CHAPTER 7

ANTIOXIDANT ACTIVITY

7.0 Introduction

7.1 Antioxidants

Antioxidants are the substance that prevent or slow the breakdown of another substance by oxidation. They donate electrons to the free radicals and convert them to harmless molecules. These are substances used by the body to protect itself from damage caused by oxidation. Oxidation is a process that causes damage in our tissues through the work of free radicals and they prevent the oxidation of other chemicals also. In the body, nutrient antioxidants such as beta-carotene (a vitamin A precursor), vitamin C, vitamin E, and selenium have been found (Gamez *et al.*, 1998).

Antioxidants neutralize highly-reactive oxygen free radicals. They act against oxidants to minimize the damage made by them. They are capable of stabilizing or deactivating, free radicals before they attack cells as they are powerful electron donors and react with free radicals before the free radical damage the biomolecules. The formed antioxidant radical is stable and unreactive. The Antioxidants belongs to various classes and act through different mechanism (Nadendla, 2002).

Free radical production is actually a normal part of life. The process of oxidation in the human body damages cell membranes and other structures including cellular proteins, lipids and DNA. When oxygen is metabolized, it creates 'free radicals' which steal electrons from other molecules, causing damage. The body can cope with some free radicals and needs them to function effectively. However, an overload of free radicals has been linked to certain diseases, including heart disease, liver disease and some cancers. Oxidation can be accelerated by stress, cigarette smoking, alcohol, sunlight, pollution and other factors. Antioxidants counteract these cellular byproducts and bind with them before they can cause damage (Corvilain *et al.*, 1991).

7.1.2 Free radicals

Free radicals can be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally to achieve stable configuration (Nadendla, 2002). Harmful free radicals are toxic molecules of oxygen that damage the body. A free radical is an unstable incomplete molecule because it is missing an electron which exists in pair in stable molecules. Free radicals steal an electron from another molecule, thereby create another free radical. This new free radical then duplicates the process, resulting in a chain reaction of events, which can ultimately damage the body. Free radicals are natural by-products of ongoing biochemical reactions in the body, including ordinary metabolic processes and immune system responses. The common free radicals are reactive oxygen species (ROS), namely, superoxide radical, hydroxyl radical and peroxyl radical which can be internally produced by cellular metabolism, inflammation by immune cells and externally by radiation, pharmaceuticals, hydrogen peroxide, toxic chemicals, smoke, alcohol, oxidized polyunsaturated fats

and cooked food. Free radicals can cause damage to parts of cells such as proteins, DNA, and cell membranes by stealing their electrons through a process called oxidation. Reactive oxygen free radicals have been known to produce tissue injury through covalent binding and lipid peroxidation and have been shown to augment fibrosis as seen from increased collagen synthesis (Geesin *et al.*, 1990). Scavenging of free radicals by antioxidants could reduce the fibrosis process in the tissues (Thresiamma *et al.*, 1996). Free radicals may also be a contributory factor in a progressive decline in the function of the immune system (Pike *et al.*, 1995). The defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes.

Free radicals such as reactive oxygen species (ROS) play important role in the etiology of number of diseases including cardiovascular, ischemic disease, and aging processes (Halliwell *et al.*, 1992; Gutteridge, 1993; Halliwell *et al.*, 1995). When antioxidants are employed in the treatment of such diseases, they participate in body defense mechanism against ROS, which include variety of enzymes such as superoxide dismutase (SOD), which produces hydrogen peroxide from superoxide radicals, catalase (CAT) and glutathione-related enzymes, which decompose hydrogen peroxide (Halliwell, 1990 and Trocino *et al.*, 1995).

7.1.3 The health benefits of antioxidants

Antioxidants work by neutralizing highly reactive, destructive compounds, the free radicals. In biological systems, the normal processes of oxidation produce highly reactive free radicals. Antioxidants work by binding to the free radicals; they transform them into non-damaging compounds or repairs cellular damage. Antioxidants are able to easily donate electrons to molecules in need of an electron, such as free radicals, before they steal one from someplace else, thus stabilize and prevent a damaging chain reaction. The antioxidant molecules interact with the oxygen free radicals and halt the spread of cancer causing cells with damaged DNA before other vital molecules are damaged. Antioxidants help neutralize the production of free radicals which are chemical complexes that cause harm to our cells and play a major role in the disease process. Antioxidants serve as a source of electrons that can be provided to free radicals without damaging the cell components. Antioxidants prevent unstable oxygen molecules (made unstable by loss of one electron) from interacting with other molecules (taking one of their electrons) and consequently causing them to become unstable, a process that starts the free-radical chain reaction. The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major role (Mukherjee *et al.*, 2003 and Rajlakshmi *et al.*, 2003). There are number of lipophilic and hydrophilic low molecular- weight antioxidants, which directly reacts and scavenge the ROS (Sharma *et al.*, 1993).

Antioxidants help alleviate the symptoms and side effects of many of these diseases. According to the free radical theory, radicals damage cells in an organism, causing aging. Antioxidants break the free radical chain reaction by sacrificing electrons, and then humbly existing without stealing more. The body naturally circulates many nutrients for their antioxidant properties, and creates antioxidant enzymes just for the purpose of controlling free radicals and their chain reactions. Antioxidants are thought to thwart heart disease by preventing oxidation. Antioxidants combat chronic inflammation. Vitamin E suppresses platelet stickiness, acting as an anticoagulant to discourage the formation of clots that lead to heart attacks. Vitamin C decreases a blood factor needed to build clots.

Antioxidants fight cancer by neutralizing DNA-damaging free radicals. Antioxidants neutralize free radicals as the natural by-product of normal cell processes. Antioxidants prevent injury to blood vessel membranes, helping to optimize blood flow to the heart and brain, defend against cancer-causing DNA damage, and help lower the risk of cardiovascular disease and dementia, including Alzheimer's disease.

7.1.4 Commonly used antioxidant supplements

Antioxidants are found in the nutrient antioxidants, vitamins A, C and E, and the minerals copper, zinc and selenium. Other dietary food compounds, such as the phytochemicals in plants and zoochemicals from animal products, are believed to have greater antioxidant effects than either vitamins or minerals. These are called the non-nutrient antioxidants and include phytochemicals, such as lycopenes in tomatoes, and anthocyanins found in cranberries. Some antioxidants are made in our cells and include enzymes and the small molecules glutathione, uric acid, coenzyme Q10 and lipoic acid. Antioxidant compounds must be constantly replenished since they are "used up" (converted) in the process of neutralizing free radicals. The repair enzymes that can regenerate some antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase and the other metalloenzymes. Their roles as protective enzymes are well known and have been investigated extensively with *in vivo* models. Several medicinal plants have been screened based on the integrative approaches on drug development from Ayurveda (Mukherjee *et al.*, 2006). Superoxide dismutase is the most important antioxidant enzyme, catalyses the removal of superoxide free radicals in all aerobic and anaerobic organisms, and catalase *in vivo* plays an important role for removal of damaging effects caused by ROS in living systems. Generation of ROS also plays an important role in the etiology of diabetic complications. Under diabetic conditions, ROS are produced via glucose autoxidation (Wolff *et al.*, 1987). Oxidative damage occurs as a consequence of an imbalance between the formation and inactivation of oxygen free radicals. This process leads to the destruction of membrane lipids and production of lipoperoxides and their products. Inactivation and removal of ROS depend on

relations with antioxidative defense mechanisms (Serafini *et al.*, 2004). The free radical nitric oxide (NO) is derived from endothelium and it is a potent vasorelaxant that elicits its effects by activating soluble guanylate cyclase (sGC), thereby stimulating the formation of cyclic guanosine monophosphate (cGMP) (Fukuto *et al.*, 1993). Since the measurement of the NO radical itself is difficult because of being a radical with poor stability with a very short half life, measurement of the end products of NO as nitrite and nitrate (NO^{2-}/NO^{3-}) is often used as a marker for the production of NO radicals (Moshage *et al.*, 1995).

7.1.4.1 Vitamin C (Ascorbic acid)

Vitamin C neutralizes potentially harmful reactions in the watery parts of the body, such as the blood and the fluid inside and surrounding cells. Vitamin C may help decrease total and LDL cholesterol and triglycerides, as well as increased HDL levels. The antioxidant activity of Vitamin C is helpful in the prevention of some types of cancers and cardiovascular diseases. The antioxidant properties of vitamin C are thought to protect smokers, as well as those exposed by passive smoking, from the harmful effects of free radicals. As a powerful antioxidant, vitamin C may help to fight cancer by protecting healthy cells from free-radical damage and inhibiting the proliferation of cancerous cells. The body does not produce vitamin C. Foods containing the highest sources of vitamin C include green peppers, citrus fruits and juices, strawberries, tomatoes, broccoli, turnip greens and other leafy greens, sweet and white potatoes, and cantaloupe.

7.1.4.2 Vitamin E

Vitamin E is the most effective, fat-soluble antioxidant known to occur in the human body. Vitamin E is an antioxidant that prevents free radical damage in biological membranes. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Vitamin E helps protect against heart disease by limiting the oxidation of LDL-cholesterol. Vitamin E helps prevent oxidation of lipoproteins, particularly in smokers, and reduces the stickiness of platelets in the bloodstream. Vitamin E as an antioxidant helps to stabilize cell membranes and protect the tissues of the skin, eyes, liver, breast, and testes, which are more sensitive to oxidation. Vitamin E is found in many common foods, including vegetable oils (such as soybean, corn, cottonseed and safflower) and products made from these oils (such as margarine), wheat germ, nuts and green leafy vegetables, although the researchers evaluated only the pill form of the vitamin.

7.1.4.3 Beta-carotene

Foods rich in beta-carotene protect the body from the damaging molecules (free radicals). The antioxidant actions of Beta-carotene make it valuable in protecting against, and in some cases even reversing, precancerous conditions affecting the breast, mucous membranes, throat, mouth, stomach, prostate, colon, cervix, and bladder. Individuals with highest levels of beta-carotene intake have lower risks of lung cancer, coronary artery heart disease, stroke and age-related eye disease than individuals with lowest levels of beta-carotene intake. The richest sources of beta-carotene are yellow, orange, and green leafy fruits and vegetables (such as carrots, spinach, lettuce, tomatoes, sweet potatoes, broccoli, cantaloupe, and winter squash).

7.1.4.4 Selenium

Selenium is a nonmetallic chemical element. Selenium is used in free radical elimination and other antioxidant enzymes and also plays a role in the functioning of the thyroid gland. Selenium is the central element in glutathione peroxidase (GPx), an antioxidant enzyme that protects cells against the oxidative damage caused by peroxides and free radicals. Selenium forms part of the structure of the important antioxidant enzyme glutathione peroxidase, which in turn recycles glutathione. Dietary selenium comes from cereals, meat, fish, and eggs. Brazil nuts are a particularly rich source of selenium.

Oxygen-dependent deterioration of lipids, known as rancidity is a major problem in the storage of oils (mainly olive oil) but was also considered useful as far back as the 15th century in preparing siccative oil, paints and printing inks. The same oxidation process is also considered important today for natural products used in human consumption such as fats, oils, dressings or margarine but also for chemical and industrial products such as paints, inks, resins, varnishes or lacquers.

The first study of this lipid oxidation problem was those of the Swiss chemist Nicolas-Théodore de Saussure who observed around 1800, using a simple mercury manometer, that a layer of walnut oil exposed to air was able to absorb about 150 times its own volume of oxygen during a one year period. Parallel with these changes, oil became viscous and had a bad smell. Later, Berzelius (who discovered selenium) suggested that this oxidation might be involved in the spontaneous ignition of wool lubrified with linseed oil in textile mills.

Systematic studies of lipid autoxidation may be considered to have begun around the 40s since (Criegee *et al.*, 1939) established that hydroperoxides are the primary products of hydrocarbon oxidation.The major credit for developing the hydroperoxide hypothesis of lipid autoxidation is due to Farmer E J, (Farmer, 1943). Bolland (Q Rev., 1949) established that the primary autoxidation

products of linoleic acid are hydroperoxides (on carbon atom 9 or 13) containing conjugated dienes (Lundberg, *et al.,* 1961). Since the early 1960's, our understanding of the oxidation of unsaturated lipids has advanced considerably as a result of the application of new analytical tools. Detailed studies of the products of polyunsaturated fatty acids were initiated in the 70's by several research groups, revealing more complex mixtures than those previously proposed (Porter, *et al.,* 1980). With the help of HPLC, several hydroperoxide products could be separated after autoxidation of arachidonic acid (Porter, *et al.,* 1980) including products of lipoxygenase action (Porter, *et al.,* 1980). The first demonstration of free radical oxidation of membrane phospholipids was given in 1980, (Porter, *et al.,* 1980) leading to a new fruitful era with a continuous flow of innumerable works devoted to chemistry, biochemistry and medicine.

7.2 Free Oxygen Radicals

Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. Their formation occurs in enzymatic or non-enzymatic reactions involving activated chemical species known as "reactive oxygen species" (ROS) which are responsible for toxic effects in the body via various tissue damages. These ROS include among others hydroxyl radicals, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxinitrite formed from nitrogen oxide (NO), all these groups of atoms behave as a unit and are now named "free radical". These chemical forms are defined as any species capable of independent existence that contains one or more unpaired electrons (those which occupy an atomic or molecular orbital by themselves). They are formed either by the loss of a single electron from a non-radical or by the gain of a single electron by a non-radical. They can easily be formed when a covalent bond is broken if one electron from each of the pair shared remains with each atom, this mechanism is known as homolytic fission. In water, this process generates the most reactive species, hydroxyl radical OH. Chemists know well that combustion which is able at high temperature to rupture C-C, C- H or C-O bonds is a free-radical process. The opposite of this mechanism is the heterolytic fission in which, after a covalent break, one atom receives both electrons (this gives a negative charge) while the other remains with a positive charge. In eukaryotic organisms, ROS are mainly generated during the normal respiration process involving oxygen, oxidases and electron transports in mitochondria or endoplasmic reticulum.

Oxygen is known since a long time as poisonous as well in plants as in animals or bacteria, mainly when supplied at concentrations greater than those in normal air. Thus, oxygen is able to damage plant tissues in inhibiting chloroplast development, seed viability and root growth. The growth of bacteria (Escherichia coli) was shown to be slowed down by pure oxygen, the toxic effect being enhanced by ionizing radiations. Very early, it appears that the effects of oxygen and those of ionizing radiations on organisms have many similarities. In man, the toxicity of oxygen was studied

in relation with diving, life in submarines and spacecrafts, treatment of some pathologies such as cancer, sclerosis or gangrene. Exposure of man to oxygen at 1 atmosphere pressure causes chest soreness, cough, sore throat, damage to lung alveoli and acute central nervous system toxicity. This toxicity was at the origin of the frequent blindness observed in the early 1940s and solved only around 1954 among infants born prematurely and kept in incubators fed with high oxygen concentrations. Several observations led Gershman R and Gilbert DL to propose in 1954, that most of the damaging effects of oxygen could be attributed to the formation of free oxygen radicals (ROS). Several reactive oxygen species (ROS) are known. Among them, the most frequently studied are given below.

7.2.1 Superoxide radical ($O_2^{\cdot-}$)

This ROS is formed when oxygen takes up one electron and as leaks in the mitochondrial electron transport but its formation is easily increased when exogenous components (redox cycling compounds) are present. Its first production site is the internal mitochondrial membrane (NADH ubiquinone reductase and ubiquinone cytochrome C reductase). This species is reduced and forms hydrogen peroxide (H_2O_2). The production of superoxide radicals at the membrane level (NADPH oxidase) is initiated in specialized cells (oxidative burst) with phagocytic functions (macrophages) and contributes to their bactericid action. The flavin cytosolic enzyme xanthine oxidase found in quite all tissues and in milkfat globules generates superoxide radicals from hypoxanthine and oxygen and is supposed to be at the origin of vascular pathologies.

7.2.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxysomes and mitochondria. Even in normoxia conditions, the hydrogen peroxide production in relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\cdot-}$ thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is also able to diffuse easily through cellular membranes.

7.2.3 Hydroxyl radical ($^{\cdot}OH$)

In the presence of Fe^{2+}, H_2O_2 produces the very active species $^{\cdot}OH$ by the Fenton reaction.

$Fe^{2+} + H_2O_2 \text{ ----> } Fe^{3+} + {}^{\cdot}OH + OH^-$

This iron-catalyzed decomposition of oxygen peroxide is considered the most prevalent reaction in biological systems and the source of various deleterious lipid peroxidation products. Another

reaction involving myeloperoxidase and Cl- ions represent an important OH- production process in neutrophils during phagocytosis.

7.2.4 Nitric oxide ($^{\cdot}NO$)

Nitric oxide is produced in various types of cells is well studied in vascular endothelium. While this species is not too reactive (poorly oxidizing function), it reacts rapidly with $O_2^{\cdot-}$ and gives the extremely reactive peroxinitrite (ONOO-). This ROS is naturally formed in activated macrophages and endothelial cells and is considered as an active agent in several pathologies based on inflammation, organ reperfusion and also may play an important role in atherosclerosis.

7.2.5 Singlet oxygen ($^1O_2$)

This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA, 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen. Several natural sensitizers are known to catalyzed oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADH). Some of these sensitizers are also found in foods and cosmetics. Some others are used for therapeutic purposes (anticancer treatments) and are sensitive to visible light. The presence of metals contributes to increase the production of singlet oxygen, as well as anion superoxide, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet O_2 may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet O_2 formation may account for the chemiluminescence observed during lipid peroxidation.

7.2.6 Ozone (O_3)

This natural compound present in the higher atmosphere and in the lower atmosphere of our polluted cities is a major pollutant formed by photochemical reactions between hydrocarbons and nitrogen oxides. Ozone is not a free radical but, as singlet oxygen, may produce them, stimulates lipid peroxidation and thus induces damages at the lipid and protein levels in vivo mainly in airways. The exact chemistry of ozone-mediated stimulation of peroxidation is not entirely known. Ozone may add on across a double bond and decomposes to form a free radical.

7.2.7 Thiyl radicals ($RS^{\cdot}$)

Thiol compounds (RSH) are frequently oxidized in the presence of iron or copper ions:

$RSH + Cu_2^+ \text{----> } RS^{\cdot} + Cu^+ + H^+$

These thiyl radicals have strong reactivity in combining with O_2:

$RS^{\cdot} + O_2 \text{---> } RSO_2^{\cdot}$

Furthermore, they are able to oxidize NADH into $NAD^{\cdot}$, ascorbic acid and to generate various free radicals ($^{\cdot}OH$ and $O_2^{\cdot-}$). These thiyl radicals may also be formed by homolytic fission of disulfide bonds in proteins.

7.2. 8 Carbon-centered radicals

The formation of these reactive free radical is observed in cells treated with CCl_4. The action of the cytochrome P450 system generates the trichloromethyl radical ($^{\cdot}CCl_3$) which is able to react with oxygen to give several peroxyl radicals (i.e. $^{\cdot}O_2CCl_3$). Reactive oxygen species (ROS) play important role in the etiology of number of diseases including cardiovascular, ischemic disease, and aging processes (Halliwell *et al.*, 1992; Gutteridge, 1993; Halliwell and Gutteridge, 1995). When antioxidants are employed in the treatment of such diseases, they participate in body defense mechanism against ROS, which include variety of enzymes such as superoxide dismutase (SOD), which produces hydrogen peroxide from superoxide radicals, catalase (CAT) and glutathione-related enzymes, which decompose hydrogen peroxide (Halliwell, 1990; Trocino *et al.*, 1995). Excess production of free radicals impacts the pathogenesis and progression of various diseases (Visioli *et al.*, 2000). Lipid peroxides, produced from unsaturated fatty acids via free radicals causes toxic effects and promote the formation of additional free radicals in a chain reaction. Deficient activity of enzymes or scavengers which takes part in neutralizing these free radicals, leads to development of oxidative stress related diseases. The rationale for the use of antioxidants is well established in prevention and treatment of chronic diseases where oxidative stress plays a major role (Mukherjee *et al.*, 2003; Rajlakshmi *et al.*, 2003). There are number of lipophilic and hydrophilic low molecular-weight antioxidants which directly react and scavenge the ROS (Sharma *et al.*, 1993).

7.3 Assay methods

7.3.1 1, 1-Diphenyl-2-picryl hydrazyl radical scavenging activity (DPPH assay)

The antioxidant activity of a substance and the standards can be assessed on the basis of the radical scavenging effect of the DPPH free radical (Gamez *et al.*, 1998; Raja *et al.*, 2005; Sundararajan *et al.*, 2006). The ability of the test substance to scavenge the free radicals can be determined by an *in vitro* assay method using a stable free radical DPPH (1,1-Diphenyl-2-picryl hydrazyl) (Soni *et al.*, 2003). In this method to the ethanolic solution of DPPH (in μM), an equal volume of the test substance is dissolved in ethanol and maintained for at least 20 minutes. The

decrease in absorbance of test mixture due to quenching of DPPH free radical is read at 517nm (Soni *et al.,* 2003).

7.3.2 Superoxide anion scavenging activity assay

The scavenging activity of a substance towards superoxide anion radicals can be measured by this method (Liu *et al.,* 1997). Superoxides are generated in a non enzymatic phenazine methosulphate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It is assayed by the reduction of Nitroblue tetrazolium (NBT). In this method the superoxide anion is generated by Tris-HCl buffer containing NBT solution, NADH solution and different concentration of the test substance. The reaction is initiated by adding PMS to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560nm is measured by spectrophotometer. The superoxide anion scavenging activity is calculated by the equation:

$$\% \text{ inhibition} = [(A_0 - A_1)/ A_0 \times 100] \quad \text{..... Eqn (1)}$$

Where A_0 is the absorbance of the control (blank, without extract) and A_1 is the absorbance of the test substance.

7.3.3 Nitric oxide radical inhibition assay

Nitric oxide radicals are generated by sodium nitroprusside in aqueous solution at physiological pH, on interaction with oxygen it produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). Griess Illosvoy reagent can be modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Maracocci *et al.*, 1994; Mukherjee, 2002; Badami *et al.*, 2003).

7.3.4 Lipid peroxidation assay

Lipid peroxidation is evaluated by measuring both thiobarbituric acid reactive substances (TBARS) generation and 4-hydroxynonenal (HNE) concentration. TBARS generation is measured spectrophotometrically at 543nm. Mesangial cells (2·5X106 cells/ml) are incubated for up to 4 h at 37^0C in a shaking bath. For the standard curve, malonildialdehyde bis-(dimethylacetal), followed by acid hydrolysis is used. The HNE concentration can be evaluated by the method described by Esterbauer *et al.* (1991).

7.3.5 Fe (II)-EDTA-H_2O_2 induced oxidative DNA damage assay

In this method the solution of DNA is prepared by dissolving 2 mg of calf thymus DNA in 1 ml of 10 mM tris-HCl pH 7.4, 500 µg DNA and varying concentration of extract (25 - 250 µg) solution, 0.08 mM EDTA, 0.08 mM $FeSO_4$, 0.03% H_2O_2 and 20 mM Na-ascorbate. All solutions are sterilized before use. After incubation at 37°C for 1 hour, DNA nuclease digestion is performed as described by Rahman *et al.*, (1989). The assay determines the acid soluble nucleotides released from DNA

because of enzymatic digestion. Acid soluble nucleotides are determined colorimetrically using the diphenylamine method of Schneider (1957). To a 1.0 ml aliquot, 2.0 ml of diphenylamine reagent (freshly prepared by dissolving 1 g of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) is added. The tubes are heated in a boiling water bath for 20 min and the intensity of blue color is read at 600 nm.

7.4 Material and methods

7.4.1 Plant materials

Methanol extracts of *Urtica parviflora, Callicarpa arborea* leaves and *Morinda citrifolia* root along with their isolated compounds (described in Chapter 3) were used as test drug in these experiments.

7.4.2 Animals

Wistar albino rats (200–250g) of either sex were maintained under standard environmental conditions and had free access to feed and water. Experiments on animals were performed based on animal ethics guidelines of Institutional Animal Ethics Committee.

7.4.3 Instruments, chemicals and drugs

UV-Visible double beam spectrophotometer (Shimadzu Corporation, Japan), cold centrifuge (Remie India Ltd.), mechanical stirrer were used in the experiment. Thiobarbituric acid was obtained from Loba Chemie, India. 1, 1-Diphynyl-2-picryl hydrazyl (DPPH), NADH and nitroblue tetrazolium (NBT) were obtained from Sigma chemicals, St. Louis, USA. Dimethyl sulphoxide, phenazine methosulphate were of analytical grade and were obtained from Ranbaxy fine chemicals.

7.4.4 Antioxidant activity of *Urtica parviflora* leaf

7.4.4.1 1, 1-Diphenyl-2-picryl hydrazyl radical scavenging activity (DPPH assay)

The antioxidant activity of the plant drugs and the standard were assessed on the basis of the radical scavenging effect of the DPPH free radical (Gamez *et al.*, 1998; Raja *et al.*, 2005; Sundararajan *et al.*, 2006). The DPPH scavenging activity of *Urtica parviflora* was measured by spectrophotometric method. To each of the nine glass tubes methanolic solution of DPPH (100 µM, 2.95ml) was taken. Tube No. 1 was treated as control without test compound where 0.05 ml of methanol was added. The second tube contained the standard compound vitamin E. To the tubes numbered from three to seven methanol extract of *Urtica parviflora* (0.05 ml) was added at concentration ranging from 200 to 1000 µg/ml at interval of 200 µg/ml between the tubes. To the tubes numbered eighth and ninth compound I was added in concentration 25 and 50 µg/ml. Absorbance of each tube was recorded at 517nm at regular intervals of 10 min up to 40 min. The

scavenging activity in percentage of inhibition was calculated by comparing the control and test samples applying the **Equation 1** mentioned in **7.3.2.**

7.4.4.2 Lipid peroxidation model

Rat liver homogenate preparation: Anaesthetized adult rats were dissected to take out the liver. The liver lobes are washed with 0.9% sodium chloride solution. Liver lobes are subjected to homogenization using Teflon homogenizer, in ratio 1: 10 of 0.05 M phosphate buffer (pH 7.4). The homogenate is used for the estimation of thiobarbituric acid reactive substances (TBARS). The extent of lipid peroxidation of the homogenate was measured *in vitro* in terms of formation of thiobarbituric acid reactive substances (TBARS) by using standard method (Okhawa *et al.*, 1979; Raja *et al.*, 2005; Sundararajan *et al.*, 2006).with minor modifications (Pandey, 1995). The content was centrifuged at 2000 rpm for 10 min and supernatant was diluted with phosphate buffer saline up to final concentration of protein 0.8-1.5 mg/0.1 ml. Protein concentration was measured by using standard method of Lowery *et al.*, (1951). To study the comparative response, the experiment was performed in nine glass Petri dishes (30 mm ID). Liver homogenate (3ml) was aliquoted to each of the petri dishes. The dish number one and two were treated as control and standard where buffer and vitamin E were added respectively. To the third to seventh dishes, different concentrations of methanol extract (200-1000 µg/ml) and the eighth and ninth dishes compound I (25, 50 µg/ml) were added. Lipid peroxidation was initiated by adding 100µl of 15mM ferrous sulphate solution to 3ml of liver homogenate in nine dishes (Sreejayan, 1997). After 30 min, 100 µl of each reaction mixture was taken into tubes containing 1.5 ml of 10% Trichloro acetic acid. After 10 minutes, tubes were centrifuged and supernatant was separated and mixed with 1.5ml of 0.67% thiobarbituric acid. The mixture was heated in a water bath at 85°C for 30 min and in a boiling water bath to complete the reaction. The intensity of pink colored complex formed was measured at 535nm in a spectrophotometer (Shimadzu model 1601). The TBARS concentration was calculated by using Equation 2 (molar extinction coefficient of TBARS) and expressed as nanomoles (nM)/mg of tissue (Ramazan, 2001). The percentage of inhibition of lipid peroxidation was calculated by comparing the results of the test with those of controls as per the **Equation 1.**

$$\textbf{nM of TBARS/mg of tissue} = \frac{\textbf{OD} \times \textbf{Volume of homogenate} \times 100 \times 10^3}{(1.56 \times 10^5) \times \textbf{Volume of extract taken}} \quad \textbf{--Eqn 2}$$

7.4.4.3 Superoxide scavenging activity

The superoxide scavenging activity of *Urtica parviflora* was determined by the method described by Nishimik *et al.*, (1972) with slight modification. To 1.0 ml of NBT solution containing 156 µM NBT dissolved in 100 mM phosphate buffer, pH 7.4, 1.0 ml of NADH solution containing 468 µM NADH dissolved in 100mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of the methanol

extract (200 to 1000 µg/ml) as well as compound I (25, 50 µg/ml) and standard compound (vitamin E) were added mixed and the reaction was started by adding 100 µl of phenazine methosulfate solution containing 60µM phenazine methosulphate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25 °C for 5 min and absorbance of the contents at 560 nm was measured against a control sample. Percent inhibition was determined by comparing the results of the test and control samples with the help of **Equation 1** mentioned in **7.3.2**.

7.4.5 Antioxidant activity of *Callicarpa arborea* leaf

The antioxidant activity of methanol extract of *Callicarpa arborea* leaf and its isolated compound II was determined as per the methods described above in **7.4.4** for *Urtica parviflora* leaf.

7.4.6 Antioxidant activity of *Morinda citrifolia* root

The antioxidant activity of methanol extract of *Morinda citrifolia* root and its isolated compound III was determined as per the methods described above in **7.4.4** for *Urtica parviflora* leaf.

7.5 Statistical analysis

All data were expressed as means ± SEM. IC_{50} values were calculated by nonlinear regression analysis after logarithmic transformation of the sample concentrations.

7.6 RESULTS

7.6.1 Antioxidant activity of *Urtica parviflora* leaf

The effect of methanol extract and compound I on scavenging of DPPH radical is presented in Table 7.1. The results showed that the DPPH scavenging capacity of the extract was found to be 75.56±2.64% at 1000 µg/ml. The compound I at 50 µg/ml concentration, on the other hand, exhibited 82.17 ± 2.74 % compared to 85.23 ± 3.23 % for the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 6.9 mg/ml. The percentage of activity was also dependent on time (**Fig 7.1**).

The results presented in **Table 7.2** showed the effect of methanol extract of *Urtica parviflora* leaf and compound I on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition percentage (71.54±3.03) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 82.01±1.08. The same results have also been presented in **Fig 7.2**, which revealed that compound I at 50 µg/ml showed 80.19±2.48% inhibition which is nearly equal to inhibition produced by vitamin E. The IC_{50} value, (concentration of the test substances at which 50 % of inhibition produced) was found to be 859.32±2.79 µg/ml. From the results of quantitative estimation of TBARS

levels it can be said that both the methanol extract at 1000 µg/ml and compound I at 50 µg/ml concentration decreases the levels of TBARS in liver homogenate and is highly comparable to the results of well known antioxidant vitamin E ($p< 0.05$).

The effect of leaf extract and compound I on superoxide scavenging model is presented in **Table 7.3**. It was found that the methanol extract at concentration of 1000 µg/ml caused significant increase of the inhibition up to 60.18 ± 1.74%. The compound I at 50 µg/ml concentration exhibited 69.01 ± 2.74% inhibition of superoxide radicals. IC_{50} was found to be 8.80.03±3.38 µg/ml. Inhibition was found directly proportional to the amount of the extract added.

Table 7.1 Free radical scavenging activity of *Urtica parviflora* leaf extract and Compound I by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	-
2	Vitamin E (5 mM)	85.23±3.23	
	Concentration of methanol extract (µg/ml)		
3	200	14.59±3.01	
4	400	39.44±3.18	
5	600	51.87±2.87	698.92±3.43
6	800	69.82±3.01	
7	1000	75.56±2.64	
	Compound I		
8	25	79.81±2.58	
9	50	82.17±2.74	

Values are mean ± SEM of 3 replicates

Table 7.2 Effect of methanol extract of *Urtica parviflora* leaf and Compound I on ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Dish Number	Treatment	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	82.01±1.08		0.73±0.01*
	Concentration of methanol extract (µg/ml)			
3	200	27.16±2.68		1.09±0.02*
4	400	41.39±2.17		1.06±0.02*
5	600	54.18±2.68	859.32±2.79	1.01±0.01*
6	800	66.34±2.82		0.98±0.01*
7	1000	71.54±3.03		0.81±0.03*
	Compound I			
8	25	69.77±3.11		0.72±0.02*
9	50	80.19±2.48		0.70±0.03*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.3 Superoxide radical scavenging activity of *Urtica parviflora* leaf extract and Compound I.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	70.2± 1.65	
	Concentration of methanol extract (µg/ml)		
3	200	15.59 ± 2.13	
4	400	29.01 ± 2.01	880.03±3.87
5	600	36.48 ± 1.93	
6	800	47.38 ± 1.82	
7	1000	60.18 ± 1.74	
	Compound I		
8	25	67.14 ± 2.62	
9	50	69.01 ± 2.74	

Values are mean ± SEM of 3 replicates

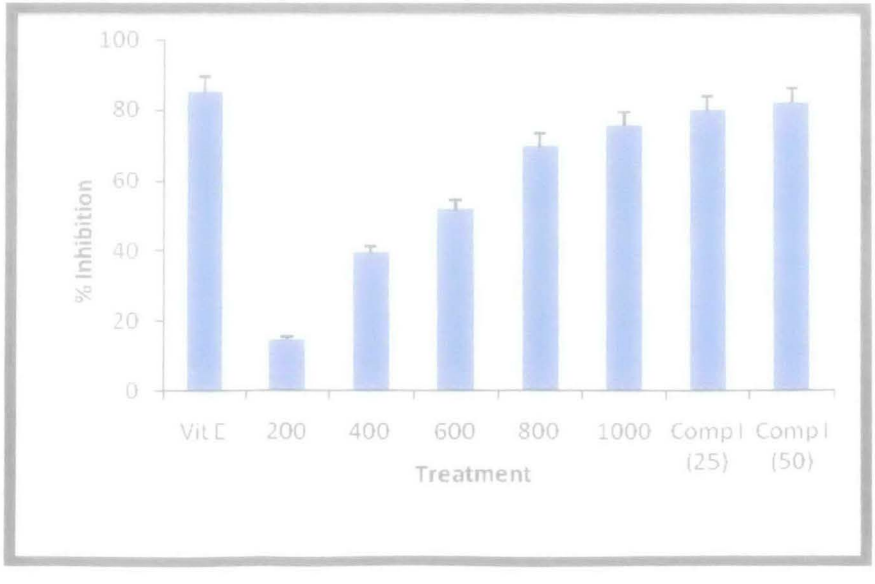

Fig 7.1 Percentage of inhibition of free radical scavenging activity of *Urtica parviflora* leaf extract and Compound I by DPPH reduction. Vitamin E (Vit E) was used as standard.

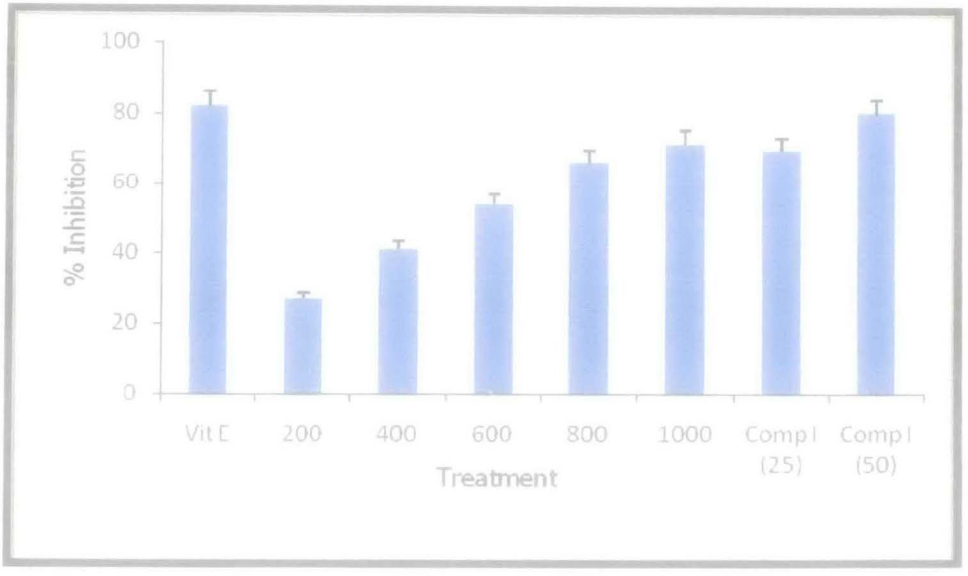

Fig 7.2 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Urtica parviflora* leaf and Compound I. Vitamin E (Vit E) was used as standard.

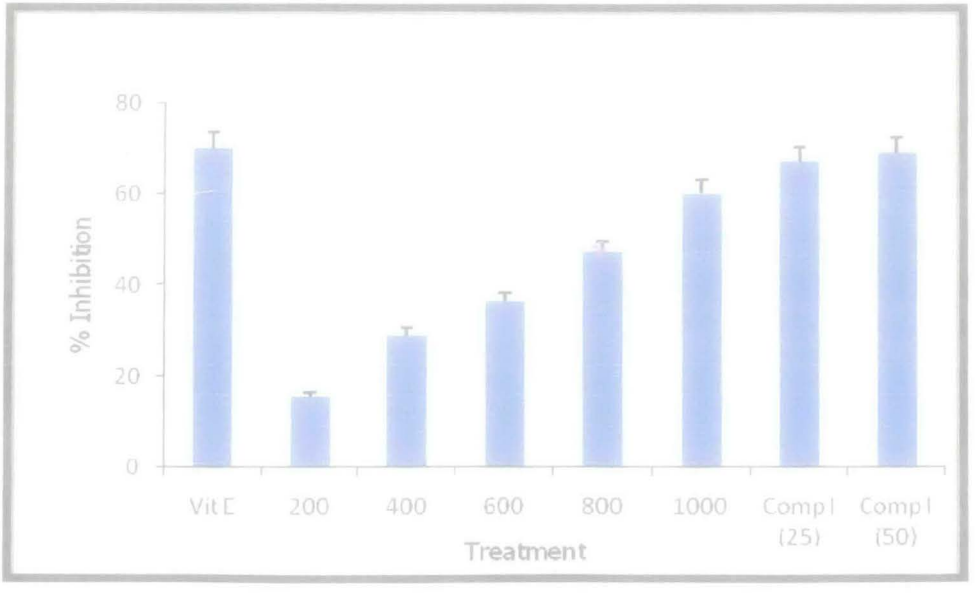

Fig 7.3 Percentage inhibition of Superoxide radical scavenging activity of *Urtica parviflora* leaf methanol extract [illegible] and Compound I. Vitamin E (Vit E) was used as standard.

7.6.2 Antioxidant activity of *Callicarpa arborea* leaf.

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in Table 7.4. The result showed that the DPPH scavenging capacity of the extract was found to be 71.22±3.41% at 1000 µg/ml concentration. The compound II at 50 µg/ml concentration showed 73.65±3.44% inhibition as compared to 83.59±3.12% of the standard drug vitamin E at 5mM. The IC_{50} value of the extract was found to be 593.52 µg/ml. The percentage of activity was also dependent on time as depicted from **Fig 7.3**.

The results presented in **Table 7.5** showed the effect of methanol extract of *Callicarpa arborea* leaf and compound II on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (61.88±2.73%) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 78.26±3.31. The same result has also been presented in Figure 7.4 which revealed that compound II at 50 µg/ml showed 75.01±4.59% inhibitions. The IC_{50} value was found to be 798.79±4.91µg/ml. From the results of quantitative estimation of TBARS levels it appears that the methanol extract at 1000 µg/ml concentration decreases the levels of TBARS in liver homogenate from 1.08±0.03 to 0.81±0.12 nmoles/mg of tissue. The compound II at 50 µg/ml concentration showed the level of TBARS at (0.72±0.03 nmoles/mg of tissue).

The effect of root extract and compound II on superoxide scavenging model is presented in Table 7.6. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 61.81±2.44%. The compound II at 50 µg/ml exhibited 66.28±2.44% inhibition of superoxide radicals. IC_{50} was found to be 891 µg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

Table 7.4 Free radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	83.59±3.12	
	Concentration of methanol extract (µg/ml)		
3	200	10.19±3.67	
4	400	31.23±4.02	593.52±3.87
5	600	[illegible]89±3.41	
6	800	*63.36*±3.68	
7	1000	71.22±3.41	
	Compound II		
8	25	72.37±2.53	
9	50	73.65±3.44	

Values are mean ± SEM of 3 replicates

Table 7.5 Effect of methanol extract of *Callicarpa arborea* leaf and Compound II on ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Dish Number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	78.26±3.31		0.71±0.02*
	Concentration of methanol extract (µg/ml)			
3	200	15.01±2.61		1.08±0.03*
4	400	28.14±3.51		1.04±0.04*
5	600	37.74±3.06	798.79±4.91	1.01±0.04*
6	800	49.62±2.91		0.95±0.03*
7	1000	61.88±2.73		0.81±0.12*
	Compound II			
8	25	71.99±4.56		0.74±0.04*
9	50	75.01±4.59		0.72±0.03*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.6 Superoxide radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidenc interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	66.38±2.29	
	Concentration of methanol extract (µg/ml)		
3	200	16.01±2.43	
4	400	31.11±3.08	891.01±2.61
5	600	49.33±2.29	
6	800	57.47±1.48	
7	1000	61.81±2.44	
	Compound II		
8	25	64.45±3.53	
9	50	66.28±2.44	

Values are mean ± SEM of 3 replicates

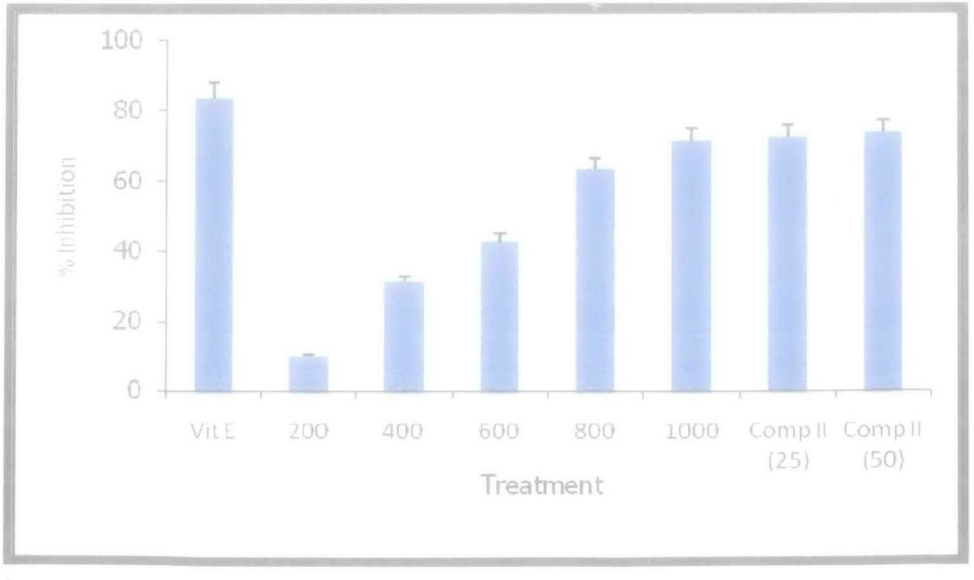

Fig 7.4 Percentage of inhibition of free radical scavenging activity of *Callicarpa arborea* leaf extract and Compound II by DPPH reduction. Vitamin E (Vit E) was used as standard.

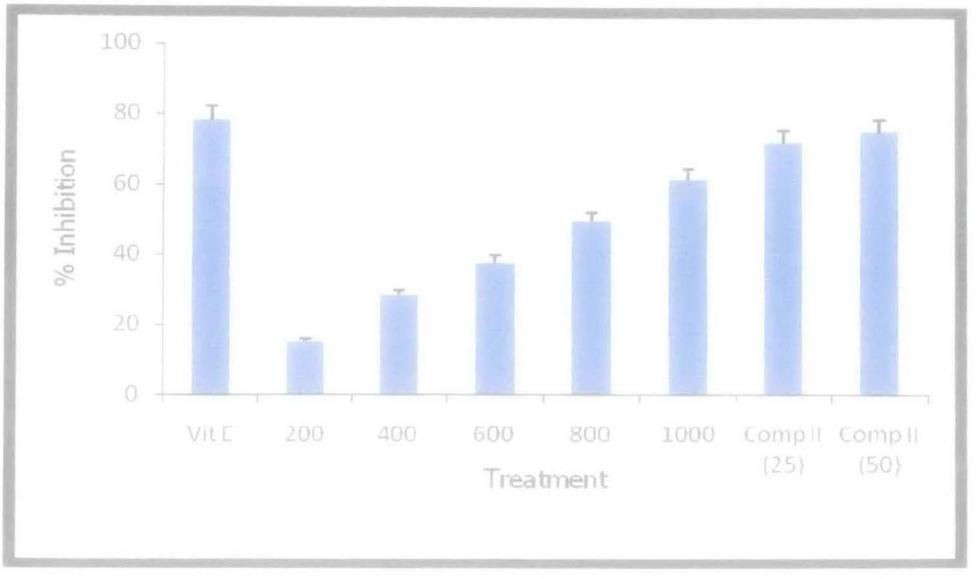

Fig 7.5 **Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Callicarpa arborea* leaf and Compound II. Vitamin E (Vit E) was used as standard.**

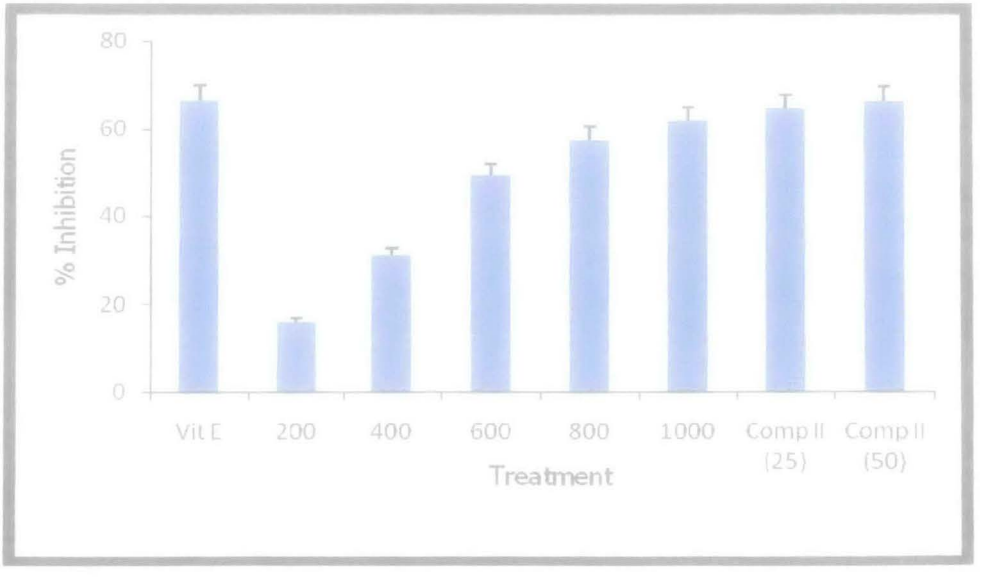

Fig 7.6 **Percentage inhibition of Superoxide radical scavenging activity of *Callicarpa arborea* leaf methanol extract [illegible] and Compound II. Vitamin E (Vit E) was used as standard.**

7.6.3 Antioxidant activity of *Morinda citrifolia* root.

The effect of methanol extract and compound II on scavenging of DPPH radical is presented in **Table 7.7**. The result showed that the DPPH scavenging capacity of the extract was found to be 74.67±3.01% at 1000 µg/ml concentration which is of similar level as compared to compound III at 50 µg/ml concentration, exhibited 74.81±3.14%. The standard drug (vitamin E) at 5mM showed 81.34±4.27 % inhibition. The IC_{50} value of the extract was found to be 591.73 µg/ml. Also the percentage of activity was time dependent **Fig 7.5**.

The results presented in **Table 7.8** showed the effect of methanol extract of *Morinda citrifolia* root and compound III on ferrous sulphate induced lipid peroxidation in rat liver homogenate. The results revealed that at 1000 µg/ml concentration of methanol extract had the maximum inhibition (63.14±2.57%) of lipid peroxidation. On the other hand the standard drug vitamin E showed that at 5mM concentration the inhibition percentage was 74.61±2.68. The same result have also been presented in **Fig 7.6** which revealed that compound III at 50 µg/ml showed 68.32±3.01% inhibitions, which is almost equal to that of the inhibition produced by vitamin E. The IC_{50} value was found to be 766.3±3.94 µg/ml. From the results of quantitative estimation of TBARS levels, it appears that the methanol extract at 1000 µg/ml concentration decreases the levels of TBARS in liver homogenate from 0.98 ± 0.01 to 0.70 ± 0.12 nmoles/mg of tissue and its compound III at 50 µg/ml concentration showed the level much below TBARS (0.57 ± 0.01 nmoles/mg of tissue), which is much higher rate of decrease than that of the results obtained from vitamin E ($p< 0.05$).

The effect of root extract and compound III on superoxide scavenging model is presented in **Table 7.9**. It was found that the methanol extract at 1000 µg/ml concentration caused significant increase of the inhibition up to 59.63 ± 2063%. The compound III at 50 µg/ml exhibited 68.01 ± 2.01% inhibition of superoxide radicals. IC_{50} was found to be 895.6 µg/ml. Inhibition was found to be directly proportional to the amount of the extract added.

Table 7.7 Free radical scavenging activity of *Morinda citrifolia* root extract and Compound III by DPPH reduction.

Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	-
2	Vitamin E (5 mM)	81.34±4.27	591.73±3.48
	Concentration of methanol extract (µg/ml)		
3	200	10.17±2.42	
4	400	34.28±2.72	
5	600	44.92±2.85	
6	800	68.49±2.91	
7	1000	74.67±3.01	
	Compound III		
8	25	72.19±3.19	
9	50	74.81±3.14	

Values are mean ± SEM of 3 replicates

Table 7.8 Effect of methanol extract of *Morinda citrifolia* root and Compound III on Ferrous sulphate induced lipid peroxidation in rat liver homogenate.

Tube Number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} value and confidence interval (µg/ml)	TBARS (n moles/mg tissue)
1	Control	-	-	-
2	Vitamin E (5 mM)	74.61±2.68		0.98±0.01*
	Concentration of methanol extract (µg/ml)			
3	200	14.68±3.11		0.97±0.01*
4	400	27.42±3.23		0.96±0.02*
5	600	37.17±3.01	766.3±3.94	0.94±0.01*
6	800	50.51±3.17		0.90±0.01*
7	1000	63.14±2.57		0.70±0.12*
	Compound III			
8	25	66.19±2.21		0.59±0.02*
9	50	68.32±3.01		0.57±0.01*

Values are mean ± SEM of 3 replicates, '*' indicates $p < 0.05$.

Table 7.9 Superoxide radical scavenging activity of *Morinda citrifolia* root extract and Compound III.

Tube number	Concentration of methanol extract (µg/ml)	Inhibition (%)	IC_{50} and confidenc interval (µg/ml)
Tube number	Treatment	Inhibition (%)	IC_{50} and confidence interval (µg/ml)
1	Control	-	
2	Vitamin E (5 mM)	67.33± 2.48	
	Concentration of methanol extract (µg/ml)		
3	200	14.01 ± 2.77	
4	400	24.32 ± 2.37	895.6±5.72
5	600	38.97 ± 2.28	
6	800	51.66 ± 2.50	
7	1000	59.63 ± 2063	
	Compound III		
8	25	67.18 ± 2.17	
9	50	68.01 ± 2.01	

Values are mean ± SEM of 3 replicates

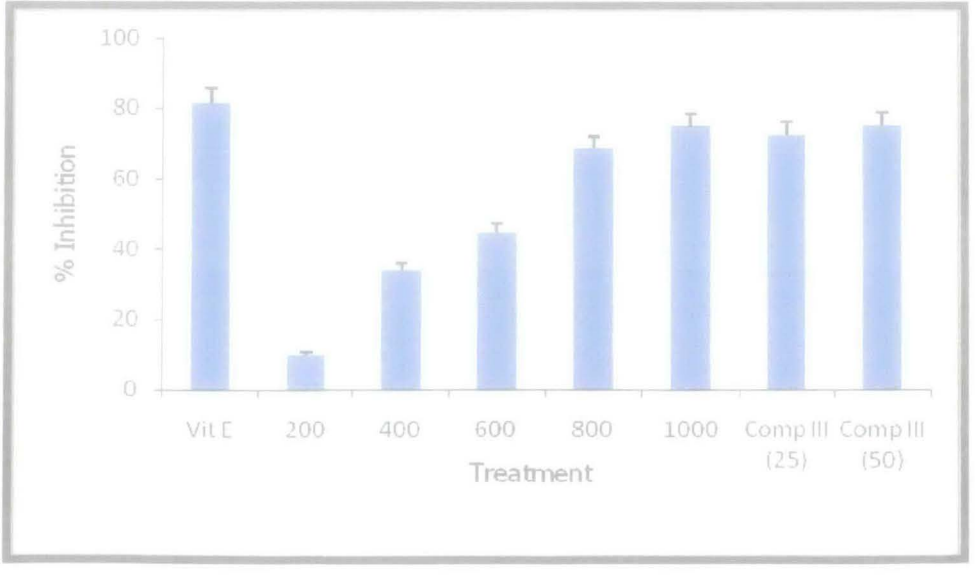

Fig 7.7 Percentage of inhibition of free radical scavenging activity of *Morinda citrifolia* leaf extract and Compound III by DPPH reduction. Vitamin E (Vit E) was used as standard.

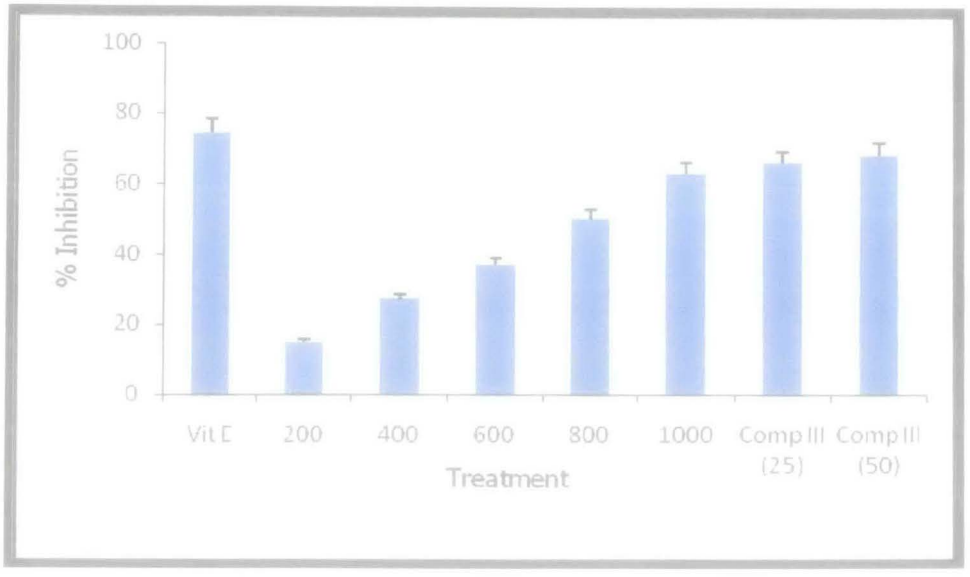

Fig 7.8 Percentage of inhibition of lipid peroxidation by different concentrations of methanol extract of *Morinda citrifolia* leaf and Compound III. Vitamin E (Vit E) was used as standard.

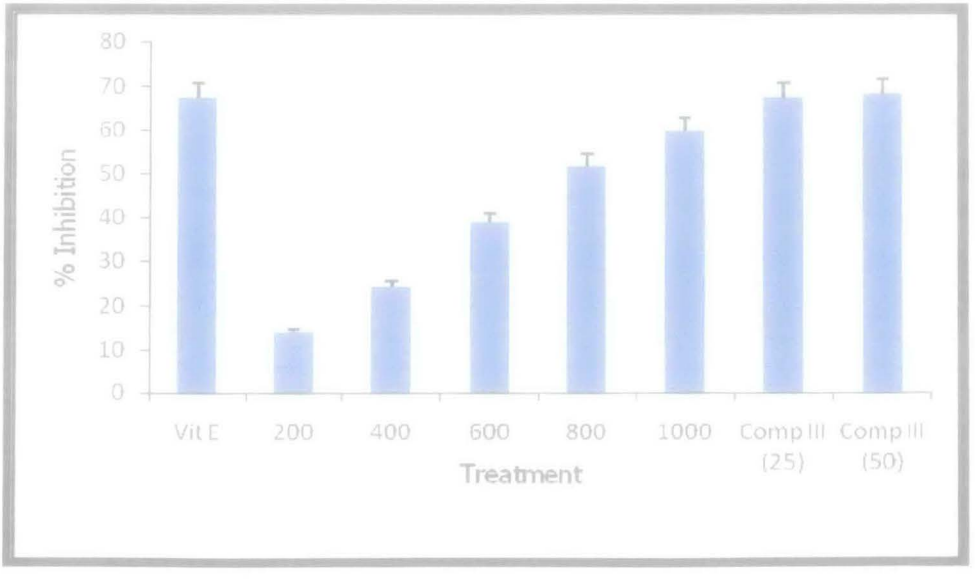

Fig 7.9 Percentage inhibition of Superoxide radical scavenging activity of *Morinda citrifolia* leaf methanol extract and Compound III. Vitamin E (Vit E) was used as standard.