responsible for the radical quenching activity of the plant extracts.

The Flavonoids were found to have a very significant positive influence in the antioxidant activity of both the methanol and acetone extracts across all the plant species (Methanol extracts: $r^2 = 0.799$ and $P < 0.01$ level; Acetone extracts: $r^2 = 0.766$ and $P < 0.01$ level). On the other hand total phenols showed no significant statistical correlation with the total antioxidant activity of the various plant extracts. Hence it can be inferred that the total phenols weren't significantly responsible for the antioxidant traits of the plant extracts. The total carotenoids however, play a significant role in the extracts' antioxidant capacity (Methanol extracts: $r^2 = 0.851$ and $P < 0.01$ level; Acetone extracts: $r^2 = 0.310$ and $P<0.05$ level). Thus carotenoids have a relatively crucial role as antioxidants. The lycopene in methanol extracts of the plants makes negligible contribution to the antioxidant activity as per the conclusions drawn from the analysis. But the acetone extracts show a statistically significant contribution of the lycopene to the total antioxidant activity (Methanol extracts: not significant; Acetone extracts: $r^2 = 0.642$ and $P<0.01$). Vitamin C does not show a positive correlation with the antioxidant activity of the methanol extracts. And its contribution is not statistically significant in the case of the acetone extracts. It has already been established that a reduction in Vitamin C content does not reduce the free radical scavenging ability of the plant leaves nor does this influence the reducing potential of the extracts (Oboh *et al.*, 2004). Hence the correlation obtained by statistical analysis of the observations conforms to the previously established result. i.e., Vitamin C may not be the major contributor of the antioxidant activity of the plant extracts. β-carotene on the other hand, displays a significant

correlation to the antioxidant activity of only the methanol extracts (Methanol extracts: $r^2 = 0.905$ and $P < 0.01$ level; Acetone extracts: not significant).

These results can be used to deduce the importance of the contribution of non-enzymatic antioxidants to the antioxidant activity of the various plant extracts.

CONCLUSION

5. CONCLUSION

Antioxidants are compounds in fruits and vegetables, which help in avoiding chronic diseases. They act as a defense system against oxidative damage in our bodies and may help in avoiding chronic diseases. The present work has been undertaken to study about the free radical scavenging ability of four species of green leaf vegetables, namely, *Trigonella foenum-graecum, Centella asiatica, Pisonia alba and Sauropus androgynus.* The primary study carried out in our work has confirmed the free radical scavenging potential of the plant extracts and also the presence of non-enzymatic antioxidants in the plant leaves. The free radical scavenging ability of the plant extracts was tested against various free radicals generated *in vitro* and the results obtained were analyzed statistically. On the whole, all the plant species exhibited a good antioxidant activity. Then, the plant species were analyzed for the presence of the various non-enzymatic antioxidants like flavonoids, total phenols, Vitamin A, Vitamin C, carotenoids and lycopene. All the plant species were found to possess significant levels of these compounds. Correlation and regression analyses were carried out to determine the components responsible for the antioxidant potential of these plant extracts. Thus from our study, we conclude that the non-enzymatic antioxidants present in the plant species make significant contributions to the antioxidant potential of the plants. A statistical analysis of ranks of the species with respect to free radical scavenging potential was carried out. The study indicated that the plant species under the present study are equally potent with no significant differences in their ability to scavenge various free radicals.

The present study was mainly aimed at studying free radical scavenging ability *in vitro*. The future perspectives of the study will be devoted to the extraction and purification of the various non-enzymatic antioxidants and also to the accomplishment of *in vivo* studies in animals and the assessment of the capability of the plants to exhibit the free radical scavenging activity in living biological systems.

APPENDICES

APPENDIX 1

ESTIMATION OF DPPH RADICAL SCAVENGING ACTIVITY

Principle

DPPH scavenging activity was measured by the slightly modified spectrophotometric method slightly modified. 1,1-diphenyl-2-picryl-hydrazyl radical is scavenged by antioxidants through the donation of protons forming reduced DPPH. The electrons become paired off and the solution loses color depending on the number of electrons taken up. The color changes from purple to yellow after reduction, which is quantified by the decrease of absorbance at 517nm (Baskar *et al.*, 2007).

Reagents

1. Methanol
2. DPPH in methanol (6×10^{-5} M)

Procedure

The plant extracts were dissolved in methanol. The acetone extract was not fully soluble in methanol. So the filtrate was used. A solution of DPPH in methanol (6×10^{-5}M) was prepared freshly. 3 ml of this solution was mixed with 100 μl of the samples of varying concentrations (50-250 μg/ml). The solution in the test tubes were shaken well and incubated in the dark for 15 min. at room temperature. The decrease in absorbance was measured at 517 nm. The control had equal volume of methanol instead of the extract. 3.1 ml of methanol was taken as the blank. The percentage inhibition of the radicals due to the antioxidant property of the extract was calculated using the formula:

$$\% \text{ inhibition} = [\ (A_{control} - A_{sample}) / A_{control}\ ] \times 100\%$$

APPENDIX 2

ESTIMATION OF ABTS CATION RADICAL SCAVENGING ASSAY

Principle

The ABTS (2,2- azobis 3- ethylbenzothiazoline-6- sulfonic acid diammonium salt) assay is based on the inhibition of the absorbance of ABTS radical cation, which has a characteristic long wavelength absorption spectrum (Baskar *et al.*, 2007).

Reagents

1. ABTS (7 mM)
2. Ammonium persulfate (2.45 mM)
3. Methanol

Procedure

The assay was performed by a slightly modified protocol. ABTS radical was produced by reacting ABTS solution (7mM) with ammonium persulfate (2.45mM) and the mixture was allowed to stand in the dark at room temperature for 12-16 hours to give a dark colored solution. The absorbance was measured at 745nm. The initial absorbance was found to be around 3.18. This stock solution was diluted with methanol to give a final absorbance value of around 0.7 (±0.02) and equilibrated at 30 °C. Different concentrations of the sample (50-250 μg/ml) were prepared by dissolving the extracts in water. About 0.3ml of the sample was mixed with 3ml of ABTS working standard in a microcuvette. The decrease in the absorbance was measured after mixing the solution in one-minute intervals up to 6min. The final absorbance was noted. A solution of ABTS working standard and 0.3ml of methanol was used as the control. About 3ml of methanol was used as blank. The percentage inhibition was calculated according to the formula:

$$\% \text{ inhibition} = [(A_{control} - A_{sample}) / A_{control}] \times 100\%$$

APPENDIX 3

ESTIMATION OF NITRIC OXIDE RADICAL SCAVENGING ASSAY

Principle

Nitric Oxide scavenging was measured spectrophotometrically. The nitric oxide generated using sodium nitroprusside is converted to nitrite ions. The chromophore is formed due to the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthyl ethylene diamine. This is measured at 546 nm (Raghavan Govindarajan *et al.*, 2003).

Reagents

1. Sodium nitroprusside (5mM)
2. Phosphate buffer saline
3. Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride).

Procedure

Sodium nitroprusside (5mM) was prepared in phosphate buffer saline. 1ml of this was mixed with 1ml of extracts of different concentrations (50-250 μg/ml). The extracts were prepared by dissolving the residue in methanol. The mixture was incubated at 25 °C for 30 min. After 30 min., an equal volume of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added to the incubated solution. The absorbance of the chromophore formed due to diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine was measured at 546nm (Raghavan Govindarajan *et al.*, 2003). Control was a solution of reagents devoid of the extracts.

APPENDIX 4

ESTIMATION OF HYDROXYL RADICAL SCAVENGING ASSAY

Principle

Hydroxyl radical scavenging activity was measured by the ability of the different fractions of plant samples to scavenge the hydroxyl radicals generated by the Fe^{3+} - ascorbate- EDTA-H_2O_2 system (Fenton reaction). These damage the substrate deoxyribose, which undergoes degradation to form malondialdehyde. This produces a pink chromogen with TBA. The antioxidants inhibit this reaction and hence there is a decrease in the color intensity of the chromogen as the antioxidant potential increases (Umamaheshwari *et al.*, 2008).

Reagents

1. 2-deoxyribose (28mM in 20mM KH_2PO_4 buffer, pH 7.4)
2. 1.04mM EDTA
3. $FeCl_3$
4. 1.0mM hydrogen peroxide
5. 1.0mM ascorbic acid
6. 1% TBA
7. 2.8% TCA

Procedure

The reaction mixture with a final volume of 1.0 ml contained 100 µl of 2-deoxy-2-ribose (28mM in 20mM KH_2PO_4 buffer, pH 7.4), 500 µl of the fractions at various concentrations (50-250 µg/ml) in water, 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$, 100 µl of 1.0mM hydrogen peroxide (H_2O_2) and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37 °C for 1 hour. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. 1ml of 1% thiobarbituric acid (TBA) and 1ml of 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100 °C for 20min. After cooling, the absorbance was measured at 532nm (Umameheshwari *et al.*, 2008) against a blank containing deoxyribose and buffer. Deoxyribose degradation was measured as TBARS against a control with buffer instead of the sample. The percentage inhibition was calculated.

APPENDIX 5

DETERMINATION OF LIPID PEROXIDATION INHIBITION ASSAY

Principle

Initiation of lipid peroxidation by ferrous sulfate takes place through the hydroxyl radical formation by Fenton's reaction. These produce malondialdehyde (MDA), which reacts with TBA to form a pink chromogen. The inhibition of lipid peroxidation could take place due to the scavenging of the hydroxyl radicals/superoxide radicals or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself (Baskar *et al.*, 2007).

Reagents

1. Goat liver
2. Phosphate buffer saline (pH 7.4)
3. 0.07M ferrous sulphate
4. 20% acetic acid (pH 3.5)
5. 0.8% TBA in 1.1% SDS
6. 20% TCA
7. butan-1-ol

Procedure

Goat liver was washed thoroughly in cold phosphate buffered saline (pH 7.4). This was then minced in a mortar and pestle with a measured volume of cold buffer in ice. The minced liver was then homogenized in a homogenizer to give a 10% homogenate. The homogenate was filtered using cheesecloth to remove unwanted residue. The filtrate was then centrifuged at 10,000rpm for 10 min. under refrigerated conditions. The supernatant was used for the assay. 0.5ml of this 10 %homogenate was taken. To this, 0.5ml of the extracts (50-250 μg/ml) in water was added. The volume of the reaction mixture was made up to 1ml with distilled water. To this, 0.05ml of 0.07 M ferrous sulfate was added. The solution was incubated at room temperature for 30min. To the incubated solution, 1.5ml of 20% acetic acid (pH3.5), 1.5ml of 0.8% TBA (in 1.1% SDS) and 0.05ml of 20% TCA were added. The tubes were vortexed to ensure appropriate mixing. Then the tubes were incubated at 100 °C

for 1 hour. The tubes were then cooled to room temperature. About 5ml of Batan-1-ol was added to each tube. The solution was mixed well and centrifuged at 3000 rpm for 10 min. The upper layer was withdrawn and used to read the absorbance at 532 nm. The control contained PBS instead of the sample. The percentage inhibition was calculated.

APPENDIX 6

TOTAL ANTIOXIDANT ASSAY ACTIVITY

Principle

The assay is based on the principle of reduction of Molybdenum (VI) to Molybdenum (V) by the extract and the subsequent formation of a green phosphate/molybdenum (V) complex at acid pH.

Reagents

1. Reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammounium molybdate)
2. Ascorbic acid

Procedure

The working standards (50-250 μg/ml) of the plant samples were prepared by dissolving the extracts in water. 0.1ml of the sample was mixed with 1ml of the reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were capped with silver foil and incubated at 95°C for 90min. The tubes were then cooled to room temperature and the absorbance was measured at 695nm against a blank. Ascorbic acid was used as the standard. Total antioxidant capacity is expressed as equivalent of ascorbic acid (Raghavan Govindarajan *et al*., 2003; Umamaheshwari *et al*., 2008).

APPENDIX 7

DETERMINATION OF FERRIC REDUCING ANTIOXIDANT POTENTIAL ASSAY

Reagents

1. Phosphate buffer (0.2M. pH 6.6)
2. 1% potassium ferricyanide
3. 10% TCA
4. 0.1% ferric chloride

Procedure

Different concentrations of the samples (50-250 μg/ml) were prepared by dissolving the extracts in water. 2.5ml of the samples were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The tubes were incubated at 50 °C for 20min. To the incubated solutions, 2.5ml of 10% TCA was added. The solutions were centrifuged at 650 g for 10min. About 5ml of the supernatant was withdrawn from each tube. To this, 1ml of 0.1% Ferris chloride was added. The absorbance was measured at 700 nm. A higher absorbance indicated a higher reducing power. The blank was chosen as 5 ml of buffer with 1ml of ferric chloride.

APPENDIX 8

ESTIMATION OF FLAVONOIDS

Reagents

1. Methanol
2. Sodium nitrite
3. Aluminium chloride
4. 1M NaOH

Procedure

1g of the leaves were weighed and ground with 20ml of 80% aqueous methanol in a mortar and pestle. The grinded sample was filtered with the help of a Whatman filter paper no.42 and a clear sample solution was obtained. 0.5ml of this sample was taken in a test tube to which 1.25ml of distilled water was added. 0.075 ml of 5% sodium nitrite was then added to the test, mix well and allow the mixture to stand for 5 min. Then 0.15ml of 10% Aluminium chloride was added. After 6min., 0.5ml of 1M NaOH was introduced into the test tube. The solution in the test tube was then diluted with 0.275ml of distilled water. The absorbance was read at 510nm .

APPENDIX 9

ESTIMATION OF TOTAL PHENOLS

Principle

Phenols react with phosphomolybdic acid in Folin-Ciocalteau reagent in alkaline medium and produce a blue colored complex (molybdenum blue) that can be estimated colorimetrically at 650nm. This assay is preferred because the plant leaves contain large quantities of polyphenols (Zao Kin *et al.*, 2004).

Reagents

1. Ethanol
2. Folin- Ciocalteau reagent
3. Sodium carbonate

Procedure

1g of the leaves were weighed and ground with 10ml of 80 % aqueous ethanol in a mortar and pestle. The ground sample was then centrifuged at 5000 rpm for 40 min/ 1000rpm for 20 min. The supernatant obtained from the centrifuge was poured into separate tubes. Re-extract the pellets in 5ml ethanol. This sample was then again centrifuged at 5000rpm for 20min. Collect and pool the supernatant. The supernatants were then allowed to boil to evaporate the ethanol. The residue obtained was then dissolved in 10ml of distilled water.

The estimation of total phenols was carried out by taking 0.1ml of the prepared sample and making it upto 3 ml with distilled water. 0.5ml of Folin – Ciocalteau reagent was added to the sample. Incubate the mixture for 3min. 2.0ml of 20% sodium carbonate is then added. Then the sample is boiled in water bath for a minute and the sample is read at 650nm.

APPENDIX 10

DETERMINATION OF LEVELS OF VITAMIN A ASSAY

Reagents

1. 2N KOH
2. 90% alcohol
3. Petroleum ether
4. Sodium sulfate (anhydrous)
5. Chloroform
6. TCA

Procedure

1.5 g of the sample was taken. It was ground to a fine paste with H_2O. 1.0 ml of 2N KOH in 90 % alcohol was added. The mixture was refluxed for 20min at 60°C. The tubes were cooled to room temperature. 20 ml H_2O was added to the test tubes and mixed well. β-carotene was extracted with 10 ml portions of petroleum ether in a separating funnel four times. The extract (organic layer) was pooled and sodium sulfate (anhydrous) was added to remove the moisture and left for 30-60 min. 5ml aliquots of the ether extract were taken and evaporated to dryness at 60°C. The dried residue was dissolved in 1.0ml chloroform.

Estimation was carried out as follows. Aliquots of the standard Vitamin A acetate were pipetted out to a series of clean, dry test tubes in concentration range (1.5 – 7.5 μg/ml). The volume in each test tube was made upto to 1.0 ml with chloroform. 2.0ml TCA was added to this. The contents were mixed well. The absorbance was recorded immediately at 520 nm. The amount of Vitamin A/g tissue of the sample was calculated.

APPENDIX 11

DETERMINATION OF LEVELS OF VITAMIN C ASSAY

Reagents

1. Oxalic acid
2. Activated charcoal solution
3. DNPH
4. 80% H_2SO_4

Procedure

5g of sample was ground in 25-50ml of 4% oxalic acid. The sample was centrifuged at 10000 rpm for 20 min. 10ml aliquots were transferred to conical flask. A few drops of activated charcoal solution were added. The solution was made up to 25 ml with 4% oxalic acid.

Estimation was carried out. 1ml aliquots of the above solution were taken and 3ml of the distilled water was added. To the above solution, 1ml of DNPH was added. This was incubated at 37°C for 3 hours.7ml of 80% H_2SO_4 (in ice) was added to the test tubes and they were incubated at room temperature for 30 min. The absorbance was read at 540nm. (Poteggente et al., 2002).

APPENDIX 12

DETERMINATION OF LEVELS OF TOTAL CAROTENOIDS AND LYCOPENE ASSAY

Principle

The total carotenoids in the sample are extracted with petroleum ether. The total carotenoids are estimated spectrometrically at 450 nm.

Lycopene has absorption maxima at 473 nm and 503 nm. A rapid method for the estimation of lycopene in plant products is based on the measurements of absorption of the petroleum ether extract of the total carotenoids at 450 nm. After measuring the total carotenoids at 450nm, the same extract can be used for estimating lycopene at 503nm.

Reagents

1. 12% KOH
2. Ethanol
3. Petroleum ether
4. Sodium sulfite

Procedure

5-10g of sample (1g of sample +10ml H2O, filtered ~ 1hr) was considered which is saponified for 30min in a shaking water bath at 37°C in 12% KOH in ethanol. Transferred the sample to a separating funnel containing 10-15ml of Petroleum ether. Mixed gently in the separating funnel. Allowed it to stand till the layers are separated completely. Collected the pigments in the petroleum layer. Transferred this to separating funnel and extract using petroleum ether- homogenous phase. Repeated the extraction of the aqueous phase with petroleum ether until it was colorless. Discarded the aqueous layer. To the extract, added a small quantity of sodium sulfide to remove turbidity. Final volume of extract noted to known volume. Read absorbance at 450nm and 503nm.

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