ANTIOXIDANT PROPERTIES OF THE LEAVES AND STEMS OF *Aristolochia indica*

BY

PRIYA.V
REG. NO: 07PB10

A THESIS SUBMITTED TO THE

AVINASHILINGAM UNIVERSITY FOR WOMEN
COIMBATORE – 641 043

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY

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Certified As Bonafide Research Work

Parvatham 30/4/09
Signature of
Head of the Department

30/04/09
Signature of the
Guide

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1.0 INTRODUCTION

Lifestyle related diseases of stroke, cancer, heart diseases, diabetes, kidney diseases and hypertension have been considered to be associated with reactive oxygen species including free radicals (Hiramatsu *et al*., 2008). In modern western medicine, the balance between antioxidants and oxidation is believed to be a critical concept in maintaining a healthy biological system (Tarhan *et al.,* 2007). Oxidation processes are very important for the living organism. Uncontrolled production of reactive oxygen species (ROS) and the unbalanced mechanism of antioxidant protection result in onset of many diseases and accelerate aging (Gulcin *et al.,* 2007).

Reactive oxygen species (ROS) including superoxide radical, hydroxyl radical, singlet oxygen and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors and molecules are responsible for cellular injury and aging processes (Wu *et al.,* 2008). ROS are generated by normal metabolic processes in all organisms utilizing oxygen (Pereira *et al.,* 2008).

Ko and Leung (2007) said that under both normal and pathological conditions, ROS are generated in all cells undergoing aerobic metabolism, particularly from mitochondria. The cell possesses two distinct antioxidant defense systems to counteract damaging ROS such as enzymic antioxidants and non-enzymic antioxidants.

Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume neutral antioxidants from food supplements and traditional medicines (Yazdanparast *et al.,* 2008).

It has long been recognized that plant polyphenols are important class of defense antioxidants. These compounds are widespread in virtually all plants, often at high level and include phenols, phenolic acids, flavonoids, tannins and lignans (Boussaada *et al.,* 2007).

Herbal formulations are getting popularity throughout the world and commercialized extensively for various medicinal properties (Rai *et al.,* 2008). The most practical and frequently used traditional remedy is preparing an infusion or a decoction from the valuable parts of plants and herbs such as flowers, leaves and roots (Buyukbalci and Nehir, 2008).

Herbals may be effective in human disease. Herbals contain a myriad of potentially biologically active compounds. Some of the

constituents of the plant such as triterpenoids and flavonoids were shown to possess antidiabetic, antioxidant and related biological activities (Vessal *et al.,* 2003). It was reported that 60-80 per cent of the population in every developing countries of the world relies on medicinal plants in the treatment of some diseases (Smith*,* 2009).

Antioxidants such as plant phenolics are sought for general health maintenance, anti-aging and chemoprevention (Kelen and Tepe, 2008). Antioxidant activity of plant is known to be mainly provided by phenolic acids and flavonoids. Phenolic acids have been repeatedly implicated as natural antioxidants in fruits, vegetables and other plants (Pyo *et al.,* 2004).

Pure compounds, found in *R. echinocarpa* fruit, have showed antioxidant, antimutagenic and anticarcinogenic activites (Suhaj, 2006). Moreover, several *Rubiaceae* plants such as *Uncaria tormentosa* and *Morinda citrifolia* have been used traditionally, around the world for the prevention of cancer (Samoylenko *et al.,* 2006).

The *Aristolochiaceae* family contains about 400 species in 7 genera of cosmopolitan distribution, many of them of economic importance due to aristolochic acids and terpenoids. The phenolic such as terpenoids are the important components present in *Aristolochia indica* (Thirugnanasampandan *et al.,* 2008).

The root of the plant is used in indigenous system of medicines as an antidote for the snake bites, gastric stimulant and bitter tonic. Among the tribal inhabitants, the roots are ground with black pepper seeds and made into pills administered to treat rheumatism and diabetes (Goverdhan *et al.,* 2008).

The present study was designed to investigate the influence of herbal medicines on the "Antioxidant properties of the leaves and stems of *Aristolochia indica*" with the following objectives

1. To assess the levels of antioxidants present in *Aristolochia indica.*
2. To analyze the extent of inhibition of lipid peroxidation in the aerial parts of *Aristolochia indica*.
3. To determine the free radical scavenging effects of *Aristolochia indica.*

2.0 REVIEW OF LITERATURE

Medicinal plants have been traditionally used in the treatment of several human diseases and their pharmacological and therapeutic properties have been attributed to different chemical composition (Mentreddy, 2007). Plants are the main source of bioactive metabolites with antimutagenic and anticarcinogenic activities (e.g. phenolics, quinines, glucosinolates, alkyl sulfides, terpenoids and alkaloids) and several attitudes have showed a relationship of these activities with antioxidant capacity (Knasmuller *et al.,* 2004). Thus the antioxidant properties of plants have a full range of perspective applications in human health care (Silva *et al.,* 2005).

The review of literature related to the study "Antioxidant properties

of the leaves and stems of *Aristolochia indica*" is discussed under the following headings

2.1 Free radical damage
2.2 Oxidative stress
2.3 Lipid peroxidation
2.4 Antioxidants
2.5 Medicinal plants and diseases

2.1 Free radical damage

A free radical is any chemical species that contain one or more unpaired electrons and they are generated as a part of the body's normal metabolic process (Saha *et al.,* 2008).

Free radicals or oxidative injury now appears the fundamental mechanism underlying number of human neurologic and other disorders (Atawodi, 2004).

When oxygen is supplied in excess or when its reduction is insufficient, reactive oxygen species such as superoxide anions, hydroxyl radicals and hydrogen peroxide are generated in high quantities (Djeridane *et al.,* 2007). They can cause the oxidation of biomolecules (protein, aminoacids, lipid and DNA) which leads to cell injury and death (Shahsavari *et al.,* 2008). The deteriorative effects of free radicals can be diminished by natural antioxidants available in foods (Sokmen *et al.,* 2004).

Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are naturally occurring intracellular metabolic byproducts that are known to cause oxidative damages to a number of cellular molecules (Chi *et al.,* 2007). ROS are toxic to cells since they oxidize cell membranes and other biological

molecules (Rio *et al.,* 2003).

ROS in the forms of superoxide anion (O_2^-) hydroxy radical ($OH^\bullet$) and hydrogen peroxide (H_2O_2) are generated by normal metabolic process or from exogenous factors and agents, and they are very unstable and react rapidly with other groups or substances in the body resulting in cell or tissue damage (Qi *et al.,* 2005).

The pathogenesis of micro and macro vascular diseases in diabetes is closely linked to mechanisms identified in injury due to hypertension and hyperlipidemia that center on ROS generation (Catharine and Whiteside, 2005).

2.2 Oxidative stress

Stressful conditions can precipitate anxiety and depression which can lead to excessive production of free radicals which in turn result in oxidative stress, an imbalance in the oxidant or antioxidant system (Zhang, 2004).

However, major phytochemicals, phenolic acid, flavonoids, coumarin derivatives are known to combat oxidative stress in the human body by helping to maintain a balance between oxidants and antioxidants (Shibano *et al.,* 2008).

A substantial amount of evidence has demonstrated that diabetes is an oxidative stress disorder (King and Loekan, 2004). Hyperglycemia can induce oxidative stress through these four mechanisms: advanced glycation end product (AGE) formation, increased flux through the polyol pathway, increased activation of protein kinase C (PKC), and increased flux through the hexosamine pathway (Nishikawa *et al.,* 2000).

2.3 Lipid peroxidation

Lipid peroxidation is a complex process ocuring in aerobic cells and reflects the interaction between molecular oxygen and polyunsaturated fatty acids (Hayet *et al.,* 2008).

Oxidative stress, leading to an increased production of reactive oxygen species (ROS) as well as lipid peroxidation. Due to their high polyunsaturated lipid content, schwann cells and axons are particulary sensitive to oxygen free radical damage. Therefore lipid peroxidation may increase cell membrane rigidity and impair cell function (Pari and Latha, 2004).

In living systems, biomembranes are composed of lipids, including unsaturated fatty acids that react easily to form lipid peroxides and free radicals. The accumulation of lipid peroxides in living systems induces functional anomalies and pathological changes (Nadaroglu *et al.,* 2007).

Lipid peroxidation in some biomembranes and suppress selectively receptor mediated phospholipadse A_2 activation (Rackova *et al.,* 2007). Of particular importance, oxidative stress has been implicated in the installation and progression of several degenerative disorders via either DNA mutation, protein oxidation or lipid peroxidation (Valko *et al.,* 2007).

Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups. In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Kumarappan and Mandal, 2008).

2.4 Antioxidants

Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions or alternatively scavenging the free radicals generated during oxidative destruction (Syvacy and Sokemen, 2004). Antioxidants neutralize direct ROS attacks, terminate free radical-mediated oxidative reaction and protect the human body from ROS damage (Gulcin *et al.,* 2003).

During lipid oxidation, antioxidants act in the various ways, binding metal ions, scavenging radicals and decomposing peroxides (Moure *et al.,* 2001). In addition under pathological conditions or oxidative stress, ROS are overproduced and cause peroxidation of membrane lipids and the accumulation of lipid peroxides. However, they are removed by antioxidant defense mechanism (Gulcin *et al.,* 2006a).

Plants have evolved different defensive systems against environmental stresses such as salinity, drought, temperature, pollutants, metal toxicity or ultraviolet-B (UV-B) radiation which generate highly reactive oxygen species (Agarwal and Pandey, 2004). The capability of antioxidants to hamper the oxidation process is determined by the fact that their composition includes weakly bound mobile hydrogen or functional groups that actively react with molecular oxygen or with free radicals that form in the oxidation process (Popovich, 2008).

Reduced glutathione and ascorbate which are found at high concentrations in chloroplasts and other cellular compartments, are crucial for plant defense against oxidative stress (Chamseddine *et al.,* 2009).

Hence, the importance of research aimed at finding natural antioxidants has greatly increased in recent years (Schaffer *et al.,* 2005). Many researches have focused on natural antioxidants and in the plant kingdom numerous crude extracts and pure natural compounds were previously reported to have antioxidant properties (Ozen and Kinalioglu, 2008).

Enzymic Antioxidants

Various herbal extracts could protect organs against CCl_4 induced oxidative stress by altering the levels of increased lipid peroxidation and enhancing the decreased activities of antioxidant enzymes (Rajesh and Latha, 2004).

The main ROS-scavenging enzymes in plants include superoxide dismutase, catalase, ascorbate peroxidase, monodehydro ascorbate reductase, glutathione peroxidase, glutathione reductase, NADPH oxidase and peroxi redoxins (a family of hydro peroxide - detoxifying enzymes recently described in plants) (Apel and Hirt, 2004).

In the presence of reduced iron (Fe^{2+}), H_2O_2 can be reduced to the highly reactive hydroxyl ($OH^{\cdot}$) radical. This radical attacks any and all molecules in the cell. Catalase and glutathione peroxidases (GPx) mediate their effects by degrading H_2O_2 and avoiding the generation of $OH^{\bullet}$ (Schriner and Linford, 2006).

Catalase

Catalase is an enzyme, mainly found in peroxisomes where H_2O_2 created during photorespiration (Chatzissavvidis *et al.,* 2008).

$$2\ H_2\ O_2 \longrightarrow 2\ H_2\ O + O_2$$

The enzyme also has peroxidase activity and reacts with organic peroxides and hydrogen donors to water and organic alcohols (Wassman *et al.,* 2006).

Peroxidase

Peroxidase belongs to a group of enzymes reducing H_2O_2 to H_2O and oxidizing co-substrates. Measurements of the antioxidant enzyme activity may provide information concerning the degree of exposes of plant tissues to reactive oxygen species and the link existing between salt stress and ROS (Erturk *et al.,* 2007).

$$H_2O_2 + AH_2 \longrightarrow 2H_2O + A$$

In the presence of peroxide, peroxides from plant tissues are able to oxidize a wide range of phenolic compounds such as guaiacol, pyrogallol, chlorogenic acid, catechin and catechol (Onsa *et al.,* 2004).

Superoxide dismutase

Superoxide dismutase converts the ROS superoxide to hydrogen peroxide, which is then transferred into water by catalase in lysosomes or by glutathione peroxidase in the mitochondria (Johansen *et al.,* 2005). The formation of ROS during the oxidative burst could cause a substrate overload of superoxide dismutase leading to its inhibition superoxide dismutse induction can also be hindered probably by other factors like the end product (Blockhina *et al.,* 2003).

Glutathione reductase

Glutathione reductase is a flavoenzyme, located in the choloroplasts, cytosol and mitochondria that catalyses the glutathione

disulfide reduction in NADPH-dependent reaction. Glutathione reductase activity supports the regeneration of both glutathione and ascorbate (Melchiorre *et al.,* 2009).

Glutathione-S-transferase

Glutathione-S-transferase catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis (Sinha *et al.,* 2007). Their reactive metabolites formed via the cytochrome P450 monooxygenase system (Andallu and Varadacharyalu, 2003).

Glutathione peroxidase

Glutathione peroxidase, a selenium-dependent enzyme, decomposes peroxides using the peptide glutathione (GSH) as their co-substrates (Ran *et al.,* 2007).

Glutathione peroxidase is also considerable to be an important hydrogen peroxide removing enzyme in mammalian cells and is more important than catalase for removing hydrogen peroxide. Glutathione peroxidase is involved in the defense mechanisms against oxidative damage, it reduces the hydrogenperoxide and hydroperoxide levels (Sivalokanathan *et al.,* 2006).

Non - enzymic antioxidants

Plants possess a complex battery of enzymic and non-enzymic antioxidants that protect cells from oxidative damage by scavenging ROS (Murgia *et al.,* 2004). Non-enzymic antioxidants include vitamins such as vitamin A, C and E found in the everyday diet. These vitamins directly detoxify free radicals (Yim *et al.,* 2007).

The function of ROS as signaling molecules is intrinsically related

to the interaction with non-enzymic antioxidants, such as ascorbate and glutathione, which are redox buffers and also signaling molecules (Foyer and Noctor, 2005).

Ascorbic acid (Vitamin C)

Ascorbic acid is a small, water-soluble antioxidant molecule, used as substrate for ascorbate peroxidase which catalyzes hydrogenperoxide detoxification. Ascorbic acid oxidation always leads monodehydro ascorbate (MDHA) which is normally converted to ascorbic acid by monodehydro ascorbate reductase (MDHAR) (Mittova *et al.,* 2000).

It is an abundant antioxidant in various plant tissues. L-Galactono-1, 4-Lactone dehydrogenase (GLDH) and ascorbate oxidase (AO), two key enzymes in ascorbic acid synthesis and degradation, as well as enzymes in ascorbic acid-reduced glutathione cycle influence the metabolism of ascorbic acid (Song *et al.,* 2006).

α-tocopherol (Vitamin E)

Vitamin-E represents a spectrum of atleast eight independent lipophilic molecules (four tocopherols and four tocotrienols) with antioxidant activity that are enriched in plant derived foods such as plant oils, seeds or grains and peanuts (Schneidar, 2005).

Tocopherols and tocotrienols are free radical scavengers that can suppress lipid peroxidation and DNA damage, but these agents in particular also influence arachidonic acid and prostaglandin metabolism. Therefore, they may also have some role in suppressing prostate inflammation (Nelson *et al.,* 2002).

In addition, protection of membrane integrity against oxidative

stress is guaranteed by the tocopherol pool, which is kept in its reduced state by the pool of ascorbic acid (Valpuesta and Botella, 2004).

Carotenoids

Carotenoids are polyisoprenoids that are invaluable for photoprotection during oxygenic photosynthesis and as antioxidants. They are responsible for the orange color of carrots and oranges, the red color of tomato, and many of the bright yellow and orange hues in flowers and serve as precursors of vitamin A and the plant hormone abscisic acid (Bartley, 2009).

Reduced glutathione

Reduced glutathione, an intermediary redox metabolite in the ascorbate-glutathione cycle of scavenging hydrogen peroxide, is maintained in the reduced state by glutathione reductase, using NADPH as a co-factor (Arbona *et al.,* 2003). Oxidative stress is paralleled by impaired antioxidant mechanisms, glutathione is one of these important systems (Borges *et al.,* 2002).

2.6 Medicinal plants and diseases

Medicinal plants are important options for developing drugs for the treatment of different diseases (Kastenholz, 2008). For example, standardized plant extracts from green leaves of the *Ginkgo biloba* tree are generally accepted in the treatment of Alzheimer's disease (Elsabagh *et al.,* 2005).

Some medicinal plants have been shown to have both chemopreventive and therapeutic effects on breast cancer (Mantle *et al.,* 2000) and skin cancer (F'guyer *et al.,* 2003). The

meadosweet flowers are officially recommended for use in medicne as an anti-inflammatory, wound-healing and astringent remedy. The results of experiments showed that extracts of this plant decrease the permeability of capillaries and produce pronounced anticoagulant, antiulcerous, antidiabetic and antitumor effects (Shilova *et al.,* 2006).

Researchers have estimated that every serving increase in fruit and vegetable consumption reduces the risk of cancer. This has been confirmed by epidemiological studies (Gupta and Prakash, 2008). Luo *et al.* (2004) reported that antioxidants from water extracts of *Lycium barburum* fruits and polysaccharides may play a synergistic role in their hypolipidemic effect. Gupta *et al.* (2005) have reported that several green leafy vegetables are rich antioxidant vitamins. Brahmi (*Centella asiatica*), curry leaf (*Murraya koenigii*), fenugreek (*Trigonella foenum graecum*) and keerae (*Amaranthus sp.)* are used in Indian culinary and are also known for their medicinal value.

3.0 EXPERIMENTAL PROCEDURE

There is an increasing interest to survive a healthy life, using traditional plants is presented as an alternative medicine and most of the people use these plants for their everyday health care needs. Although some of the therapeutic properties attributed to plants have been proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years (Fakim *et al.,* 2005). *Aristolochia indica* is commonly known as Garudakkodi in the southern part of India. This plant is known to possess certain medicinal properties and is used in the preparation of ayurvedic products.

The present study was undertaken to analyse the levels of enzymic and non-enzymic antioxidants and to determine the *in vitro* free radical scavenging activity in the leaves and stems of *Aristolochia indica*. The methodology adopted for the study is given below

3.1 Collection of sample

3.2 Biochemical analysis

3.3 Determination of the activities of enzymic antioxidants

3.4 Assessment of the levels of non-enzymic antioxidants

3.5 Determination of *in vitro* free radical scavenging activity

3.6 Statistical analysis

3.1 COLLECTION OF SAMPLE

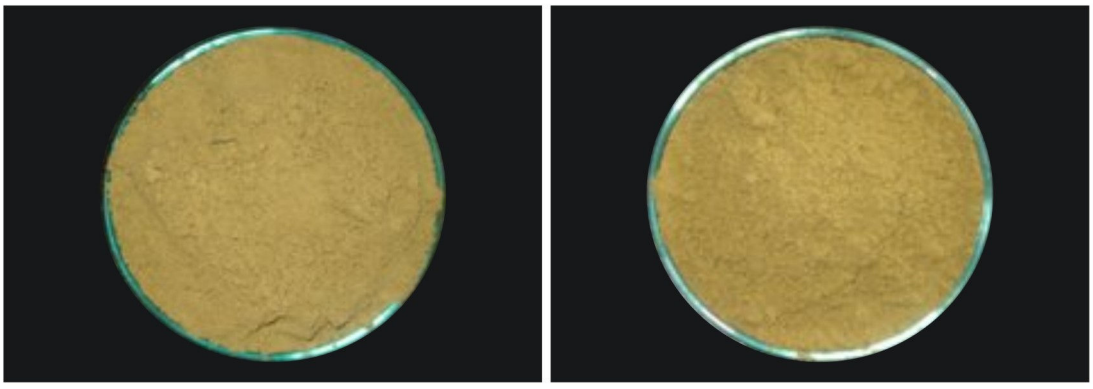
The plant sample of both leaves and stems were collected from local market in Coimbatore. The plants obtained were washed, shade dried, powdered to coarse size and stored at room temperature. Dried leaf powder and stem powder were taken separately for the experiment (Plate 1 and Plate 2).

Plate 1

Aristolochia indica

Plate 2

Leaf and stem powder of *Aristolochia indica*



Leaf powder Stem powder

3.2 BIOCHEMICAL ANALYSIS

3.2.1 Estimation of carbohydrate

Estimation of carbohydrate was done according to the method of Hedge and Hofrieter, 1962. The steps involved in the procedure are given in appendix- I.

3.2.2 Estimation of protein

Protein was estimated by the method of Lowry *et al.* 1951 as explained in appendix- II.

3.3 DETERMINATION OF THE ACTIVITIES OF ENZYMIC ANTIOXIDANTS

The activity of antioxidant enzymes can be considered as an important mechanism in the cellular defense strategy against oxidative stress (Shi *et al.,* 2006). Antioxidant enzymes such as superoxide dismutase, peroxidase and catalase are considered to be the main protective compounds engaged in the removal of free radicals and active oxygen species (Khan and Panda, 2002).

3.3.1 Catalase

The enzyme catalase catalyzes the conversion of H_2O_2 into water. Catalase activity in the sample was assessed by the method of Luck (1974). The detailed procedure is given in appendix- III.

3.3.2 Superoxide dismutase

Superoxide dismutase, a suppressing enzyme of superoxide anions, was determined by the method of Misra and Fridovich (1972). The procedure for the determination of activity of superoxide dismutase is presented in appendix- IV.

3.3.3 Glutathione reductase

Glutathione reductase catalyzes the reduction of the oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH+H^+ as substrate (Caballero *et al.,* 2006). Glutathione reductase was estimated by the method of David and Richard (1983). The steps involved in the procedure are given in appendix- V.

3.3.4 Glutathione-S-transferase

Glutathione-S-transferase bind to liophilic compounds and acts as an enzyme for reduced glutathione conjugation reactions (Manna *et al.,* 2006). Glutathione-S-transferase was determined by the method proposed by Habig *et al.* (1974). The detailed procedure is given in appendix- VI.

3.3.5 Glutathione peroxidase

The method of Rotruk *et al.* (1973) was followed to assess the activity of glutathione peroxidase and it is given in appendix- VII.

3.4 ASSESSMENT OF THE LEVELS OF NON-ENZYMIC ANTIOXIDANTS

Non-enzymic antioxidants ascorbate (ASA) and reduced glutathione (GSH) react with singlet oxygen, superoxide and hydroxyl radicals (Agarwal, 2007) and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Miller *et al.,* 2000).

3.4.1 Ascorbic acid

Ascorbic acid, which is a H_2O soluble antioxidant forage free radical protect the biological system from oxidative stress (Nirmal *et al.,* 2008). Ascorbic acid, a scavenger of oxyradicals, was

estimated by the method of Roe and Kuether (1953) as explained in appendix-VIII.

3.4.2 α-tocopherol

α-tocopherol was estimated by the method proposed by Rosenberg (1992). The steps involved in the procedure are given in appendix- IX.

3.4.3 Total carotenoids

A modified version of the procedure proposed by Zakaria *et al.* 1979 was used for the estimation of total carotenoids in the plant. The detailed procedure is given in appendix- X.

3.4.4 Flavonoids

Flavonoids are one of the most numerous and widespread group of phenolics in higher plants (Tepe *et al.,* 2005). Pietta (2000) suggesting that plant flavonoids, which show potent activity *in vitro*, also function as antioxidants *in vivo* and their protective effects may be attributed to their scavenging ability towards free radicals. The flavonoid content of the sample was estimated by the method of Cameron *et al,* (1943), as it is described in appendix- XI.

3.4.5 Reduced Glutathione

Estimation of glutathione was done according to the procedure described by Moron *et al.* 1979. The details of the procedure are given in appendix- XII.

3.5 DETERMINATION OF in *vitro* FREE RADICAL SCAVENGING ACTIVITY

Free radicals are highly reactive molecules with an unpaired electron and are produced by radiation or as byproducts of metabolic

processes (Devi *et al.,* 2008). Antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus reduce the level of oxidative stress and prevent the development of complications associated with oxidative stress-related diseases (Wu and Hansen, 2008).

3.5.1 Preparation of plant extract

The 5 g of powdered aerial parts of *Aristolochia indica* was extracted with petroleum ether, benzene, chloroform, acetone, methanol and water. The extract was evaporated to dryness under boiling water bath to obtain the dry extracts. Each extract were diluted in dimethyl sulfoxide and stored in the refrigerator until use. 0.001g of exracts per 20 µl were taken for the assay.

3.5.2 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) scavenging activity

1, 1-Diphenyl-2-picryl hydrazyl is a stable nitrogen centered free radical which can be effectively scavenged by antioxidants (Villano *et al.,* 2007). The leaves and stems of *Aristolochia indica* were checked for their antioxidant property using the biological end point of *in vitro* DPPH scavenging activity by the method of Mensor *et al*. (2001) as described in appendix- XIII.

3.5.3 Determination of inhibition of *in vitro* lipid peroxidation

Lipid peroxide is a complex process. It involves the formation and propagation of lipid peroxide and the eventual destruction of membrane lipids, producing the breakdown products such as malondialdehyde in microsomes (Zhang *et al.,* 2003). Lipid peroxides were estimated *in vitro* using the method of Okhwa *et al.* (1979). Appendix- XIV gives the details of the method.

3.5.4 Determination of inhibition of superoxide generation

Superoxide anion is also very harmful to cellular components (Hazra *et al.,* 2008). Inhibition of superoxide generation was estimated by the method of Mc Cord and Fridovich (1968). The procedure of the method is presented in appendix- XV.

3.5.5 Determination of inhibition of nitric oxide generation

Nitric oxide is an important bioregulatory molecule. Low concentration of nitric oxide is sufficient in controlling blood pressure, neural signal transduction, platelet function, antimicrobial and antitumor activity. However, during infectious and inflammations, formation of nitric oxide is elevated and may bring about some undesired deleterious effects like renal dysfunction and tumor growth (Kumaran and Karunakaran, 2006). Inhibition of nitric oxide generation was determined by the method of Green and Hill (1984). The details of the procedure are given in appendix-XVI.

3.5.6 Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups (Devi *et al.,* 2008). Hydrogen peroxide scavenging activity was estimated *in vitro* using the method of Ruch *et al.* (1989) as described in appendix- XVII.

3.6 Statistical analysis

All data were expressed as mean ± SD. The results were subjected to statistical evaluation with student's t- test for significance between the examined antioxidant activities in leaves and stems of *Aristolochia indica.*

4.0 RESULTS AND DISCUSSION

Reactive oxygen species (ROS) are generated in aerobic organisms during physiological or physiopathological oxidative metabolism of mitochondria. ROS may react with a variety of biomolecules, including lipids, carbohydrates, proteins, nucleic acids and macromolecules of connective tissue, thereby interferring with all function. Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems. Impairment in the oxidant or antioxidant equilibrium provokes a situation of oxidative stress and generally results from hyperproduction of ROS. Oxidative stress is known to be component molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases (Valko *et al.,* 2007).

The use of natural antioxidants for the treatment and prophylaxis of free radical induced pathology has certain advantages. Plants are sources of natural antioxidants and known to be rich in biologically active substances such as flavonoids, phenolic acids, anthocyanins, ethereal oils and tannins (Korotkova *et al.,* 2003).

Aristolochia indica was reported to have various biological properties, including potent antioxidant activity. However, no information about the antioxidant activity of *Aristolochia indica* is available in the literature. Hence, the present study was carried out to analyse the "Antioxidant properties of leaves and stems of *Aristolochia indica*". The results pertaining to the study is discussed as follows

4.1 Quantification of nutritional components

The nutritional components such as carbohydrate and protein content in *Aristolochia indica* were quantitatively determined. The results obtained are given in Table 1 and Figure 1.

TABLE 1

NUTRITIONAL COMPONENTS IN *Aristolochia indica*

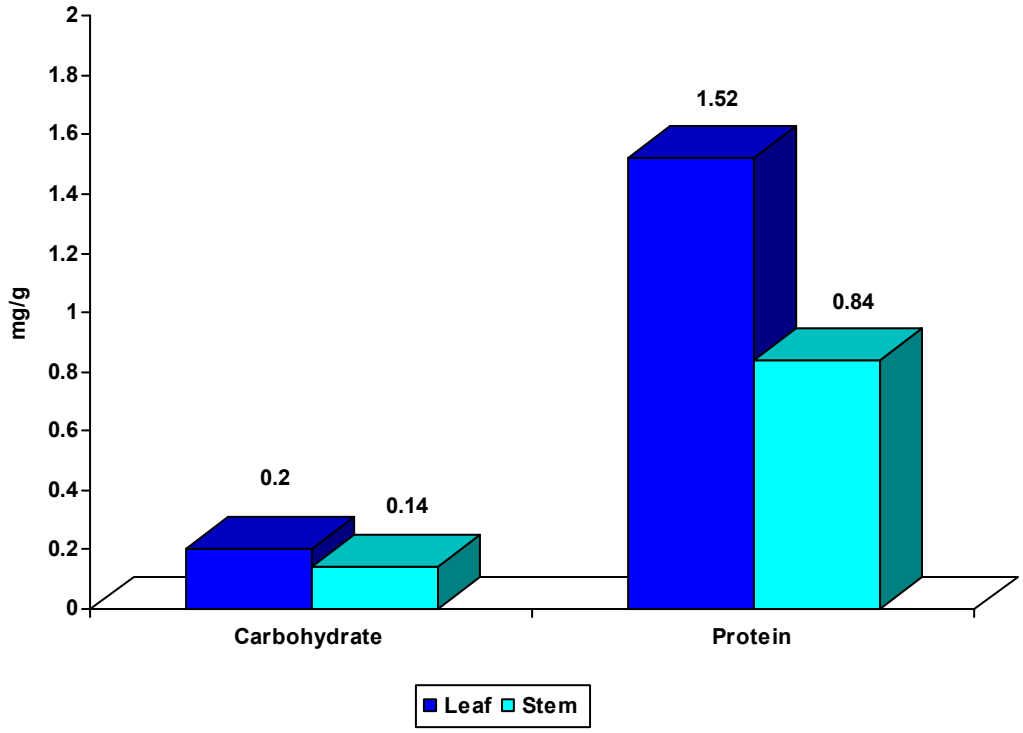
Components (mg/g)	*Aristolochia indica*		
	Leaf	**Stem**	**t value**
Carbohydrate	0.20 ± 0.06	0.14 ± 0.004	1.29^{ns}
Protein	1.52 ± 0.38	0.84 ± 0.05	2.13*

Values are mean ± SD of triplicates * - Significant at $p < 0.05$ level

ns - not significant

FIGURE 1

NUTRITIONAL COMPONENTS IN *Aristolochia indica*



The carbohydrate and protein content of leaf was higher than that of the stem. Certain carbohydrate derivatives are used as drugs like cardiac glycosides or antibiotics and proteins are the main structural components of the cytoskeleton. *Aristolochia indica* is found to contain moderate quantities of carbohydrate and protein which are necessary for human health maintenance.

4.2 Antioxidant potential of the plant samples

Antioxidants occur in all higher plants in all parts of the plant, such as wood, bark, stems, pods, leaves, fruits, roots, flowers and seeds. Thus development of safer natural antioxidants that can replace synthetic ones has been of interest (Pathirana and Shahidi, 2006).

The antioxidant activity of plants are mainly contributed by phenolic and flavonoid compounds (Syvacy and Sokmen, 2004). Plants evolved protective enzymic and non-enzymic mechanisms to scavenge ROS and to alleviate their deleterious effects. The enzymic mechanism includes catalase, peroxidase and superoxide dismutase, while several molecules such as glutathione, ascorbate and carotenoids provide non-enzymic protection (Chatzissavvidis *et al.,* 2008).

4.2.1 Enzymic antioxidants

Activities of various enzymic antioxidants like catalase, superoxide dismutase, glutathione reductase, glutathione-S- transferase and glutathione peroxidase in *Aristolochia indica* were tested and the results obtained are depicted in the following tables and figures.

4.2.1.1 Catalase and superoxide dismutase

Activites of catalase and superoxide dismutase are presented in Table 2 and Figure 2.

TABLE 2

ACTIVITIES OF CATALASE AND SUPEROXIDE DISMUTASE IN *Aristolochia indica*

Enzymic antioxidants (U/g)	*Aristolochia indica*		
	Leaf	Stem	t value
Catalase[1]	235 ± 13.9	257 ± 27	1.02^{ns}
Superoxide dismutase[2]	6.5 ± 2.1	11.5 ± 0.4	9.01*

Values are mean ± SD of triplicates * - Significant at 0.05 level

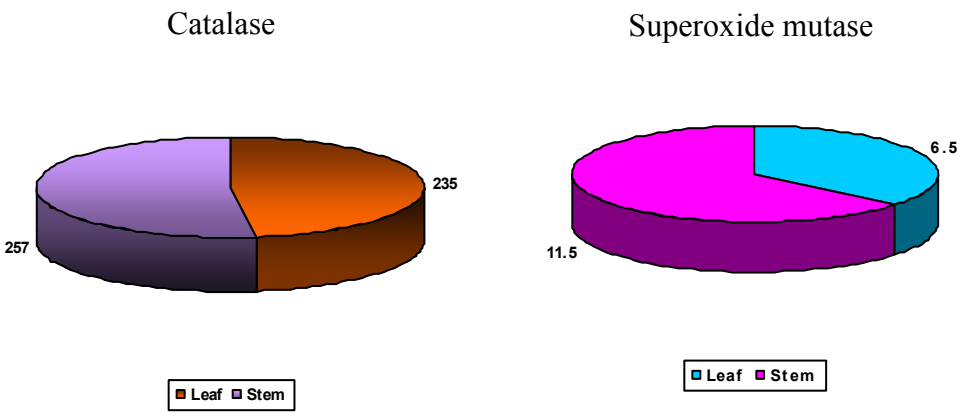
ns - not significant

1 - Amount of enzyme required to the optical density by 0.05

2 - Amount that cause 50 per cent reduction in the extent of NBT oxidation.

FIGURE 2

ACTIVITIES OF CATALASE AND SUPEROXIDE DISMUTASE IN *Aristolochia indica*



From the above table and figure, it is clear that *Aristolochia indica* has considerable activity of catalase than that of superoxide dismutase. Among the leaves and stems, the stems shows higher activity of catalase

than that leaves. Leaves of *Aristolochia indica* exhibited the minimum activity of superoxide dismutase (6.5 ± 0.5U/g).

Catalase is responsible for the destruction of hydrogen peroxide generated by superoxide dismutase (Hu *et al.,* 2007) and superoxide dismutase is a metallo enzyme containing Cu/Fe or Mn in its prosthetic groups. Superoxide dismutase catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen (Ali *et al.,* 2002).

Catalase activity was found to be decreased significantly in the skin of mice which received *Azadirachta indica* leaf extract. Activity of superoxide dismutase decreased significantly in tumorous tissues (Koul *et al.,* 2006).

Catalase (Sharma *et al.,* 2004), superoxide dismutase and peroxidase (Wu *et al.,* 2003) have been shown to be activated by cadmium treatment. Studies revealed that intracellular protection of cytoplasmic components against phagocyte derived oxidative injury is metabolized predominantly by antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase (Irshad and Chaudhari, 2002).

The alcoholic and aqueous extract of *Plagiochasma appendiculatum*, which is used by treating skin diseases by Gaddi tribe in Kangra valley has antioxidant activity. The results indicated that *Plagiochasma appendiculatum* extract possesses potent antioxidant activity by inhibiting lipid peroxidation and increase in superoxide dismutase and catalase activity (Singh *et al.,* 2006).

4.2.1.2 Xenobiotic enzymes

The xenobiotic enzymes such as glutathione reductase, glutathione-S-transferase and glutathione peroxidase catalyzes the conjugation of variety of phase I metabolites to the reduced glutathione producing non reactive water soluble and readily excretable substances, thereby preventing the xenobiotic-induced cell toxicity (Forbes *et al.,* 2003). The activites of these enzymes in *Aristolochia indica* were assessed and the results obtained are presented in Table 3 and Figure 3.

TABLE 3

ACTIVITIES OF XENOBIOTIC ENZYMES IN *Aristolochia indica*

Enzymic antioxidants (U/g)	***Aristolochia indica***		
	Leaf	**Stem**	**t value**
Glutathione reductase [1]	11.3 ± 2.5	5.6 ± 1.04	2.98*
Glutathione-S-transferase[2]	0.6 ± 0.2	0.3 ± 0.05	2.134*
Glutathione peroxidase[3]	0.5 ± 0.04	0.4 ± 0.01	3.38*

Values are mean ± SD of triplicates * - Significant at 0.05 level

ns - not significant

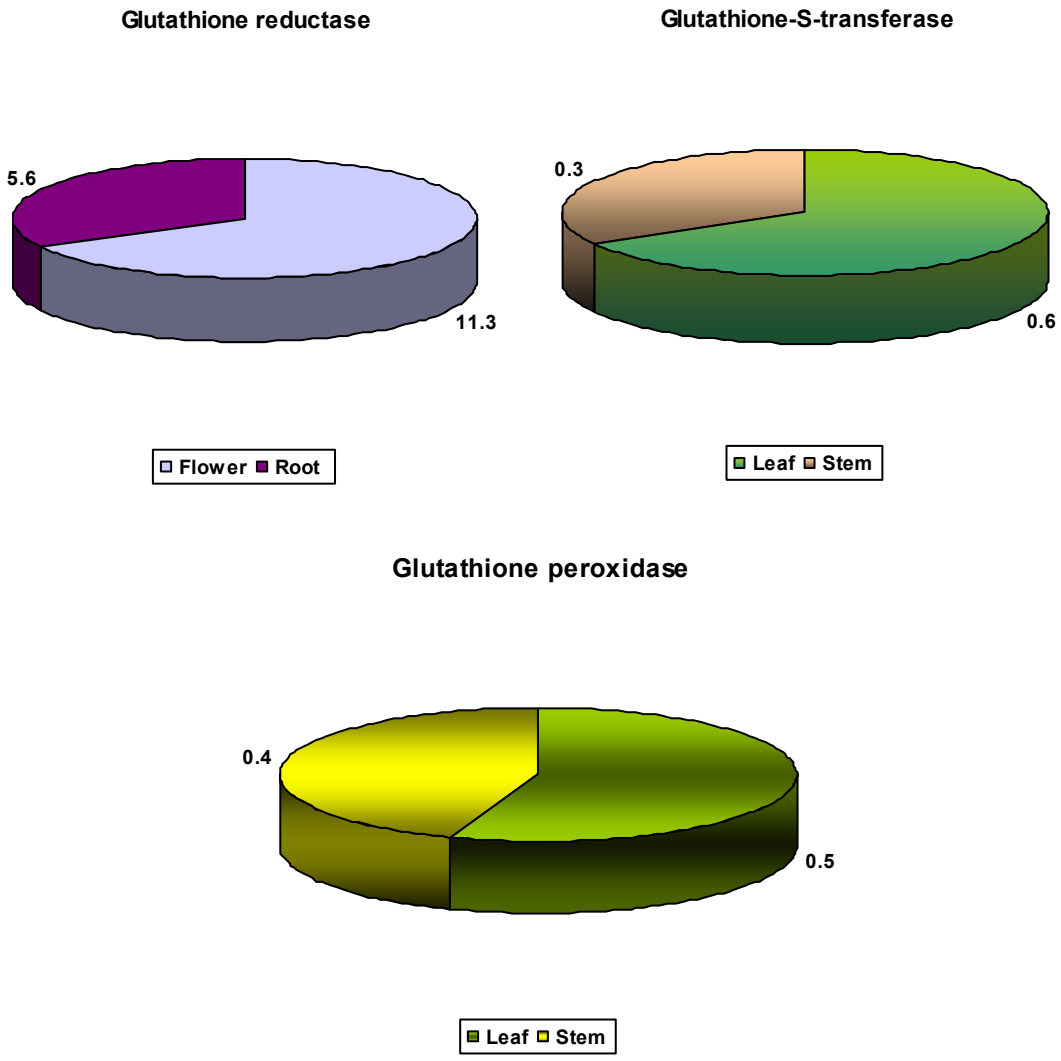
1 - μ moles of CDNB conjugated / minute

2 - μ moles of NADPH utilized

3- μ moles of GSH utilized / minute.

FIGURE 3

ACTIVITIES OF XENOBIOTIC ENZYMES IN *Aristolochia indica*



From the above table and figure, it is clear that the activity of glutathione reductase is higher than that of glutathione-S-transferase and glutathione peroxidase. The leaves exhibited the highest activity of glutathione reductase (11.3 ± 2.5U/g). The extracts of leaf and stem exhibited moderate glutathione peroxidase activity.

Reduced glutathione - depending antioxidant reactions are likely influenced by changes in glutathione reductase activity, eliciting alterations in the cell oxidative state (Nusetti *et al.,* 2005). In comparison to catalase, glutathione peroxidase is part of a more complex enzyme system. Inorder to degrade H_2O_2, these proteins require another compound, reduced glutathione, which participates in its own reactions independent of glutathione peroxidase (Thongsook and Burret, 2005).

The phytotoxicity imposed by cadmium (Cd) and its detoxifying responses of *Bacopa monnieri* L. have been investigated. Induction of phytochelatins and enzymic and non-enzymic antioxidants were monitored as plants primary and secondary metal detoxifying responses, respectively. Enzyme viz., superoxide dismutase, glutathione reductase and ascorbate peroxidase which showed stimulation except catalse which showed declining trend. Besides synthesis of phytochelatins, availability of reduced glutathione and concerned activity of glutathione reductase seem to play a central role for Bacopa plants to combat oxidative stress caused by metal and to detoxify it (Mishra *et al.,* 2006).

Since, *Aristolochia indica* contains significant activities of all the enzymic antioxidants analysed, it may reduce the risk of serious diseases caused by reactive oxygen species like malignancy, cardiovascular diseases and severe neural diseases.

4.2.2 Non-enzymic antioxidants

Non-enzymic defense involves glutathione, ascorbic acid, α-tocopherol, β-carotene and other compounds capable of quenching reactive oxygen species (Razinger *et al.,* 2007).

Table 4 and Figure 4 presents the levels of the non-enzymic antioxidants such as ascorbic acid, α-tocopherol, total carotenoids and reduced glutathione in the leaves and stems of *Aristolochia indica.*

TABLE 4

EFFECT OF NON-ENZYMIC ANTIOXIDANTS IN *Aristolochia indica*

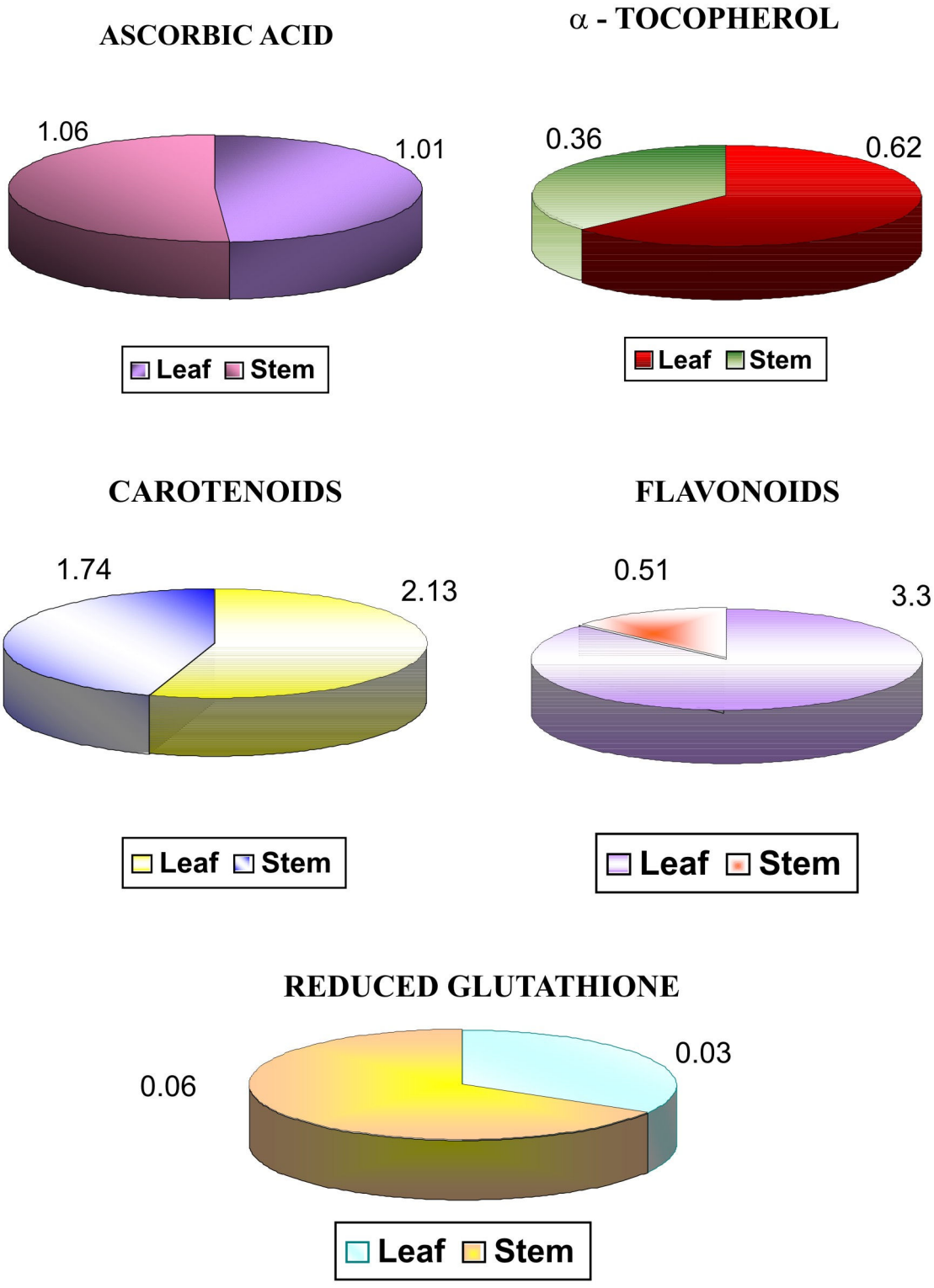
Non-enzymic antioxidants (mg/g)	***Aristolochia indica***		
	Leaf	**Stem**	**t value**
Ascorbic acid	1.01 ± 0.01	1.06 ± 0.04	1.14^{ns}
α-tocopherol	0.62 ± 0.04	0.36 ± 0.01	5.78*
Carotenoids	2.13 ± 0.02	1.74 ± 0.04	33.97*
Flavonoids	3.30 ± 0.99	0.51 ± 0.25	3.10*
Reduced glutathione	0.03 ± 0.01	0.06 ± 0.01	2.21*

Values are mean ± SD of triplicates * - Significant at 0.05 level

ns - not significant

Figure 4

EFFECT OF NON ENZYMIC ANTIOXIDANTS IN *Aristolochia indica*



The above table and figure reveals that *Aristolochia indica* contains ascorbic acid, α-tocopherol and reduced glutathione in moderate levels. There was a significant difference in the levels of α-tocopherol, carotenoids, flavonoids and reduced glutathione was seen between the leaves and stems of *Aristolochia indica*.

The total antioxidant capacity of *Ficus glomerata* was found to be 0.32 ± 0.02mM of ascorbic acid per gram of the root extract (Channbasavaraj *et al.,* 2008).

Jayasinghe *et al.* (2003) showed the existence of a synergistic antioxidant effect in sweet basil extract between rosmarinic acid and α-tocopherol. Increased vitamin C levels showed an improvement of endothelial function, a main surrogate end point for cardiovascular disease in multiple disease models of oxidative stress (Yim *et al.,* 2007).

The bark powder of *Terminalia arjuna* has also been found to improve antioxidant status in the patients of coronary heart disease and these beneficial effects may be related to its high flavonoid content (Chander *et al.*, 2004).

Flavonoids, polyphenols, α-tocopherol and ascorbate reduce the LDL oxidation and quench reactive oxygen radicals, thereby decreasing the risk of cancer (Jae and Mark, 2000). β-carotene, ascorbic acid, α-tocopherol and multivitamin as food supplements may modify the oxidation of LDL cholesterol although this protective effect does not occur under all conditions (Kaikkonen *et al.,* 2006).

Epidemiological studies have indicated that vitamin C and E exert protective effect against cardiovascular disease. Low plasma level or low dietary intake of vitamin C is associated with high blood pressure and

unstable coronary syndrome. Low plasma levels of α-tocopherol were associated with risk of angina (Kechnie *et al.,* 2002).

Due to high oxygen consumption and low glutathione (antioxidant status) content in the neuronal cells, it appears to be particularly vulnerable to oxidative stress. This have role in the genesis and progression of Alzeimer's disease, Parkinson's disease, brain neoplasm and also some other neurodegenerative disorders (Pratico *et al.,* 2002).

Platelet adhesiveness to the blood vessel wall is profoundly reduced in healthy and diseased individuals when tocopherol is taken in doses around 400 mg daily (Asplund, 2002).

Hence, *Aristolochia indica* may have the ability to reduce the risk of cardiovascular diseases, cancer, high blood pressure, coronary syndrome and angina.

4.3 *in vitro* FREE RADICAL SCAVENGING ACTIVITY

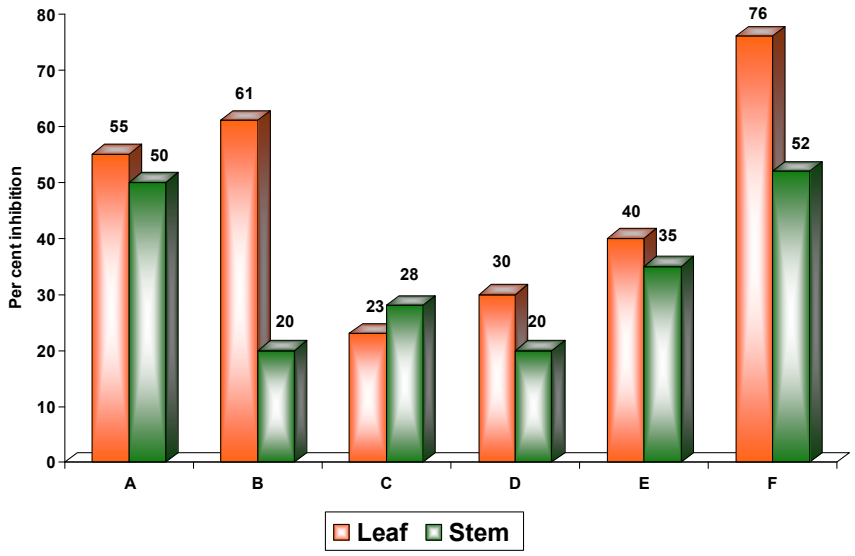
Free radical often exist in the form of peroxyl ($ROO^{\bar{}}$), alkoxyl (RO), hydroxyl ($OH^{\bullet}$) and nitric oxide (NO), all of which retain surplus non-paired electrons being highly reactive to macromolecular compounds including proteins and nucleic acids (Chi *et al.,* 2007). They play an important role in chronic diseases related to oxidative stress such as diabetes, cancer and cardiovascular pathologies (Fresco *et al.,* 2007).

4.3.1 DPPH Radical Scavenging Activity of *Aristolochia indica*

Figure 5 presents the DPPH radical scavenging activity of different extracts of the *Aristolochia indica*.

FIGURE - 5

DPPH RADICAL SCAVENGING ACTIVITY OF *Aristolochia indica*



A - Petroleum ether

B - Benzene

C - Chloroform

D - Acetone

E - Methanol

F - Aqueous

All the extracts of *Aristolochia indica* were capable of scavenging 1, 1-Diphenyl-2- picryl hydrazyl (DPPH) radicals. The aqueous extract of the leaves of *Aristolochia indica* was found to have a strong inhibition (76 per cent) against DPPH radical, whereas the chloroform fraction showed only 23 per cent of inhibition when compared with other extracts. The aqueous extract of stem showed a good radical scavenging activity of about 52 per cent, whereas the benzene and acetone fractions were found to have 20 per cent of inhibition against DPPH radicals when compared to other fractions.

DPPH is a nitrogen-centered free radical. It reacts similar to the peroxyl radical and the reaction rates correlate directly with the antioxidant activity (Tenpe *et al.,* 2008). The free radical scavenging properties of the extracts were determined by the DPPH assay, where the DPPH radical is reduced by the antioxidant compound to its hydrazine derivative (Rocha *et al.,* 2008).

Neill *et al.* (2000) had also observed a similar type of results for *Elatostema rugosum* which showed 50 per cent inhibition concentration against DPPH radicals. Wang *et al.* (2004) stated that the ethanolic extracts of both heartwood and bark of *Calocedrus formosana* exhibited a significant inhibitory activity against DPPH radical, whereas the leaf extract was found to have much less effect on free radical inhibition.

Falleh *et al.* (2008) pointed out the correlation co-efficient between phenolics (total polyphenol, flavonoids, and condensed tannins) and DPPH, indicating that polyphenolics may play an important role in free-radical scavenging.

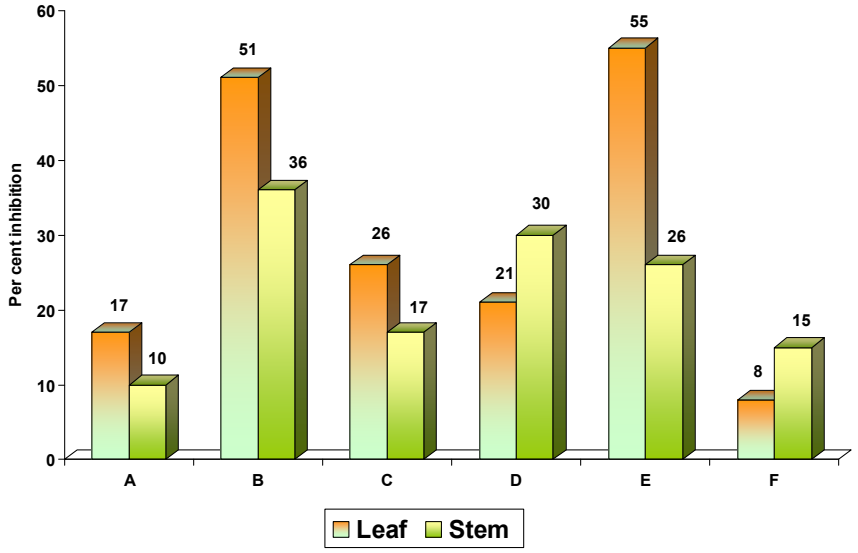
In general, the antioxidant activities of the plant extracts are ascribed to the pheolic contents (Miliauskas *et al.,* 2004). Hence the presence of phenols in *Aristolochia indica* may be responsible for the inhibition of DPPH radicals.

4.3.2 Inhibition of *in vitro* lipid peroxidation of *Aristolochia indica*

Figure 6 shows the percentage inhibition of *in vitro* lipid peroxidation of different extracts of the leaves and stems of *Aristolochia indica*.

FIGURE - 6

INHIBITION OF *in vitro* LIPID PEROXIDATION OF *Aristolochia indica*



A - Petroleum ether

B - Benzene

C - Chloroform

D - Acetone

E - Methanol

F - Aqueous

The percentage inhibition of *in vitro* lipid peroxidation exerted by the leaves of *Aristolochia indica* in the extracts of petroleum ether, benzene, chloroform, acetone, methanol and aqueous was 17, 51, 26, 21, 55 and 8.4 per cent respectively. Among the six extracts, the methanol extract was found to possess a strong inhibitory action on *in vitro* lipid peroxidation than that of the others. The benzene extract of the stem of *Aristolochia indica* showed the highest (36 per cent) antioxidant activity, whereas the petroleum ether extract showed a lower activity in comparison to other extracts.

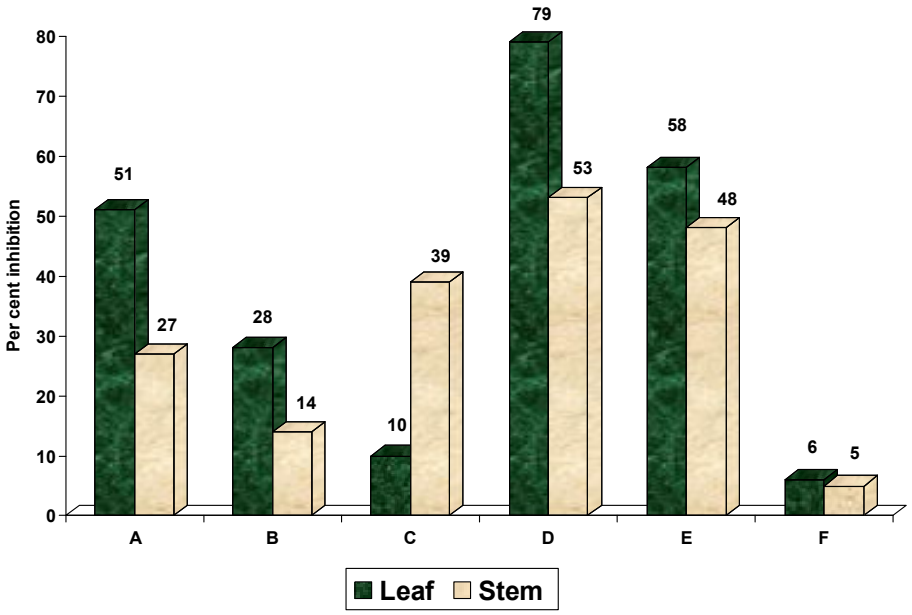
Many scientific studies showed that sweet basil extract is a strong radical scavenger and can be considered as a good source of natural antioxidants (Abas *et al.,* 2006).

4.3.3 Inhibition of superoxide generation

Figure 7 presents the superoxide scavenging activity of the *Aristolochia indica* extracts.

FIGURE - 7

INHIBITION OF SUPEROXIDE GENERATION OF *Aristolochia indica*



A - Petroleum ether
B - Benzene
C - Chloroform
D - Acetone
E - Methanol
F - Aqueous

The acetone (stems) extracts showed a strong inhibitory activity against superoxide radicals than the other solvent extracts. The aqueous

extract of *Aristolochia indica* stems showed the lowest activity on superoxide radical inhibition.

The scavenging effects of different extracts of leaves were in the order of: acetone > methanol > petroleum ether> benzene > chloroform> aqueous which were 79, 58, 57, 28, 10, and 6 respectively.

Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (Gulcin *et al.,* 2006). Superoxide anions ($O2^{-}$) are precursors for active free radicals that have potential for reacting with biological macromolecules and thereby inducing tissue damage.

Studies conducted by Ani *et al.* (2006) revealed that bitter cumin seed extract exhibited significant antioxidant activity at microgram quantities as quenchers of DPPH radicals, lipid peroxy radicals, hydroxyl radicals and superoxide anion radicals in different antioxidant systems.

Parmelia saxatilis extracts had strong superoxide radical scavenging activity. The results were found statistically significant at $p< 0.01$ level (Behra *et al.,* 2006). Wang *et al.* (2004) results demonstrate that the ethanolic extracts of *Calocedrus formosana* heartwood show a better effect against superoxide radicals as compared with the well known standard (+)-catechin.

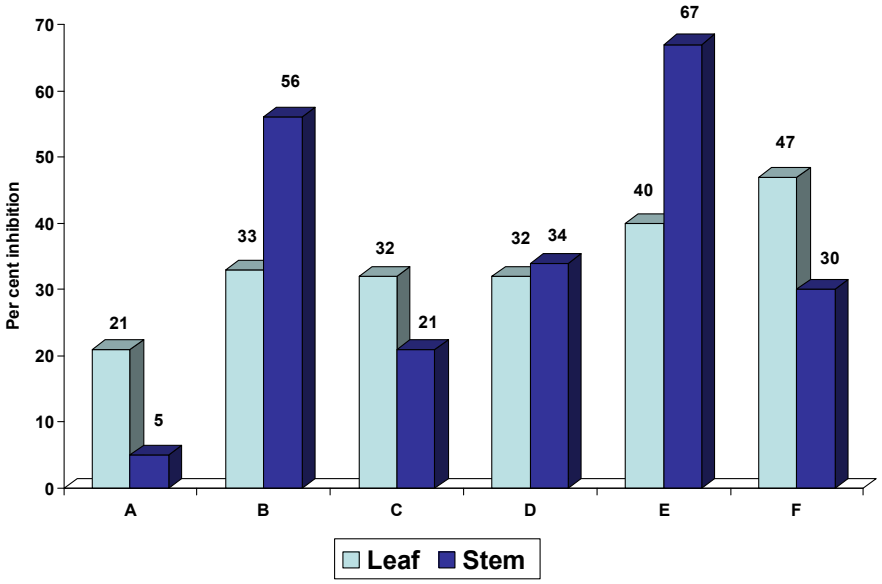
4.3.4 Inhibition of nitric oxide generation of *Aristolochia indica*

Figure 8 shows the nitric oxide scavenging activity of the leaf and stem of *Aristolochia indica* with six different extracts.

FIGURE – 8

INHIBITION OF NITRIC OXIDE GENERATION OF

Aristolochia indica



A - Petroleum ether
B - Benzene
C - Chloroform
D - Acetone
E - Methanol
F - Aqueous

The aqueous extract of leaves shows more inhibitory (47 per cent) activity against nitric oxide radicals. The methanol extract of stem shows high inhibitory activity. The lowest inhibitory activity was found to be in leaf extracts of petroleum ether and stem extracts of chloroform.

A strong inhibitory action was observed against the nitric oxide generation in the methanol extract, whereas a least inhibition was observed in petroleum ether extract of stem of *Aristolochia indica.*

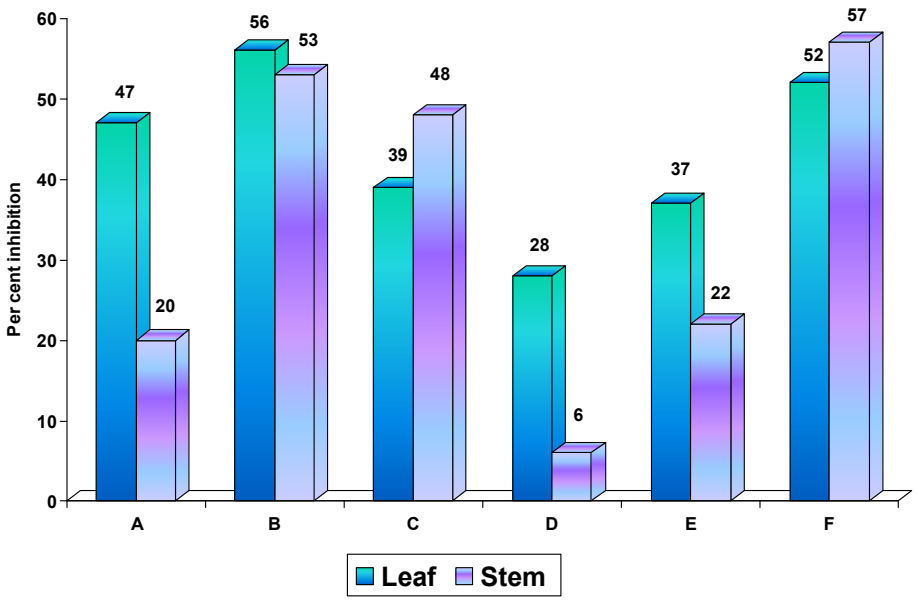
Devi *et al.* (2008) suggests that *Gelidiella acerosa* and *Haligra sp* might be potential novel therapeutic agents for scavenging of nitric oxide and the regulation of pathological conditions caused by excessive generation of nitric oxide and its oxidation product, peroxynitrite.

4.3.5 Hydrogen peroxide scavenging activity of *Aristolochia indica*

The ability of *Aristolochia indica* to scavenge hydrogen peroxide is shown in Figure 9.

FIGURE - 9

HYDROGEN PEROXIDE SCAVENGING ACTIVITY OF *Aristolochia indica*



A - Petroleum ether
B - Benzene
C - Chloroform
D - Acetone
E - Methanol
F - Aqueous

The results showed that both aqueous (52 per cent) and benzene (56 per cent) extracts of leaf sample produce strong hydrogen peroxide scavenging activity.

The hydrogen peroxide scavenging effect of the extracts of *Aristolochia indica* is shown in order

aqueous > benzene > chloroform > methanol> petroleum ether > acetone

Hydrogen peroxide can be formed *in vitro* by many oxidizing enzymes such as superoxide dismutase, cross membranes and slowly oxidize a number of compounds (Ozen and Kinalioglu, 2008).

Results of Nadaroglu *et al.* (2007) showed that both aqueous and methanol extracts of *Iris germanica* produces a strong hydrogen peroxide scavenging activity.

Since, *Aristolochia indica* is exhibiting significant *in vitro* free radical scavenging activity, it may be effective in acting against cell or tissue damage which are caused by lipid peroxidation and therefore reduce the risk of aging, cardiovascular disease and cancer.

5.0 SUMMARY AND CONCLUSION

It is now recognized that reactive oxygen species (ROS) may play major roles in tumor promotion and progression, chronic diseases and aging. In order to maintain cellular health, it is essential to have a specific and effective chemical scavenger to target multiple types of radicals. Most of the commercially based antioxidant supplements are single oxidant. Therefore it is important to find specific scavenger to efficiently and effectively reduce multiple reactive oxygen species.

The use of natural antioxidants as a potential preventive for free radical mediated diseases has become a very important issue for improving the quality of life. Recently, various phytochemicals like polyphenols, which are widely distributed in plants, have been reported to act as free radical scavengers.

Aristolochia indica is a plant belonging to the family of *Aristolochiaceae* is widely distributed throughout the India is commonly called Garudakkodi in Tamil. Hence the present study was carried out to analyse the "Antioxidant properties of leaves and stems of *Aristolochia indica*".

The nutritional components like carbohydrates and proteins were quantitatively analysed in the sample. Among the leaves and stems, the leaves show higher carbohydrates and proteins than that of stems.

Enzymic antioxidants (catalase, superoxide dismutase, glutathione reductase, glutathione-S-transferase and glutathione peroxidase) and non-enzymic antioxidants (ascorbic acid, α-tocopherol, reduced glutathione, flavonoids and carotenoids) were analysed in the leaves and stems of *Aristolochia indica* to ascertain antioxidant activities.

The results for antioxidant potential reveals that the enzymic antioxidants like catalase, peroxidase, glutathione-S-transferase, glutathione reductase and glutathione peroxidase activities were found to be higher in the leaves of *Aristolochia indica* and superoxide dismutase activity is rich in stems. Thus, *Aristolochia indica* could be used to treat malignancy, cardiovascular diseases and neural diseases. The non-enzymic antioxidants such as α-tocopherol, carotenoids and flavonoids were found to be higher amounts in the leaves of *Aristolochia indica*, when compared to stems of *Aristolochia indica*. Thus, *Aristolochia indica* can deduce the risk of cardiovascular diseases, cancer, high blood pressure, coronary syndrome and angina.

The free radical scavenging activity such as DPPH radical and hydrogen peroxide radical scavenging activity, inhibition of nitric oxide, superoxide generation and *in vitro* lipid peroxidation of different extracts with solvents like petroleum ether, benzene, choloroform, acetone, methanol and aqueous of *Aristolochia indica* was also observed. Among these, the aqueous leaf extract of *Aristolochia indica* shows more inhibitory (76 per cent) activity against DPPH radicals. The methanol extract was found to possess a strong inhibitory action on *in vitro* lipid peroxidation than that of the others. The acetone (stems) extracts showed a strong inhibitory activity against superoxide radicals than the other solvent extracts. The aqueous extract of leaves shows more inhibitory (47 per cent) activity against nitric oxide radicals. The results showed that both aqueous (52 per cent) and benzene (56 per cent) extracts of leaf sample produce strong hydrogen peroxide scavenging activity.

RECOMMENDATION

- The leaf powder of *Aristolochia indica* was found to be rich in antioxidant activity than that of the stem. Hence, the active phytochemical constituents in the leaves of *Aristolochia indica* could be identified, isolated and studied in detail.
- Utilization of locally available herbal plants by human beings against the human disorder is cost effective. So the rural people can be educated to use these herbal plants.

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APPENDIX - I

ESTIMATION OF TOTAL CARBOHYDRATE
(Hedge and Hofreiter, 1962)

Principle

Carbohydrates are first hydrolyzed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxy furfural. This compound forms a green coloured product with anthrone which has an absorption maxima at 630nm.

Reagents

1. 2.5N HCl
2. Anthrone reagent: Dissolved 200mg anthrone in 100 ml of ice cold 95% H_2SO_4 (Prepared fresh before use)
3. Stock standard: Dissolved 100mg of glucose in 100 ml distilled water
4. Working standard: 10 ml of stock solution is made up to 100 ml of distilled water.

Procedure

Weighed 100mg of the sample in a boiling tube. Hydrolyzed by keeping it in a water bath for 3 hours with 5 ml of 2.5N HCl and cooled to room temperature. Neutralized it with solid sodium carbonate until the effervescence ceases. Made up the aliquots for analysis. Prepared the standards by making 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standards. '0' served as the blank. Made up the volume to 1 ml in all the test tubes including the sample tubes by adding distilled water. Then added 4.0 ml of anthrone reagent. Heated for eight minutes in boiling water bath. Cooled rapidly and read the green to dark green colour at 630nm.

APPENDIX - II

ESTIMATION OF PROTEIN

(Lowry *et al*., 1951)

Principle

The amino acid tyrosine and trytophan present in the protein will react with the Folin-Ciocalteau reagent. By the reduction of phosphomolybdic phosphotungstic components it will produce blue colour. Also the colour developed by the Biuret

reaction of the protein with the alkaline cupric tartarate is measured in Micro Lowry's method.

Reagents

1. Solution A: 1% copper sulphate
2. Solution B: 2% sodium potassium tartarate
3. Solution C: 2% sodium carbonate in 0.1N NaOH
4. Solution D: Mixed just before use, 1 ml of solution A, 1ml of solution B and 100ml solution C.
5. Solution E: 1N Folin-Ciocalteau reagent (stored protected from light)

Procedure

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard and known volume of the sample in duplicates to different tubes. Made up the volume to 1 ml with 0.1N NaOH. Added 3.0 ml of solution D, followed by 0.3 ml of solution E to each tube, mixed well and incubated for 3 min at 37^0C. Read the colour developed at 750nm against a reagent blank.

APPENDIX- III

ESTIMATION OF CATALASE ACTIVITY
(Luck, 1974)

The enzyme catalase has a double function and it catalyses the following

a). It decomposes hydrogen peroxide to give water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

b). It oxidizes H^+ donors, for example methanol, formic acid, phenol with the consumption of one mole of peroxide.

$$ROOH + AH_2 \xrightarrow{\text{Catalase}} H_2O + ROH + A$$

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only to enzyme solution, which do not absorb strongly at 230-250nm.

Reagents

1. Phosphate buffer 0.067M (pH 7.0)

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

2. Hydrogen peroxide – Phosphate buffer

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

Procedure

Enzyme extract:

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

Assay

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

Calculation

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

APPENDIX - IV

ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY
(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating system and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can be assayed spectrophotometrically.

Reagents

1. 50mM potassium phosphate buffer, pH 7.8
2. 45µM methionine
3. 5.3µM riboflavin
4. 84µM nitro blue tetrozolium (NBT)
5. 20mM potassium cyanide

Procedure

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45µM methionine, 5.3µM riboflavin, 84µM NBT and 20mM potassium cyanide. The tubes were placed in an aluminum Foil – lined box maintained at 25^{o}C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophotometrically at 600nm after exposure to light for ten minutes. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50% inhibition of the reduction of NBT.

APPENDIX – V

ASSAY OF GLUTATHIONE REDUCTASE
(David and Richard, 1983)

Glutathione reductase catalyses the conversion of oxidized glutathione to reduced glutathione employing NADPH as a substrate. The amount of NAPDH utilized is a direct measure of enzyme activity.

Reagents

1. 0.12M phosphate buffer, pH 7.2
2. 15mM EDTA
3. 10mM sodium azide

4. 6.3 mM oxidized glutathione

5. 9.6 mM NADPH

Procedure

20% aqueous extract was prepared in 0.12 M phosphate buffer (pH 7.2) was used as the source of enzyme. The assay system contained 1 ml of 0.12M potassium phosphate buffer, 0.1 ml of 15mM EDTA, 0.1ml of 10mM sodium azide, 0.1 ml 0f 6.3mM oxidized glutathione and 0.1ml of enzyme source and distilled water in the final volume of 2 ml. Kept for 3 minutes. The 0.1 ml of NADPH was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 2 to 3 minutes. For each series of measurement controls were done that contained water instead of oxidized glutathione. The enzyme activity was expressed as milli moles of NADPH oxidized/minutes/ g sample.

APPENDIX – VI

ASSAY OF GLUTATHIONE-S-TRANSFERASE
(Habig *et al*., 1974)

The enzyme was assayed by the conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

Reagents

1. 1mM –Chloro 2,4-dinitrobenzene (CDNB) in ethanol

2. 1mM – Glutathione

3. 0.1 M phosphate buffer

Procedure

The assay was done at 5^{o}C under condition giving activities linear with respect to incubation time and protein concentration for at least 3 minutes. The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. GSH and CDNB (0.1 ml) was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9 ml.

The reaction was started by the addition of 0.1 ml of sample to this mixture; the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the sample served as the control to

monitor non-specific binding of the substrate. Care was taken to ensure that final concentration of ethanol in the mixture was always less than 4%.

Calculation

GST activity was calculated using the extinction coefficient of the product formed and the values have been expressed as nmoles and CDNB conjugated/minutes/ g sample.

APPENDIX-VII

ASSAY OF GLUTATHIONE PEROXIDASE

(Rotruck *et al.*, 1973)

Principle

A known amount of enzyme preparation was used to react with H_2O_2 in the presence of GSH for a specified time period. Then the screening GSH was measured by the method of Ellman.

$$2GSH+H_2O_2 \xrightarrow{\text{Se- GPx}} GSSG+2H_2O_2$$

Reagents

1. 0.4 M Tris buffer
2. 10 µM Sodium azide
3. 10% TCA
4. 0.4 µM EDTA
5. 10 µM Hydrogen peroxide
6. 2 µM Glutathione

Procedure

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

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

APPENDIX – VIII
ESTIMATION OF ASCORBIC ACID
(Roe and Kuether, 1953)

Principle

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

Reagents

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

Procedure

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000 rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up 2.0 ml with 4% TCA. The working standard solution 0.2 to 1.0 ml containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0 ml with 4% TCA. Added 0.5 ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37^{o}C for 3 hours.

The osazones formed were dissolved in 2.5 ml of 85% sulphuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH

reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophotometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX – IX
ESTIMATION OF TOCOPHEROL

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

Principle

Tocopherol can be estimated using Emmerie-Engel reaction which is based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'- dipyridyl. Tocopherol and carotenes are first extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2'-dipyridyl
4. Standard solution: Dissolved 10 mg / 10 ml of α-tocopherol in absolute alcohol. 91mg of α-tocopherol is equivalent to 100 mg of tocopherol acetate.

Extraction

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

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of extract, 1.5 ml of standard, 1.5 ml of water respectively. To the test and blank, added 1.5 ml of ethanol and to the standard, added 1.5 ml of water. Added 1.5 ml

xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0 ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0 ml of 2, 2'-dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5 ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33 ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{AMOUNT OF TOCOPHEROL} = \frac{\text{Reading at 520nm - Reading at 460nm}}{\text{Reading of standard at 520nm x 0.29 x 15}}$$

APPENDIX – X

EXTRACTION AND ESTIMATION OF TOTAL CAROTENOIDS (Zakaria *et al*., 1979)

Principle

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in a UV/visible spectrophotometer at 450nm.

Reagents

1. Petroleum ether
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10 g of the sample. Saponified for about 30 minutes in a shaking water bath at 37^{o}C after extracting the sample in 12% alcoholic KOH. Transferred the saponified extract into a separating funnel packed with glass wool and $CaCO_3$ containing 10 to 15 ml of petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and the petroleum ether extract containing the carotenoid pigments to amber coloured bottle. Repeated the extraction of the aqueous phase. To the petroleum ether extract, added a small quantity of anhydrous Na_2SO_4 to remove the turbidity. Noted the final volume of the petroleum ether extract and diluted if

needed by a known dilution factor. The absorbance of the extract at 450nm and 503nm was noted in a spectrophotometer.

$$\text{Amount of total carotenoids present} = \frac{P \times 4 \times V \times 100}{W}$$

P = Optical Density of the sample

V = Volume of the sample

W = Weight of the sample

APPENDIX-XI

ESTIMATION OF FLAVONOIDS

(Cameron *et al.*, 1943)

Reagents

1. Vanillin reagent: 1% vanillin in 70% con. H_2SO_4.
2. Catechin standard (110 µ/ ml): 0.011 g of catechin in 100 ml of water.

Extraction

A portion of the ground plant material was weighed out and extraction was carried out in two steps. Firstly, with methanol: water (9:1) and secondly with methanol: water (1:1). At each step, sufficient solvent was added to make liquid slurry and the mixture was left for 6- 12 hrs. Filtration to separate the extract from the plant material was carried out rapidly by using a glass wool or cotton plug in the ring of a filter funnel. The two extracts were then combined and evaporated to about $1/3^{rd}$ of the original volume or until most of the methanol had been removed. The resultant aqueous extract was cleared of low polarity contaminants such as fats, terpenes, chlorophyll and xanthophylls by extraction (in a separating funnel) with hexane or chloroform. This was repeated several times and the extracts combined. The solvent extract aqueous layer containing the bulk of the flavonoids was then concentrated.

Procedure

An aliquot of the extract was pipetted into a test tube and evaporated to dryness. Then added 4 ml of vanillin reagent and heated for 15 minutes in a boiling water bath. A standard was also treated in the same manner. Then the optical density was read at 340 or 360 nm.

APPENDIX – XII

ESTIMATION OF REDUCED GLUTATHIONE

(Moron *et al*., 1979)

Principle

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

Reagents

1. DTNB
2. 5% TCA
3. 0.2M Sodium phosphate buffer

Procedure

One gram of the sample was homogenized in 5% TCA to give a 20% homogenate. The precipitated protein was centrifuged at 1000 rpm for 10 minutes. The homogenate was cooled on ice and 0.1 ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0 ml with 0.2M Sodium phosphate buffer (pH 8.0), 2 ml of freshly prepared DTNB solution (0.6mM) in 0.2M phosphate buffer (pH 8.0), was added to the tubes and intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentration ranging from 2 to 10 nmoles of GSH in 5% TCA.

APPENDIX – XIII

DPPH SCAVENGING ACTIVITY

(Mensor *et al*., 2001)

Principle

DPPH radical reacts with an antioxidant compound, which can donate hydrogen and gets reduced. The change in colour from deep violet to yellow can be measured at 515nm.

Reagents

1. DPPH – 2, 2'-diphenyl-2-picryl hydrazyl hydrate (0.3mM)
2. Methanol

Procedure

A methanolic solution of 0.3 mM DPPH (0.5 ml) was added to equal volume of sample homogenate (20% homogenate was prepared in Tris EDTA buffer, pH 7.2) and allowed to react at room temperature. DPPH in methanol without plant extracts served as positive control. After 30 minutes, the mixture was centrifuged and the absorbance of the supernatant was measured at 515nm and converted into percentage radical scavenging activity as follows.

$$\text{Scavenging activity (\%)} = 100 \quad \frac{A_{518}\,(\text{sample}) - A_{518}\,(\text{blank})}{A_{518}\,(\text{blank})} \times 100$$

APPENDIX XIV
ASSAY OF LIPID PEROXIDATION
(Okhawa *et al*., 1979)

Principle

Malondialdehyde formed (MDA) from the break down of polyunsaturated fatty acid serves as a convenient index for the extent of per oxidation reaction. MDA a product of lipid peroxidation that reacts with TBA give a pink colour product having absorbance maximum at 535nm.

Reagents

1. 10% TCA
2. 0.1M Thiobarbituric acid

Procedure

0.1 ml of homogenate was treated with 1ml of 10% TCA and 1 ml of 0.1M TBA and mixed thoroughly. The mixture was heated in a boiling water bath for 20 minutes. Centrifuged at 1000 rpm for 10 minutes and the absorbance were read at 535nm against blank that contains all reagents minus homogenate. The MDA equivalent of the sample calculated using the extinction coefficient 1.56×10^5 µM/cm.

Calculation

$$\text{Concentration of MDA} = \frac{A}{E \times L}$$

Where,
A = O.D at 535nm
E = Extinction coefficient of MDA
L = Length of cuvette (1cm)

APPENDIX – XV

DETERMINATION OF INHIBITION OF SUPEROXIDE GENERATION

(McCord and Fridovich, 1968)

The extent of superoxide generation was studied on the basis of inhibition in the production of Nitro Blue Tetrazolium (NBT) formazon of the superoxide ion on the plant sample measured colorimetrically at 560nm.

Reagents

1. EDTA (0.1 M containing 1.5 mg NaCN/100 ml)
2. NBT (1.5mM)
3. 0.12mM riboflavin
4. 0.067M phosphate buffer, pH 7.8
5. Dimethylsulfoxide

Procedure

The assay tubes contained test sample (20 mg concentration) with 0.2 ml of EDTA, 0.1 ml NBT, 0.05 ml riboflavin and 2.55 ml of phosphate buffer. The control tubes were also set up in DMSO and were added instead of sample.

All the tubes were vortexed and measured the initial optical density at 560nm. After that, these tubes were placed in an area where they received uniform illumination for 30 minutes. Again the optical density was measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the percentage of inhibition by the test sample was calculated by comparing with the optical density of control.

APPENDIX – XVI

DETERMINATION OF INHIBITION OF NITRIC OXIDE GENERATION

(Green and Hill, 1984)

Aqueous solution of sodium nitroprusside spontaneously generates nitric oxide (NO) at physiological pH, which interacts with oxygen to produce nitrite ion, which is measured colorimetrically.

Reagents

1. Phosphate buffered saline
2. Sodium nitroprusside (100mM)

3. Griess reagent (1% sulfanilamide, 2% H_3PO_4, 0.01% Naphthalene diamine dihydrochloride)

Procedure

Three ml of reaction mixture containing sodium nitroprusside in PBS and extract was incubated at 25^oC for 150 minutes. Controls were kept without test compound in an identical manner. After incubation, 0.5 ml of Griess reagent was added. The absorbance of the chromopore formed was read at 546 nm.

The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

APPENDIX – XVII

HYDROGEN PEROXIDE SCAVENGING ASSAY

(Ruch *et al*., 1989)

The ability of the plant extract to scavenge H_2O_2 was determined according to the method of Ruch *et al*., (1989). A solution of H_2O_2 (4 mM) was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically from its absorption at 230nm was determined after 10min against a blank solution containing phosphate buffer without H_2O_2.

The scavenging activity of H_2O_2 by plant extract and the standard compounds was calculated using the formula

$$\% \text{ Scavenging } H_2O_2 = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 = Absorbance of control

A_1 = Absorbance in presence of sample of plant extract and standards.