Phytochemical Screening and Antioxidant Activity of the Stem of *Amaranthus spinosus* Linn. (Family- Amaranthaceae)

Thesis submitted in partial fulfilment for the requirement of the
Degree of Master of Pharmacy
Faculty of Engineering and Technology

By
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Examination Roll No.: **M4PHA19024**
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Certificate of Approval

This is to certify that **Suparna Ghosh, (Examination Roll No.: M4PHA19024, Registration No.: 140848 of 2017-18)** has carried out the project work on the subject entitled **"Phytochemical Screening and Antioxidant Activity of the Stem of *Amaranthus spinosus* Linn. (Family- Amaranthaceae)"** under the supervision of **Prof. (Dr.) Tapan Kumar Maity**, Division of **Pharmaceutical chemistry**, Department of Pharmaceutical Technology, Jadavpur university. This project work submitted by her in partial fulfilment of the requirements for the degree of **Master of Pharmacy** (Pharmaceutical Chemistry) of Jadavpur University. She has carried out her work independently, with proper care and attention to our entire satisfaction.

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DECLARATION OF THE ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research as part of my work on **"Phytochemical Screening and Antioxidant Activity of the Stem of *Amaranthus spinosus* Linn. (Family- Amaranthaceae)".**

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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SIGNATURE WITH DATE:

Dedicated to
my guide and parents

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CHAPTER 1

INTRODUCTION

INTRODUCTION

According to the World Health Organization, traditional medicines are the type of drugs that refer to health practices, approaches, knowledge and beliefs in which plant, animal, mineral based medicines, spiritual therapies and manual techniques applied individually or in combination for the treatment, diagnosis and prevention of illness[1]. During the last decade traditional medicines have gained popularity all over the world. A large portion of the population relies on traditional medicines and herbal plants in order to meet health care needs. Herbal medicines have often maintained their popularity for historical and cultural reasons and herbal products have become more widely available commercially, especially in developed countries. Herbal medicines also known as phytomedicine or phytotherapy, is an alternative of crude plant extracts used as health promoting agent. Consumers believe that herbal medicines are safe because they are natural product, although herbal medicines have adverse effects and may cause toxicity to human being[2]. Many antioxidant compounds can be found in fruits and vegetables including phenolics, carotenoids, anthocyanins and tocopherols. Approximately 20% of known plants have been used in pharmaceutical studies, impacting the healthcare system in positive ways such as treating cancer and harmful diseases. Plants are able to produce a large number of diverse bioactive compounds. High concentrations of phytochemicals, which may protect against free radical damage, accumulate in fruits and vegetables. Plants containing beneficial phytochemicals may supplement the needs of the human body by acting as natural antioxidants. Various studies have shown that many plants are rich source of antioxidants. For example, vitamin A, C, E and phenolic compounds such as flavonoids, tannins and lignins found in plants and all act as antioxidants. The consumption of fruits and vegetables has been linked with several health benefits, a result of medicinal properties and high nutritional value. Antioxidants control and reduce the oxidative damage in foods by delaying or inhibiting oxidation caused by reactive oxygen species (ROS), ultimately increasing the shelf-life and quality of these foods. β-carotene, ascorbic acid and many phenolics play dynamic roles in delay of aging, reducing inflammation and preventing certain cancers. Increasing the consumption of fruits and vegetables has been recommended by many agencies and health care[3].

ANTIOXIDANT: Substances that at low concentration delays the oxidation of protein, carbohydrate, lipid and DNA are known as antioxidants. More biologically it can be said that antioxidants are synthetic or natural substances those are added to products to prevent their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene that are capable of preventing the damaging effects of oxidation in animal tissues. In chemical industry there are some products like rubber, plastic etc. in which auto-oxidation process retards due to presence of antioxidants[4].

CLASSIFICATION OF ANTIOXIDANT: Antioxidants can be classified into two groups. They are-

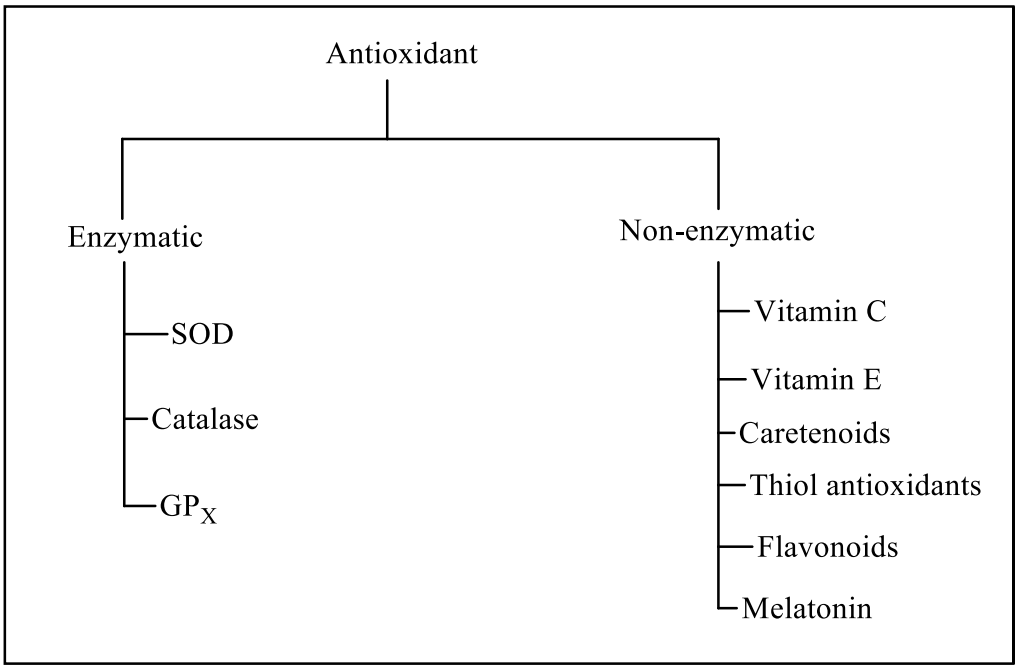


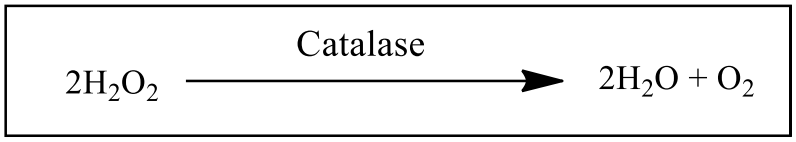
Figure 1: Classification of antioxidant

A. ENZYMATIC ANTIOXIDANT:

They are natural antioxidants. Some examples of enzymatic antioxidants are: superoxide dismutase, catalase and glutathione peroxidase.

i. Superoxide dismutase (SOD): Superoxide dismutase is an enzyme that is present in the body and catalyzes the superoxide dismutation. Hydrogen peroxide is produced from this reaction which helps to transmit the injury caused by free radicals. The enzyme SOD has three variants. One is predominant copper-zinc containing enzyme that is found in the cytoplasm. Another one is manganese SOD that is located in the mitochondria and a third type SOD is present extracellularly[5].

ii. Catalase: Catalase is an enzymatic antioxidant that acts as a catalyst. It converts hydrogen peroxide to oxygen and water and prevents the effect of hydrogen peroxide that is present intracellularly. Most of the catalase present in cytoplasm is lost during tissue manipulation[6].



iii. Glutathione peroxidase: Glutathione peroxidase and glutathione reductase are enzymes that act as an enzymatic antioxidant. The reduced form of glutathione is protective because it helps to neutralize hydrogen peroxide produced in the cell and the oxidized form is not protective. These enzymes maintain a key role in preventing increased level of oxidative stress. It is a free radical scavenger[7].

B. NON-ENZYMATIC ANTIOXIDANT:

Non-enzymatic antioxidants are: vitamin C, vitamin E, α-lipoic acid, N-acetyl cysteine, taurine, carotenoids, flavonoids etc.

i. Ascorbic acid (vitamin C): Ascorbic acid is a very important and powerful antioxidant that works in aqueous environment of the body. Ascorbic acid behaves as a vinylogous carboxylic acid where the double bond (vinyl) transmits electron pairs between the hydroxyl and the carbonyl group. Ascorbate acts as an antioxidant by being available for energetically favorable oxidation. Reactive oxygen species oxidize ascorbate first to monodehydro ascorbate and then dehydro ascorbate. The reactive oxygen species are reduced to water while the oxidized forms of ascorbate are relatively stable and unreactive and do not cause cellular damage[8]. Vitamin C scavenges the aqueous reactive oxygen species by very rapid electron transfer that inhibits lipid peroxidation[9].

ii. Vitamin E: Vitamin E exists in eight different isomeric forms of two substructures: tocopherol and tocotrienol. Both structures are similar except the tocotrienol structure that has double bond on the isoprenoid unit[10]. Alpha tocopherol is the most active form of vitamin E in human and is a powerful biological antioxidant which is considered to be the major membrane bound antioxidant[11]. Its' main antioxidant function is protection against lipid peroxidation. During the antioxidant reaction, α-tocopherol is converted to a α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical[12]. Vitamin E performs a unique function by interrupting

free radical chain reactions via capturing the free radical. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties. The hydrogen from this group is donated to the free radical, resulting in a relatively stable free radical form of the vitamin. The antioxidant function of this micronutrient enhances immunity by maintaining the functional and structural integrity of important immune cells[13]. Vitamin E has the ability to prevent cell injury and also has protective activity against arsenic toxicity. It can prevent inhibition of blood amino-levulinate-dehydratase (ALAD) activity by intramuscular administration[14].

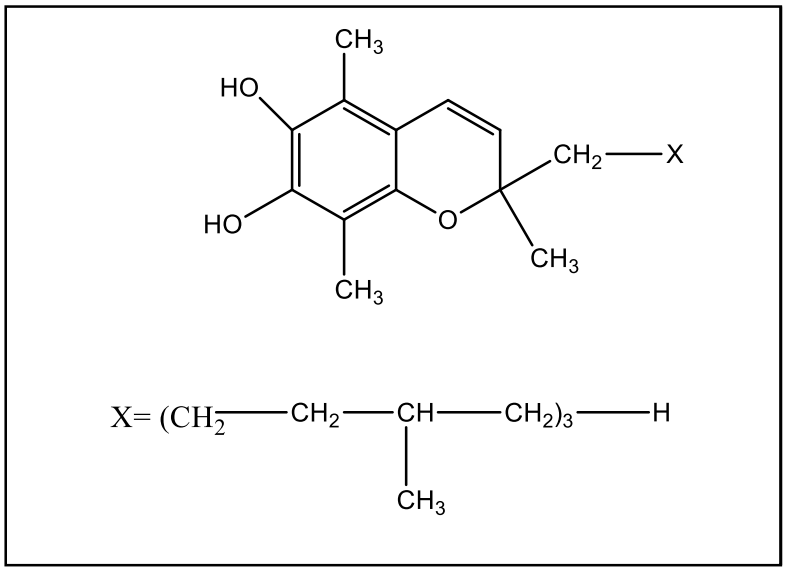
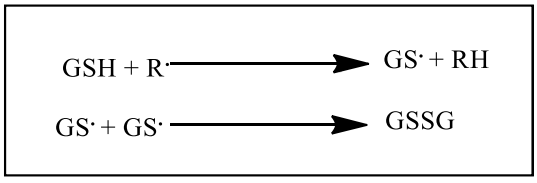


Figure 2: Chemical structure of α-tocopherol.

iii. Glutathione (GSH): The major thiol antioxidant is tripeptide glutathione. GSH is a multifunctional intracellular non-enzymatic antioxidant which is considered to be the major thiol-disulphide redox buffer of the cell[15]. Generally the antioxidant capacity of thiol compounds is due to the sulfur atom which can easily accommodate the loss of a single electron[16]. The reaction of glutathione with the radical R• as well as combination with another thiol radical is shown in the above reaction:



Oxidized glutathione GSSG is accumulated inside the cells and the ratio of GSH:GSSG is a good measure of oxidative stress of an organism[17].

iv. α-lipoic acid (LA): α-Lipoic Acid (1,2-dithione-3-pentanoic acid) is a sulfur containing antioxidant with metal chelating and antiglycation capabilities. Lipoic acid

is active in both lipid and aqueous phases[18]. LA is readily absorbed from diet and is rapidly converted to dihydrolipoic acid (DHLA) by NADH or NADPH in most tissues. DHLA possess metal chelating properties which help the body to get rid of accumulated ingested toxins[19]. Exogenous administration of LA has been found to have therapeutic potential in neurodegenerative disorders. LA can also cross the blood-brain barrier and is taken up by all areas of the central and peripheral nervous system[20].

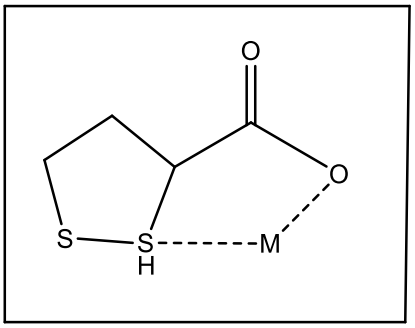


Figure 3: Reduced form of lipoic acid showing metal chelation at thiol groups (M=Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Fe^{3+})

v. Carotenoids: Carotenoids are pigments that are found in plants and micro-organisms. Various studies have indicated that carotenoids may prevent or inhibit certain types of cancer, artherosclerosis, age-related muscular degeneration and other diseases. The antioxidant activity of carotenoids arises primarily as a consequence of the ability of the conjugated double-bonded structure to delocalize unpaired electrons[21]. Carotenoids have anti-proliferative effect on various cancer cell lines. Among them, lycopene has been shown to inhibit cell cycle progression in breast, lung and prostate cell lines. β-carotene inhibits the expression of antiapoptotic protein Bcl-2 in cancer cells, thus reducing growth of the cells[22].

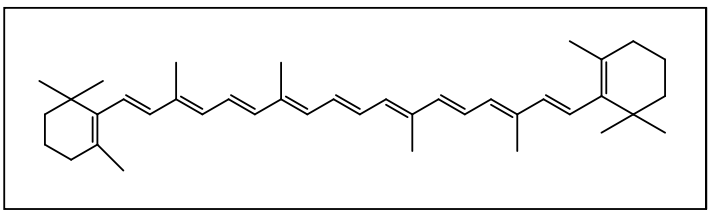


Figure 4: β-Carotene

vi. Flavonoids: Polyphenolic compounds constitute one of the most commonly occurring groups of plant metabolites and represent an integral part of human[23]. Their common structural feature is the diphenylpropane moiety, which consists of two aromatic rings linked through three carbon atoms that together usually form an

oxygenated heterocycle. Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation[24]. One of the most active properties of flavonoids is their protection against oxidative stress. For example, flavonoids are ideal scavengers of peroxyl radicals due to their favorable reduction potentials relative to alkyl peroxyl radicals and thus, they are effective inhibitors of lipid peroxidation[25].

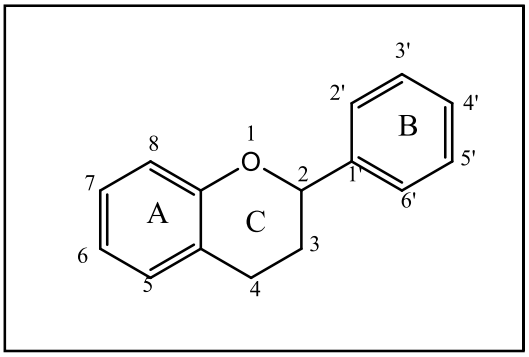


Figure 5: Diphenylpropane

MECHANISM OF ACTION OF ANTIOXIDANTS[26]:

The mechanism of action of antioxidants are as follows:

i. Chain breaking reaction. Like the antioxidant α-tocopherol act in lipid phase and trap free radical.

ii. Reduction of reactive oxygen species concentration. Eg.: glutathione.

iv. Chelation of transition metal catalyst. In this way transferrin, lactoferrin and ferritin reduces iron induced oxidative stress and ceruloplasmin and albumin as copper sequestrants.

FREE RADICAL:

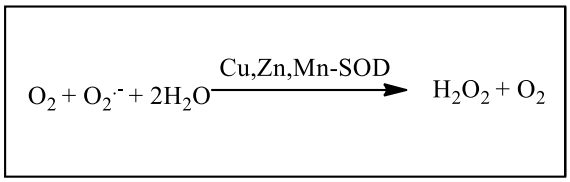
Free radical is an atom that loses an electron from the orbital of an outer shell. Free radicals are highly reactive and unstable because of this unpaired electron. They react with their nearby molecule and cellular constituents such as protein, membrane and nucleic acid. The free radical can react with any molecule and convert it to a free radical to initiate a reaction. Thus a single free radical can cause various reactions. Antioxidant supplementation is needed for protection against free radical production[27].

Free radicals can be classified into 2 groups: Reactive oxygen species and Reactive nitrogen species.

A. REACTIVE OXYGEN SPECIES (ROS):

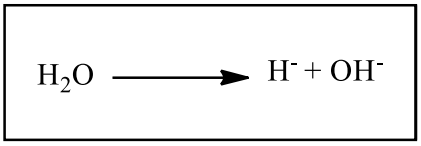
Reactive Oxygen Species are different kind of reactive molecules or free radicals that are derived from molecular oxygen. They are formed by the mitochondrial electron transport of aerobic respiration or by the oxidoreductase enzymes. ROS has an important role in cell signaling, such as gene expression, apoptosis etc. They also can activate the cell signaling cascades. ROS can act as both intra-cellular and inter-cellular messengers. Some example of reactive oxygen spices are: superoxide radical, hydroxyl radical, alkoxyl radical and peroxyl radical[28].

i. Superoxide ion radical ($O_2^{\cdot-}$): Some enzymatic process and auto-oxidation reactions are responsible for the production of superoxide ion radical. Also a non-enzymatic process is responsible for this radical formation in which an electron is transferred to molecular oxygen. It has disadvantages because of their damaging effect in DNA, mitochondria and other molecules. There are some enzymes like lipo-oxygenase, cyclooxygenase, xanthine-oxidase and NADPH dependent oxidase that can produce this type of radicals. Superoxide radicals produce hydrogen peroxide in a dismutation reaction[29].



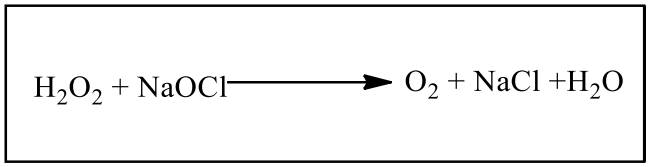
In the above reaction one radical is oxidized to oxygen and another one is reduced to hydrogen peroxide[29].

ii. Hydroxyl radical ($^{\cdot}OH$): Neutral form of hydroxide ion is hydroxyl ion. They can damage the organic molecules like carbohydrate, lipid, protein, DNA etc. At diffusion controlled rate it reacts with living cells. Exposure to ionizing radiation can cause harmful effects and most of them are caused by the attack of OH^-.

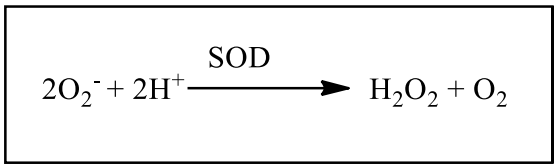


Above reaction shows that ionizing radiation causes fission of OH bonds in water to give H^- and OH^-[30].

iii. Singlet oxygen ($^1O_2$): It is known as dioxygen or dioxidene. By irradiation of oxygen gas methylene blue (an organic dye) generates singlet oxygen. Hydrogen peroxide reacts with hypochlorite to give singlet oxygen[31].



iv. Hydrogen peroxide (H_2O_2): Hydrogen peroxide is produced by dismutation reaction in which superoxide dismutase is involved[31].



They promote the disproportionation of superoxide into oxygen and hydrogen peroxide, which is then rapidly decomposed by the enzyme catalase to oxygen and water. Hydrogen peroxide can cause cell damage at a low concentration[31].

v. Peroxyl radical ($ROO^{\cdot}$): Peroxyl radicals are produced by the metabolism of hydroperoxides. Peroxyl radical play an important role in biological system, including lipid peroxidation, DNA cleavage and protein backbone modification. Peroxyl radicals are formed by the decomposition of lipid and protein peroxides or by the addition of transition metal ions. It can also be generated by the addition of O_2 in carbon centered radicals[31].

B. **REACTIVE NITROGEN SPECIES (RNS):**

Reactive nitrogen species are derived from nitric oxide. Example of some reactive nitrogen species are nitroxyl anion, nitrosonium cation, higher oxides of nitrogen, s-nitrosothiols and dinitrosyl iron complexes[32].

i. Nitric oxide (NO): Nitric oxide is a gaseous molecule. They are synthesized by NO-synthase (NOS). It contains one atom of oxygen and one atom of nitrogen thus they contain one unpaired electron. It acts as a second messenger and modulates biological processes such as inflammation, cytotoxicity, endothelial function, neuroplasticity etc. It can also generate other reactive nitrogen species by reacting with oxygen and thiols[33].

ii. Peroxynitrite: It forms in an aqueous environment. It can rapidly decompose into nitrogen dioxide and hydroxyl radical which is a highly reactive species. It can remove electrons from any biological molecule. By reacting with CO_2, peroxynitrite produce nitrosoperoxycarbonate ($ONOOCO_2$). Peroxynitrite reacts with metal centers that create cytotoxic circumstances which can be showed by the production of

carbonate and hydroxyl radical[34]. RNS acts on a variety of targets responsible for their pleiotropic effects.

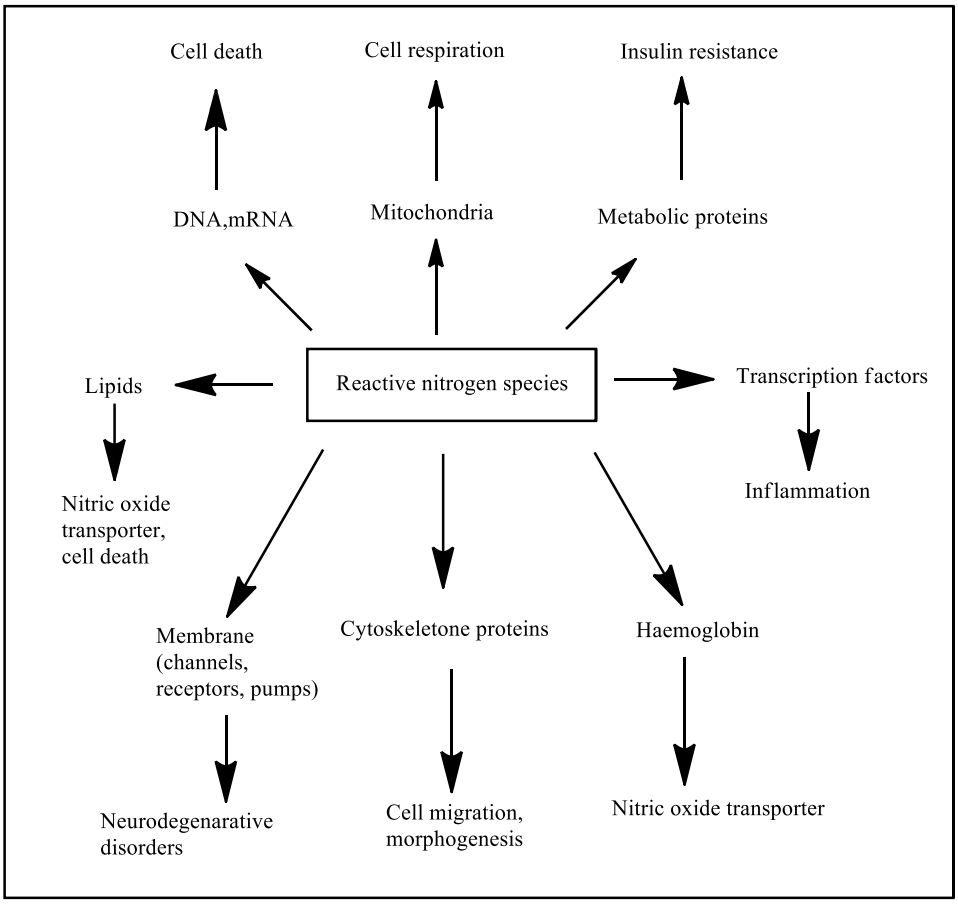


Figure 6: Molecular targets of reactive nitrogen species (RNS)[35].

FORMATION OF FREE RADICALS:

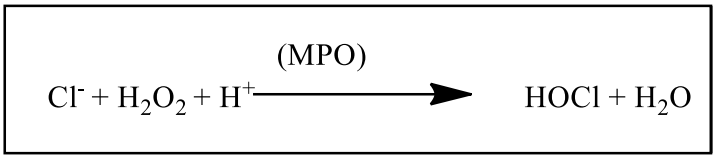
ROS is generated by the rapid uptake of oxygen. Superoxide anion radical is produced by the activation of NADPH oxidase[36].

$$2O_2 + NADPH \xrightarrow{(Oxidase)} 2O_2^{\cdot -} + NADP^+ + H^+$$

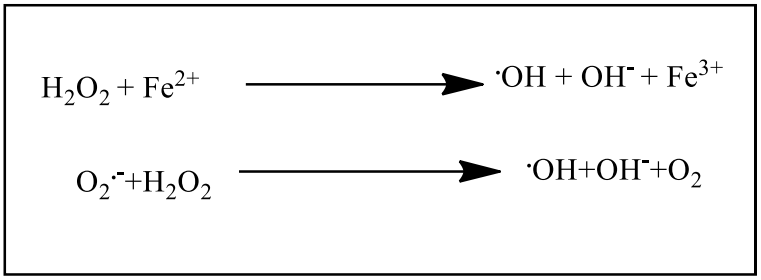
The superoxide anion radical ($O2^{\cdot -}$) is then converted to hydrogen peroxide by superoxide dismutase[36].

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{(SOD)} H_2O_2 + O_2$$

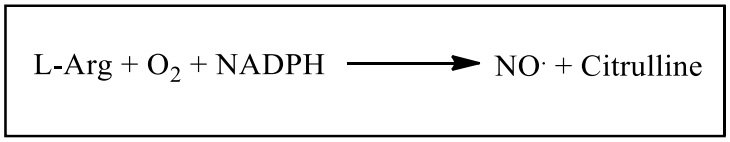
The reactive species can also be produced by myeloperoxidase-halide-hydrogen-peroxide system. Myeloperoxidase is present in neutrophil cytoplasmic granule. Hydrogen peroxide is converted to hypochlorous acid when chloride ions are present which is a potent oxidant and antimicrobial agent[36].



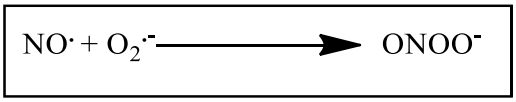
Reactive oxygen species are also generated by respiration burst process from $O_2^{\cdot -}$ and H_2O_2. This reaction is known as Haber-Weiss reaction[37].



Nitric oxide is a reactive nitrogen species which is produced in a large amount by the enzyme nitric oxide synthase from arginine[38].



The $NO^{\cdot}$ and $O_2^{\cdot -}$ reacts together and produces peroxynitrite which is a very strong oxidant. Peroxynitrite is a potent and versatile oxidant that can attack a wide range of biological targets[38].



Peroxynitrite reacts with the aromatic amino acid residues in the enzyme resulting in the nitration of the aromatic amino acids. This change in the amino acid residue can result in the enzyme inactivation. Nitric oxide is an important cytotoxic effector molecule in the defense against tumor cells, various protozoa, fungi, helminthes and mycobacteria[39]. Cyclooxygenation, lipooxygenation, lipid peroxidation, metabolism of xenobiotic and ultraviolet radiations also can produce free radicals[40].

DAMAGING REACTIONS OF FREE RADICALS:

Free radicals can cause oxidative stress that may lead to various chronic diseases like cancer, coronary heart disease, osteoporosis etc. Free radicals attack all major classes of biomolecules, mainly the polyunsaturated fatty acids (PUFA) of cell membranes. The oxidative damage of PUFA, known as lipid peroxidation is particularly destructive, because it proceeds as a self-perpetuating chain reaction[41].

There are some diseases related with free radicals:

Free radicals and aging: Aging is a progressive decline in the efficiency of biochemical and physiological process after the reproduction phase of life. Aging process rapidly increase with age because of the exponential nature. Many theories have been proposed to explain aging. Among this "free radical theory of aging" has gained universal acceptance and is supported by the fact of production of free radicals and free radical damage increases with age. Free radicals cause oxidative damage to cellular components that results in altered cellular function and finally death occur. Oxidative phosphorylation is a process that occurs in mitochondria and by which body can produce energy and other metabolic reactions generate free radicals imposing oxidative stress on proteins, DNA and lipids. The restriction of caloric intake in rodents has been shown to increase lifespan, increase free radical defenses and reduce oxidative damage. The tissues of species which live longer are also less susceptible to oxidative damage then tissue of species which have shorter life spans and this is supported by the fact that conditions which generate increased free radical production such as a high metabolic rate are associated with a shorter lifespan[42].

OXIDATIVE STRESS:

Oxidative stress occurs during an imbalance between free radicals and antioxidants. Oxygen free radical can attack many biological molecules like lipids, proteins, DNA etc. Some biomarkers like lipid hydro peroxidase, isoprostanand 8-hydroxyguanine can cause oxidative stress. Oxidative stress also can cause some diseases that are related to our lifestyle such as atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, malignancies etc[43].

Oxidative stress and rheumatoid arthritis: Rheumatoid arthritis (RA) is an autoimmune inflammatory disorder. Highly reactive oxygen free radicals are involved in the pathogenesis of the disease. Oxidative stress can be observed in patients having rheumatoid arthritis by increased intracellular ROS production, increased lipid

peroxidation, protein oxidation, DNA damage and impaired enzymatic and non-enzymatic antioxidant defense system of the body. DCFH-DA (Dichloro-dihydro-fluorescein diacetate) is used for detecting intracellular ROS Production as it can easily pass the cell membrane[44]. DCFH-DA is hydrolyzed to DCFH by intracellular esterase which on reacting with ROS forms a highly fluorescent DCF. DCF formation was increased in RA patients relative to control due to enhanced ROS formation thereby indicating insufficient antioxidant defense system in RA patients. ROS formation has also found to be increased in the liver and brain of rats with adjuvant arthritis[45]. Oxidative stress, in addition impairs DNA mismatch repair system which results in an increase in the formation of DNA adducts in the joints and hence in the augmentation of the disease[46].

Oxidative stress in angiogenesis: VEGF (vascular endothelial growth factor) is the most potent and primary endothelial specific angiogenic growth factor. VEGF signaling is required for normal vascular development and homeostasis but it is also actively engaged in tumor progression by promoting growth of tumor vasculature[47]. This signaling pathway affected by reactive oxygen species (ROS). Exogenous ROS stimulate the induction of VEGF expression in various cell types such as endothelial cells, smooth muscle cells and macrophages. VEGF induces endothelial cell migration and proliferation through an increase of intracellular ROS. Thus there is a relation between oxidative stress and angiogenesis on the VEGF signaling pathway. Hydrogen peroxide induces VEGF expression in vascular smooth muscle cells as well as endothelial cells and thereby promotes angiogenic responses. Physiological angiogenesis in adult organisms is required for tissue repair and remodeling process such as wound healing, skeletal remodeling and female reproduction. In wound healing, angiogenesis is induced by tissue hypoxia and ROS which either stimulate macrophages, fibroblasts, endothelial cells and keratinocytes to produce VEGF or operate in a VEGF independent fashion. During endochondrial ossification during longitudinal bone growth increasing ROS promote chondrocyte hypertrophy which in turn induces angiogenesis. Antioxidant treatment inhibits these processes. Angiogenesis is important for cyclical regeneration of endometrium in the menstrual cycle and is also regulated by ROS[48].

Effects of oxidative stress on lipid (Lipid Peroxidation): Reactive oxygen species can induce lipid peroxidation and disrupt the membrane lipid bilayer arrangement that

may inactivate membrane-bound receptors and enzymes and increase tissue permeability[49]. Lipid peroxidation can be described generally as a process under which oxidants such as free radical or non-radical species attack lipid containing carbon-carbon double bond especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxide radicals and hydroperoxides[50]. Lipid also can be oxidized by enzymes like lipoxygenases, cyclooxygenases and cytochrome P450. Under physiological or low lipid peroxidation rate the cells stimulate their maintenance and survival through constitutive antioxidant defense system or signaling pathway activation that upregulate antioxidant proteins resulting in an adaptive stress response. By contrast, under medium or high lipid peroxidation rates the extent of oxidative damage overwhelms repair capacity and the cells induce apoptosis or necrosis that causes cell death. Both processes eventually lead to molecular cell damage which may facilitate development of various pathological states and accelerated aging[51]. Products of lipid peroxidation such as unsaturated aldehydes are capable of inactivating many cellular proteins by forming protein cross-linkage. Isoprostanes and thiobarbituric acid reactive substances have been used as indirect biomarkers of oxidative stress and increased levels were shown in the exhaled breath condensate or bronchoalveolar lavage fluid or lung of chronic obstructive pulmonary disease patients or smokers[52].

Effect of oxidative stress on DNA: Reactive oxygen species can lead to DNA modifications in several ways which involves degradation of bases, single or double stranded DNA breaks, purine, pyrimidine or sugar bound modifications, mutations, deletions or translocations and cross-linking with proteins. Most of these DNA modifications are highly relevant to carcinogenesis, aging and neurodegenerative, cardiovascular and autoimmune diseases. Tobacco smoke, redox metals and nonredox metals, such as iron, cadmium, chrome and arsenic are also involved in carcinogenesis and aging by generating free radicals or binding with thiol groups. Formation of 8-OH-G is the best-known DNA damage occurring via oxidative stress and is a potential biomarker for carcinogenesis[52]. Promoter regions of genes contain consensus sequences for transcription factors. These transcription factor binding sites contain GC rich sequences that are susceptible for oxidant attacks. Formation of 8-OH-G DNA in transcription factor binding sites can modify binding of transcription

factors and thus change the expression of related genes for AP-1 and Sp-1 target sequences. The TATA-binding protein initiates transcription by changing the bending of DNA. The binding of TATA-binding protein may be impaired by the presence of cyclo-dA[53]. Oxidative stress causes instability of microsatellite regions. Redox active metal ions, hydroxyl radicals increase microsatellite instability[54].Methylation at CpG islands in DNA is an important epigenetic mechanism that may result in gene silencing. Oxidation of 5-MeCyt to 5-hydroxymethyl uracil can occur via deamination or oxidation reactions of thymine or 5-hydroxymethyl cytosine intermediates[55].In addition to the modulating gene expression, DNA methylation also seems to affect chromatin organization. Aberrant DNA methylation patterns induced by oxidative attacks also affect DNA repair activity. Oxidative stress is also responsible for the oxidation of DNA and increased oxidative DNA damage has been found in many autoimmune disorders[56].

Role of oxidative stress in air: Ambient air contains a range of pollutants, the exact combination of which varies from one microenvironment to the next. Many of the individual pollutants that make up this ambient mix are free radicals (for example, nitrogen dioxide) or have the ability to drive free radical reactions (for example, ozone and particulates). The three major pollutants that cause oxidative stress are: Ozone, nitrogen dioxide and particulates[57].

i. Ozone: Ozone is a relatively insoluble gas. It is very reactive and uptake is directly related to reactions with substrates present in the lung lining fluid, the first compartment it encounters on entering the lung. **Langford** *et al.*, referred to this mechanism as reactive absorption. The uptake of ozone is thus related not only to its concentration but also availability of substrates within the lung lining fluid compartment. Following reaction with a target substrate, ozone is consumed, disabling it from transiting the lung lining fluid compartment. Cellular responses to ozone are therefore not the result of direct reaction of ozone with cell surface components but are mediated through a cascade of secondary, free radical derived ozonation products[58].

ii. Nitrogen dioxide: Nitrogen dioxide is a nitrogen centered free radical with limited solubility in aqueous solutions. Like ozone, it reacts with substrates present in the lung lining fluid compartment and is therefore unlikely to interact directly with the pulmonary epithelium. Instead, it is the oxidized species arising from a reaction

between nitrogen dioxide and the lung lining fluid compartment that is responsible for initiating the signaling cascade which brings the inflammatory cells into the lung[59].

iii. Particulates: Ambient particles contain a large number of soluble metals including transition metals that are capable of redox cycling. The idea has therefore developed that oxidative stress underlies much of the toxicity of ambient particles. Organic components carried on the particle surface play an important role in mediating the toxic effect. For example, polycyclic aromatic hydrocarbons (PAHs) can induce oxidative stress indirectly, through biotransformation by cytochrome P450 and dihydrodioldehyrrogenase to generate redox active quinones that act as catalyst for free radical production[60].

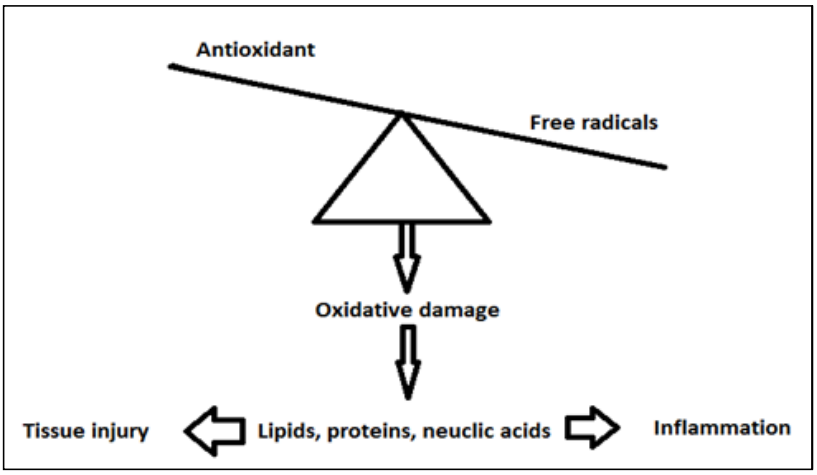


Figure 7: Existence of oxidative stress

MANAGING AND PREVENTION OF OXIDATIVE STRESS[61]:

It is impossible to avoid free radical exposure and oxidative stress. One method of preventing oxidative stress is getting enough antioxidants in diet. Example of fruits and vegetables that should be added in a diet list are: berries, cherries, citrus fruits, prunes, carrots, tomatoes, olives. Other healthy lifestyle choices can also prevent or reduce oxidative stress. They are:

- A regular, moderate exercise routine.
- Avoidance of smoking and exposure to secondhand smoke.
- Avoidance of unnecessary radiation exposure and awareness of other sources of chemical exposure such as pesticides used in food and gardening.
- Use of sunscreen as it prevents ultraviolet light to damage skin.
- Decrease of alcohol intake.

- Avoidance of overeating.

FOOD SOURCES OF FREE RADICAL[62]:

Overeating further increases free radical production. As we eat more, our mitochondria release more activated oxygen than normal during energy consumption, thus generating higher levels of free radicals and risk of oxidative stress is greater when certain type of foods are consumed and the degree of danger can be influenced by the way in which they are prepared or cooked. We should avoid some food which have free radicals. They are:

i. High glycemic foods or foods that is rich in refined carbohydrates and sugars.

ii. Processed meats such as sausages, bacon and salami. They contain preservatives which lead to the production of free radicals.

iii. Red meat. It is particularly more vulnerable to oxidation because of its high iron content.

iv. Cooking fats and oils. Heating fats and oils during cooking oxidizes them that generate free radicals.

v. Alcohol. Alcoholic drinks not only are high in calories but also can produce free radicals in the body.

IMPORTANT ROLE OF FREE RADICALS:

Although free radicals are traditionally regarded as harmful products of aerobic cellular metabolism, this view has recently essentially changed and it is now evident that production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are strongly regulated process that plays central roles in most cell signaling. During physiological and pathophysiology process ROS and RNS can act as secondary messengers and control gene expression, apoptosis, cell growth, cell cycle, cell adhesion, chemo taxis, protein-protein interactions and enzymatic functions, Ca^{2+} and redox homeostasis[63]. Cancer is a multistage disease including initiation, promotion and progression. The increased ROS causes DNA damage or gene mutation resulting in the progression of cancer. Increased generation of ROS and an altered redox status have observed in cancer cells and investigations suggest that this biochemical property of cancer cells can be exploited for cancer therapy. For treatment of cancer since high levels of ROS can induce cell death, treatment of radiation, chemotherapy and molecule compounds all can increase the level of intracellular ROS to induce

cancer cell death and apoptosis. The increased intracellular ROS level could make cancer cells more vulnerable than normal cells to oxidative stress induced cell death. Arsenic trioxide (ATO) is used in the treatment of acute promyelocytic leukemia (APL). It was reported that ATO induces apoptosis signaling in several cancer cells such as lung, leukemia and myeloma cancer through the induction of ROS. The mechanism by which ATO cause increased ROS generation is not completely well known. The most recent investigations indicated that ATO can impair the function of respiratory chain in the mitochondria, leading to increased generation of superoxide, likely by causing leakage of electrons from the mitochondrial respiratory chain complexes. On the other hand, ATO could be used in mixture with several anticancer drugs, which play a role through increasing ROS production. The doxorubicin, daunorubicin and bleomycin are anthracycline antibiotics, cisplatin is a platinum compound and amitriptyline as a tricyclic antidepressant is used in the treatment of several types of cancer. The mechanism of doxorubicin, bleomycin, cisplatin and amitriptyline in the ROS production is the same of ATO. These drugs with impair the function of respiratory chain in the mitochondria, leading to increase generation of superoxide. These compounds due to this mechanism (ROS generation) are used for the treatment of several types of cancers[64].

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CHAPTER 2

LITERATURE SURVEY

LITERATURE SURVEY

GENERAL DESCRIPTION:

A*maranthus spinosus* Linn. is one such plant that has been frequently used in traditional system of medicine. Family of *A.spinosus* Linn. is Amaranthaceae. In Hindi it is commonly known as ‘Kate wali chaulai’ or ‘kanatabhajii’ and in Bengali it is known as ‘Kantanote’. This plant is mostly used as vegetable and cultivated throughout India, Sri Lanka and many tropical countries. *Amaranthus spinosus* derived from the Greek word "Amarantos" which means "unfading" that is a reference to the persisting color of certain Amaranth flowers.[1]

MORPHOLOGY:

Amaranthus spinosus has different morphological characteristic. The characteristics were affected by plant adaptation and genetic variation among them. *Amaranthus spinosus* can be adapted in the different eco geographic and wide range of edaphic factor (environmental heterogeneity). Amaranth genus has capability to interbreed among species in the same genus. The interbreeding also causes different morphological characteristic of *A.spinosus*[2].

This is an erect, many-branched annual herb. The stem is smooth, robust, cylindrical and often reddish. The leaves are simple and alternate, glabrous or with sparse hairs on the main veins below, often diamond shaped, long petiolate, up to 9cm. They are dotted with numerous translucid spots and the venation is well marked. The leaf axis bears pairs of fine and slender spines. The small green flowers are grouped in clusters in the axis of the leaves and in branched terminal spikes. The fruit is a dehiscent capsule that splits open at maturity; it contains a single smooth, black, lens shaped seed. It can grow upto1.5 meter[3].

Figure 1: *Amaranthus spinosus* Linn.

SCIENTIFIC CLASSIFICATION / TAXONOMY[4]:

- Kingdom: Plantae
- Subkingdom: Viridaeplantae
- Phylum: Magnoliophyta
- Subphylum: Euphyllophytina
- Class: Magnoliopsida
- Subclass: Caryophyllidae
- Order: Caryophyllales
- Suborder: Chenopodiineae
- Family: Amaranthaceae
- Genus: Amaranthus
- Species: spinosus L.
- Botanical name: *Amaranthus spinosus* Linn.

VERNACULAR NAMES[5]:

- English: Spiny amaranth, Pig weed, Thorny amaranth, Prickly Amaranth, Needle burr.
- Sanskrit: Meghanada, Alpamarisha, Tandula, Tanduliuyah.
- Kannada: Mulla-dantu, Mullaharavesoppu.
- Hindi: Kantamiris, Kantabhaji, Kataili-chaulai, Kantanatia.
- Gujarati: Kantalodhimdo, Kantanudant, Tandaljo.

- Telugu: Mundlakura, Nalladoggali, Mullatotakura.
- Tamil: Mud-kerrai, Mullukeerai.
- Malayalam: Kattumullenkeera, Mullan-cheera.

GEOGRAPHICAL SOURCE:

Amaranthus spinosus is widely distributed throughout the tropics and warm temperate regions of Asia from Japan to Indonesia to India, Bangladesh, the pacific islands and Australia as a weed in cultivated as well as fallow lands. This plant is widely distributed in roadsides, waste places and fields in Southeastern North America, also found in Cambodia, Philippines and Maldives as a valued food Plant[1]. This weed has a wide distribution including the United States of America and all tropical and sub tropical regions of Africa. *Amaranthus spinosus* grows annually as an erect, monoecious herb, up to 100-300cm tall, much branched[6].

PHYTO CONSTITUENTS:

A study by **Damini** *et al.*, suggested that *Amaranthus spinosus* is a very good dietary source for the presence of pro-vitamin A and carotenoid (β-carotene). The highest content of carotenoid is in the leaves, seeds, stem and roots. Canthaxanthin is the major carotenoid, identified in the leaves, which is an antitumor agent. Lutein is found in the plant which can be used for the retardant purpose of age related eye diseases. Chemical analysis of aerial parts of various *Amaranthus spinosus* has showed the presence of various active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, saponins, amino acids, vitamins, minerals, terpenoids, lipids, betaine, and tannins. Amaranthoside is a lignan glycoside and amaricin is a coumaroyl adenosine is present in this plant. Flavonoids and phenolic acids are also identified. Amaranth extracts isolated sequentially by acetone and methanol or water from defatted plant leaves, flowers, stem and seeds which yields rutin, nicotiflorin, isoquercitrin, 4-hydroxybenzoic and p-coumaric acids as major components[1]. The betalains in the stem of *Amaranthus spinosus* were identified as amaranthine and iso-amaranthine along with hydroxycinnamates, quercetin and kaempferol glycoside at a concentration of 305 mg/100 g. The plant has high nutritive values due to the presence of high concentration of antioxidant components, fibers, proteins and essential amino acids, especially lysine[7].

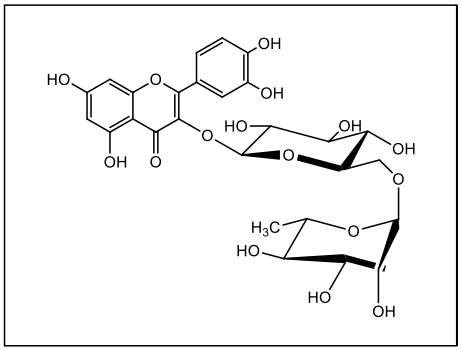

Figure 2: Rutin

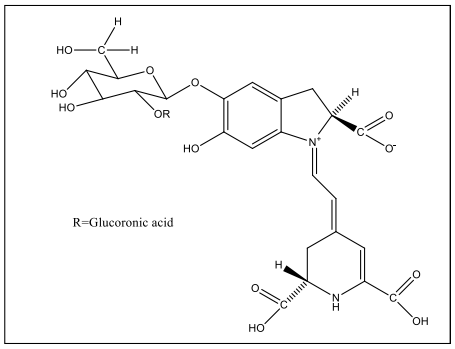

Figure 3: Amaranthin

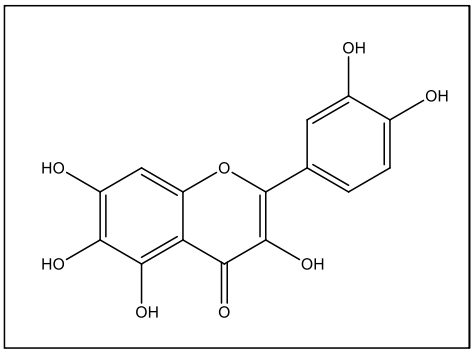

Figure 4: Quercetin

PHARMACOGNOSTIC DESCRIPTION:

Terpene is present in all extracts of *Amaranthus spinosus*. The alcohol extract also showed the presence of alkaloids, glycosides and sugars. The TLC of Petroleum ether (60 – 80°C) extract of the drug was done on silica gel 60 F254 pre-coated sheets by using benzene and ethyl acetate in a ratio of 6:1 which showed six spots in iodine vapor. In the chloroform extract, benzene and ethyl acetate in a ratio of 4:1 showed nine spots. In ethanol extract chloroform and methanol solvent system is used in a ratio of 93:7, which showed only four spots in the same viewing medium[1]. Hence, it is desirable to pursue a study on pharmacognostical investigation of *Amaranthus spinosus* to supplement useful data in regard to its correct identity[8]. Ash values are helpful in determining the quality and purity of crude drug, especially in the powder form. Total ash reflects the care taken in its preparation as all traces of organic matters were removed during ash formation and usually consists of carbonates, phosphates and silicates of sodium, potassium, calcium and magnesium. A higher limit of acid insoluble ash reflects the cases where silica may be present or when the calcium oxalate content of the drug is very high extractive values of selected medicinal plants like *Amaranthus spinosus* were observed. The total percentage of ash values, acid

insoluble ash, water soluble ash, percentage yield of extractives in various solvents are constant features of a part of the plant which may constitute individual drug[9].

TRADITIONAL USES:

Ethno medically the plant is used as a source to treats several disorders. The leaves are used as laxative and applied as an emollient poultice to abscesses, boils and burns. The juice of the root is used to treat fevers, urinary troubles, diarrhea and dysentery. The seed is used as a poultice for broken ribs[10]. The plant is used as a sudorific and febrifuge and is recommended for eruptive fevers. The leaves are considered a good emollient, lactogogue and a specific treatment for colic. Externally, the bruised leaves are applied to treat eczema. In Kenya, leaves of *Amaranthus hybridus* are eaten as spinach or green vegetables. In Nigeria, Amaranthus leaves combined with condiments and used to prepare soup. These leaves are boiled and mixed with a groundnut sauce and eaten as salad in Mozambique or pureed into a sauce and served over vegetables in West Africa. The plant is used in the treatment of intestinal bleeding, diarrhoea and excessive menstruation[11].

LITERATURE REVIEW:

1. Hepatoprotective activity:

The flavonoids and phenolic compounds present in *Amaranthus spinosus* extract may be responsible for hepatoprotective activity. It was suggested by **Zeashan H** *et al*. The hepatic damage was induced by carbon tetrachloride (CCl_4) in five groups of rat. In the 1st normal group hepatic cord cells were present around the central vein. The 2nd groups were treated with CCl_4 that showed liver cell necrosis with inflammatory conditions around central vein. The remaining three groups received *Amaranthus spinosus* extract, showed absence of cell necrosis with minimal inflammatory conditions around the central vein[7].

Rjeibi I *et al.*, used methanolic extract of *Amaranthus spinosus* seed and showed hepatoprotective activity on liver injury induced by deltamethrin (DLM). The hepatic lipid peroxidation level increased in DLM receiving groups. *Amaranthus spinosus* seed extract reduced the effect of DLM[12].

2. Antibacterial activity:

Ten bacterial strains of Gram-positive and Gram-negative were used by **Harsha VS** *et al*., to evaluate the antibacterial activity of aqueous and ethanol root extract of *Amaranthus spinosus*. The ethanol extract showed complete inhibition of microbial growth and the aqueous extract showed inactivation against two bacteria[13].

3. Anthelmintic activity:

To evaluate anthelmintic activity **Baral M** *et al*., used piperazine citrate as reference standard. *Amaranthus spinosus* aqueous extract showed shortest time of paralysis and death on *Pheritima posthuma* and *Tubifex tubifex* earthworms. The higher concentration extract produced much earlier effect[14].

4. Anti inflammatory activity:

Baral M *et al.,* evaluated the anti-inflammatory activity of petroleum ether and ethanolic extract of *Amaranthus spinosus* whole plant at a dose of 250, 500 and 750 mg/kg body weight which was comparable with that of ibuprofen. He induced paw edema by carrageenan which was inhibited by the both extract. The first phase is attributed to the release of histamine, 5-hydroxytryptamine and kinins and the second phase is related to the release of prostaglandins[14].

The methanol extract of *Amaranthus spinosus* leaves for anti-inflammatory activities in different animal models was reported by **Olajide OA** *et al.* Paw edema was induced by carrageenan and writhing was induced by acetic acid in mice. 25, 50, 100 mg/kg extract inhibited the edema by 25%, 37.5% and 53.3%, respectively and there was a reduction in writhing. The highest dose of the extract that was 100 mg/kg exhibited an inhibition of writhing comparable to 5mg/kg indomethacin[15].

5. Antimalarial activity:

Hilou A *et al.,* used two plants, *Amaranthus spinosus* and *Boerhaavia erecta* and investigated their antimalarial activity. To induce malaria the mice were inoculated with red blood cells. The blood cells were parasitized with *Plasmodium berghei*. The plant extract showed significant reduction of malaria on 4^{th} day in the mice. ED_{50} values were also calculated[16].

6. Anti gastric ulcer activity:

Root, stem and leaves of *Amaranthus spinosus* were used by **Mitra PK.,** and he reported the anti gastric ulcer activity of those parts. Ethanol, hydrochloric acid, indomethacin, stress was used to induce gastric ulcer in mice. Pretreatment with A*maranthus spinosus* gave significant protection to the animals from gastric ulcers. Protections were 52.52%, 42.09% and 38.04% respectively. Root of the plant showed highest activity and that was comparable with omeprazole[17].

The anti gastric ulcer activity of A*maranthus spinosus* in rats were reported by **Mitra P***et al*. The aspirin induced gastric ulcer was produced by the method of Parmar and Desai with slight modification. Results showed that the plant leaves decreased the gastric volume and acidity. The results were comparable to that of ranitidine, a standard anti ulcer drug[18].

Ghosh D *et al.,* isolated a compound AS-1 from the leaves of *Amaranthus spinosus* and studied the anti-gastric ulcer effect of this compound. Indomethacin was used to produce gastric ulcer in rats. Pretreatment of rats with AS-1 produced dose dependent reduction of ulcer index in indomethacin treated rats when compared to control[19].

7. Antioxidant activity:

The antioxidant activity of methanolic extract of *Amaranthus spinosus* leaves was investigated by **Bagepalli SAK***et al.* He measured DPPH radical scavenging activity, nitric oxide scavenging activity, superoxide anion scavenging assay, ABTS scavenging assay, hydroxyl radical scavenging activity, nitric oxide radical scavenging activity, ABTS radical scavenging activity to evaluate the antioxidant properties of the plant. Methanolic extract of *Amaranthus spinosus* showed potent antioxidant activity by giving significant value of IC_{50}[20].

The total antioxidant and free radical-scavenging activities and total phenolic content of *Amaranthus spinosus* was determined by **Amin I** *et al*. Total antioxidant activity of water-soluble components in raw spinach was in the order of BI(*Amaranthus blitum*)>BM(*Amaranthus gangeticus*)> BPG *Amaranthus viridis*)> BP (*Amaranthus paniculatus).* The free radical-scavenging activity was in the order of BI> BPG > BM > BP. The total phenolic contents of BM and BP were significantly higher than other samples[21].

The antioxidant activity of *Amaranthus spinosus* was evaluated by **Kumar BSA** *et al.,* by non-enzymatic haemoglycosylation method. Colorimetric method at 520 nm was used to know the degree of haemoglycosylation in the presence of different extracts of *Amaranthus spinosus.* Methanol extract showed more antioxidant activity than petroleum ether, chloroform, and aqueous extract. Phytochemical analysis showed the presence of flavonoids in the plant having antioxidant properties and α-tocopherol (vitamin E) was used as a standard antioxidant compound[22].

Kumar ASB *et al.,* evaluated the in vitro α-amylase enzyme inhibition by CNPG3 (2-chloro-4-nitrophenol a-D-maltotrioside) and in vivo antioxidant potential of malondialdehyde (MDA), glutathione (GSH), catalase (CAT) and total thiols(TT) in alloxan-induced diabetic rats of a methanolic extract of *Amaranthus spinosus*. Result showed that methanol extract of *Amaranthus spinosus* at a concentration of 10, 50, 100 g/ml showed 38.02 ±0.1%, 55.9 ±0.11% and 63.14± 0.1% of α-amylase enzyme inhibition. The IC_{50} value was 46.02 lg/ml. Here acarbose used as a reference standard[23].

8. Antipyretic activity:

Yeast was induced to increase the body temperature in rats and to investigate the antipyretic activity of *Amaranthus spinosus* by **Bagepalli SAK***et al*. The body temperature reduced while the rats received *Amaranthus spinosus* methanolic extract. Thus the anti pyretic properties were present in the plant which was comparable to the standard antipyretic drug paracetamol. Phytochemical study showed the presence of alkaloids, steroids, glycosides, flavonoids, phenolic compounds, terpenoids, proteins and carbohydrates which may be responsible for the antipyretic activity[20].

9. Antihyperglycemic activity:

The methanolic extract of the stem of *Amaranthus spinosus* for its anti-hyperglycemic and anti-hyperlipidaemic effects was investigated by **Balakrishnan S** *et al.,* in male wister albino rats. Alloxan monohydrate was administered to induce diabetes and the plant extract was administered daily at single dose of 250 and 500 mg/kg, to diabetes-induced rats for 15 days. Standard anti diabetic agent, glibenclamide was used to compare the anti hyper-glycaemic activities. The methanolic plant extract produced significant decrease in blood glucose levels from 327.17±8.42 to 175.2±2.2 and from 325.00±9.10 to 170.6±1.2 mg/dl, respectively after 15 days[24].

Md. AS *et al.*, did oral glucose tolerance tests in glucose-loaded mice to investigate the anti hyper glycaemic effect. The methanol extract of the leaf of *Amaranthus spinosus* resulted in significant dose-dependent anti hyperglycemic activity, when administered to mice at doses of 50 mg and 500 mg extract per kg body weight. The glycemic status of the treated mice varied considerably from the untreated ones[25].

Theantidiabetic potential of *Amaranthus spinosus* ethanolic extract was investigated by **Bavarva JH** *et al*. When the extract was administered to type-1 and type-2 diabetic rats, it significantly decreased plasma glucose levels, hepatic glucose-6-phophatase activity and increased the hepatic glycogen content with a concurrent increase in hexokinase activity. As a standard drug, glibenclamide and metformin were used for comparison[26].

10. Antihyperlipidaemic activity:

Balakrishnan S *et al.,* used*Amaranthus spinosus* stem to investigate anti-hyperglycemic and antihyperlipidaemic effects in male wister albino rats. Diabetes was induced in the albino rats by administration of a single dose of alloxan monohydrate. The methanol extract of *A. spinosus* was administered daily at single doses of 250 and 500 mg/kg, to diabetes-induced rats for a period of 15 days. In alloxan induced diabetic control rats there was a significant increase in total cholesterol, TG (Tri-glyceride), LDL (Low-density lipoprotein) and VLDL (Very lo-density lipoprotein) cholesterol. In addition, there was a significant decrease in HDL (High-density lipoprotein) cholesterol in diabetic control rats compared with normal control. The extract-treated rats had significant decrease in their total cholesterol, TG, LDL and VLDL cholesterol, and significantly increased HDL cholesterol[24].

11. Anti diarrhoeal activity:

The extract of *Amaranthus spinosus* was orally administered to animals in different groups by **Olajide AO** *et al.,* which exerted anti-diarrhoeal activity. Animals in the reference group received atropine, whereas animals in the control group received tween 80. One hour later, castor oil was administered orally to induce diarrhoea. He observed that the number of feces produced by rats was significantly reduced in the group of rats, when compared with rats that received tween 80 prior to castor oil administration[15].

12. Antimicrobial activity:

The antimicrobial activity of the extracts of *Amaranthus spinosus* was reported by **Pannu J***et al.*, and compared the activity using in-vitro and in-vivo systems. Five microbial strains were used including *Escherichia coli*, *Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus mutans* and *Bacillus subtilis*. The highest callus induction from leaf explants of *A.spinosus* was obtained in MS medium. Also the best direct adventitious shoot regeneration from nodal explants was obtained in MS medium. The rooting was obtained from elongated shoots in half MS medium. There was no significant difference was observed in antimicrobial activity of the plants regenerated through in-vitro and in vivo grown plants[6].

13. Cytotoxic and apoptosis induced activity:

Prajitha V *et al.,* examined the apoptosis inducing effects of *Amaranthus spinosus* aqueous extract in *Allium cepa* root meristematic cells and human erythrocytes. In case of RBC assay the treated RBCs show membrane blebbing and shrinkage features typical for apoptosis. *Amaranthus spinosus* aqueous extract induced membrane damage and subsequent cell death by using evans blue staining method in treated *Allium cepa* root meristematic cells[27].

14. Effects at haematological parameters:

The effect of whole plant aqueous extract against albino rats at hematological levels was reported by **Balkrishnan S** *et al.* The aqueous extract was administered orally at 125, 250, 500 and 1000 mg/Kg body weight respectively for 60 days that did not show any mortality in all the treatment groups throughout the study period. The significant reduction in RBC, hemoglobin, PCV and MCHC and significant increase in WBC and MCV was observed at the dose level 1000 mg/Kg body weight. After 90 days of treatment withdrawal, the hematological parameters regained to control levels[24].

Ezekwe CI *et al.***,** investigated the effect of methanol extract of *Amaranthus spinosus* leaves at hematological parameters and on kidney. The hematological test carried out, showed that there was a decrease in PCV and an increase in RBC level in the blood and it has the ability to reverse anemia and other blood related diseases in the rat. The extract does not show any significant difference in the levels of creatinine and urea

when compared to the control. Thus the kidney can be able to perform its filtration function without being impaired by the level of creatinine and urea in the blood[28].

Bhande SS *et al*., has been carried out a study to verify the toxic effect of aqueous extract of *Amaranthus spinosus* whole plant against albino rats at hematological levels. The aqueous extract was administered orally at 125, 250, 500 and 1000 mg/kg body weight respectively for 60 days that did not show any mortality in all the treatment groups. The significant reduction in RBC, hemoglobin, PCV, and MCHC and significant increase in WBC and MCV was observed at the dose level 100mg/kg body weight[29].

15. Analgesic activity:

The analgesic activity of *Amaranthus spinosus* leaves was reported by **Kumar MKS** *et al*. Analgesic activity was assessed by glacial acetic acid-induced writhing test. The writhing response was induced by intraperitoneal administration of acetic acid solution and a dose dependent reduction was produced after administration of methanolic extract of the plant. The acute oral toxicity of methanol extract of *Amaranthus spinosus* leaves did not show any abnormality in the skin, fur, eyes, mucous membrane, respiratory, circulatory, autonomic, central nervous system and behavioral pattern[30].

Petroleum ether, ethyl acetate and methanol extracts of the whole plant of *Amaranthus spinosus* was used to investigate the analgesic activity by **Md. TAJ***et al*. Two models of mice were used. In one group acetic acid was administered to induce writhing. Other group was radiant heat tail-flick model. The result showed writhing inhibition as well as the elongation of tail-flick time at a dose of 500 mg/kg body weight. A linear dose response relationship was also observed[31].

16. Diuretic activity:

The diuretic activity of *Amaranthus spinosus* aqueous extract was evaluated by **Amuthan A***et al.,* in wistar rats. Different concentrations of the extract were orally administered to rats and their urine output was collected after 24 h. The volume, pH and the Na^+, K^+ and Cl^- concentrations of urine were estimated. An increase in Na^+, K^+, Cl^- excretion occurred that caused alkalinization of urine and showed strong saluretic activity and carbonic anhydrase inhibition activity[32].

17. Bronchodilator activity:

Chaudhary MA *et al.,* reported that the administration of *Amaranthus spinosus* increased fecal output at doses of 100 and 300 mg/kg showing laxative activity. It also inhibited carbachol-induced bronchospasm in anesthetized rats at 1, 3, 10 and 30 mg/kg. When tested on isolated gut preparations, the plant extract showed a concentration-dependent spasmogenic effect in spontaneously contract in rabbit jejunum and guinea-pig ileum. The spasmolytic effect was mediated through calcium channel blocking (CCB), while bronchodilator activity through a combination of β-adrenergic and CCB pathways[33].

18. Genotoxic activity:

Prajitha V *et al.,* investigated the possible genotoxic effects of *Amaranthus spinosus* leaf extracts on *Allium cepa* root meristematic cells and its antigenotoxic effects against hydrogen peroxide-induced genetic damage in *Allium cepa*. The excessive reduction in mitotic index (MI) and extremely significant levels of clastogenicity was observed after genotoxicity assay. In antigenotoxicity studies, initial toxicity was induced by two concentrations of hydrogen peroxide. The results demonstrated that the aqueous plant extract of *Amaranthus spinosus* have the ability to inhibit the oxidative damage induced by the direct-acting mutagen at a precise concentration[34].

19. Nephroprotective activity:

The ethanol extract of *Amaranthus spinosus* root by histological assessment was investigated by **Kengar S** *et al.* CCl_4 was administered to induce nephrotoxicity. The intoxicated rats were treated with ethanolic extract at a dose of150, 300 and 450 mg/kg body weight. That protects the kidney by improving disrupted metabolisms especially at 450 mg/kg body weight[35].

20. Antitumour activity:

Arijit M *et al*., isolated fatty acid to examine the anti-proliferative and pro-apoptotic effects of *Amaranthus spinosus* against HepG2 human liver cancer cells. They used 3-(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell viability, flow cytometry assay for cell cycle analysis, and Western analysis to measure protein expression of (dc2), cyclin B1, Bcl-2-associated X protein (Bax), and B-cell lymphoma 2 (Bcl-2). The MTT assay resulted in inhibition of the

proliferation of HepG2 cells in a dose dependent manner, with a half maximal inhibitory concentration (IC50) value of 25.52 μmol/L. The fatty acid also induced apoptosis mediated by down regulation of cyclin B1, up regulation of Bax, and down regulation of bcl-2, resulting in the G2/M transition arrest[36].

The study of anti-tumour potentials of *Amaranthus spinosus* against EAC bearing swiss albino mice was carried out by **Joshua LS** *et al*. The ethanol extract of the leaves given orally to mice at the dose of 100 and 200 mg/kg body weight for 16 days resulted in decrease of tumour volume and viable cell count with increase in mean survival time. Restoration of hematological and biochemical parameters towards normal was also observed[37].

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CHAPTER 3

AIM AND OBJECTIVES

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From literature survey, it was observed that *Amaranthus spinosus* pocesses many activities. Among them, **Bagepalli SAK** *et al.,* **Amin I** *et al.,* **Kumar BSA** *et al.,* evaluated that *A.spinosus* has significant antioxidant activity.

Beside antioxidant activity *A.spinosus* also has other properties like, hepatoprotective activity, anti-bacterial activity, anti-malarial activity, anti-cancer activity, anti-diarrheal activity, anti-hyperglycemic activity, analgesic activity, diuretic activity etc. Thus, this plant could be used as a traditional medicine to treat various diseases and in health care needs.

Herbal therapy is a holistic therapy in which life style, emotional, mental and spiritual considerations are involved. The use of herbs generally does not have adverse effects. Though herbal medicines usually tend to have several broad complementary or synergistic actions on physiological systems at the same time which are usually in the same general therapeutic direction and often non-specific. Furthermore, these actions are rarely adverse effects.

People every year turn to herbal medicine because they believe plant remedies are free from undesirable side effects. In the United States of America approximately 100,000 people each year die due to the toxicities showed by synthetic drugs. Synthetic drugs not only cure disease but also cause severe side effects to human body. A lot of examples reported in literature which is related with the side effects cause by the synthetic drugs e.g. Paracetamol is well known antipyretic drug but it can also cause liver damage as major side effect, naproxen causes gastrointestinal side effect and ibuprofen is another antipyretic drug which causes nephrotoxicity including renal failure.

Based on the evidence, in the present study we were focused on the below points:

- Collection of *Amaranthus spinosus* plant, proper authentication of the plant, drying, powdering and extraction of the stem by various solvents.
- Phytochemical screening of secondary metabolites.
- Evaluation of antioxidant activity of the stem extracts of *A.spinosus.*

CHAPTER 4

MATERIALS AND METHODS

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CHEMICALS:

- Standard- Vitamin C
- Riboflavin
- Ethylene diamine tetra acetic acid (EDTA)
- Nitro blue tetrazoleum (NBT)
- Sodium nitrite ($NaNO_{3)}$
- Sodium carbonate (Na_2CO_3)
- Sodium nitroprusside ($Na_2[Fe(CN)_5]NO$)
- Potassium ferricyanide [$k_4Fe(CN)_6$]
- Trichloroacetic acid (TCA)
- Hydrogen peroxide (H_2O_2)
- Ferric chloride ($Fecl_3$)
- Petroleum ether
- Chloroform
- Ethyl acetate
- Methanol

Petroleum ether, chloroform, ethyl acetate and methanol of analytical grade were purchased from Merck India Ltd.

PLANT MATERIAL:

The plant *Amaranthus spinosus* Linn. (Family- Amaranthaceae) was collected from the rural areas of Nadia district (West Bengal) and was identified by Botanical Survey of India, Howrah. The specimen No. is CNH/18/2011/Tech. II/419. The fresh stems were separated from the whole plant and subjected to air drying at room temperature. After drying stems were powdered and packed in a plastic container.

PREPARATION OF THE PLANT EXTRACTS:

Stored and dried stems were converted to powder material by grinder. After sufficient grinding, the weight of the powdered material obtained was 360g. The powdered material was packed in a paper thimble (made by filter paper) and was inserted into the body of a soxhlet apparatus. After that the powder was extracted successively by solvent of lower to higher polarity, heating with each solvent for 12 hours and

siphoned over 6 times. The ascending order of the polarity of the solvent is: Petroleum ether>Chloroform>Ethyl acetate>Methanol. The solvents were evaporated by a rotary evaporator (EYELA, CCA-1110) to prepare concentrated extract and recollected. The concentrated extracts were air dried for 5-7 days and then weighed and their percentage yields were calculated. Weights of the extracts are:

Petroleum ether: 1.120g

Chloroform: 2.410g

Ethyl acetate: 2.150g

Methanol: 0.440g

PHYTOCHEMICAL ANALYSIS OF THE PLANT EXTRACTS:

The qualitative chemical tests are performed to establish the profile of the extracts of *Amaranthus spinosus* Linn. and to know the nature of the chemical composition.

The various chemical tests are:

Test for alkaloid:

i. **Mayer's test-** Test solutions were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow colored precipitate showed the presence of alkaloid[3].

ii. **Wagner's test-** Test solutions were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown or reddish precipitate showed the presence of alkaloid[3].

iii. **Hager's test-** Test solutions were treated with Hager's reagent (saturated picric acid solution). Formation of yellow precipitate showed the presence of alkaloid[3].

iv. **Dragendrof's test-** Test solutions were treated with dragendrof's reagent (solution of potassium bismuth iodide). Formation of red precipitate showed the presence of alkaloids[3].

Test for carbohydrate:

i. **Molisch's test-** Filtrates were treated with few drops of alcoholic α-napthol and then few drops of concentrated sulphuric acid added through side of test tubes. Purple to violet color ring appeared at the junction, that showed the presence of carbohydrate[3].

ii. **Fehling's test-** Test solutions were hydrolyzed by dilute hydrochloric acid, neutralized with alkali and heated with Fehling's A and Fehling's B solution. Formation of red precipitate showed the presence of carbohydrate[3].

Test for glycoside:

i. **Borntrager's test-** The test materials were boiled with 1ml of sulphuric acid in a test tube for 5 minute. The hot solutions were filtered and the filtrate were cooled and shaken with equal volume of chloroform. The lower layer of chloroform were separated and shaken it with half of its volume of dilute ammonia. Rose pink to red color is produced in the ammoniacal layer. That showed the presence of glycoside[3].

ii. **Keller-killiani test-** The extracts were treated with 1ml of glacial acetic acid, few drops of ferric chloride and 0.5ml of concentrated sulphuric acid. Formation of reddish brown color at the junction of two layers and bluish green color in upper layer showed the presence of glycoside[3].

iii. **Legal's test-** The test solutions were treated with pyridine and alkaline sodium nitroprusside. Formation of orange color showed the presence of glycoside[3].

Test for tannin:

i. **Ferric chloride test-** To the extract, same volume of ferric chloride solution was added. Formation of blue-black color indicates tannin[3].

ii. **Lead acetate test-** To the extracts, lead acetate solution was added. Formation of white precipitate indicates presence of tannin[3].

iii. **Chlorogenic acid test-** The test solutions were treated with aqueous ammonia solution and expose to air gradually. Green color is developed, which showed the presence of tannin[4].

Test for flavonoid:

i. **Shinoda test-** To the test solutions, few magnesium turnings and drop wise concentrated hydrochloric acid were added. Pink or crimson red color or occasionally green to blue color is appeared after few minutes, that showed the presence of flavonoid[3].

ii. **Alkaline reagent test-** To the test solutions, few drops of sodium hydroxide solution was added. Intense yellow color is formed which showed the presence of flavonoid[4].

Test for saponin:

i. **Foam test-** Test solutions were shaken vigorously with 20ml of water and observed for persistent foam. It showed presence of saponin[3].

Test for steroids:

i. **Salkowaski test-** To the test solutions, few drops of concentrated sulphuric acid was added. Red color at lower layer showed the presence of steroids[3].

ii. **Libermann-burchard test**- The extracts were treated with few drops of acetic anhydride. Then concentrated sulphuric acid were added from the side of the test tube. Brown ring is formed at the junction of two layers. Upper layer turned green which showed the presence of steroid[3].

Test for protein:

i. **Hydrolysis test-** The test solutions were hydrolyzed with hydrochloric acid or sulphuric acid. In this solution ninhydrine solution were added and boiled. Formation of violet color showed the presence of protein[3].

ii. **Xanthoprotein test-** To the test solutions, 1ml of concentrated nitric acid were added and boiled. Yellow precipitate is formed. After cooling it, sodium hydroxide solution was added. Orange color is formed, which showed the presence of protein[3].

DETERMINATION OF ANTIOXIDANT ACTIVITY:

Plants having antioxidant capacity helps to prevent many lifestyle diseases by removing free radicals. In the present study in-vitro models were used to determine the antioxidant activity of the stem of *Amaranthus spinosus* Linn. Percentage inhibition and IC_{50} value was calculated and compared with standard ascorbic acid.

1. HYDROGEN PEROXIDE SCAVENGING ACTIVITY:

The ability of *A.spinosus* stem extracts to scavenge hydrogen peroxide was estimated according to the method reported by **Ruch** *et al*[5]., with slight modification.

A solution of hydrogen peroxide (40 mM) and phosphate buffer (0.1 M, pH 7.4) were prepared. Different concentration of sample (10-200 mg/ml) was added to hydrogen

peroxide solution (0.5 ml) and 2.5 ml of phosphate buffer solution. Absorbance of the mixture was determined at 230 nm after 10 minute against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. The free radical scavenging activity was determined by evaluating % inhibition.

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

Where,

$A_{control=}$ Absorbance of control.

$A_{sample=}$ Absorbance of sample.

2. DPPH RADICAL SCAVENGING ASSAY:

DPPH radical scavenging assay is a widely used method to evaluate the free radical scavenging ability of natural compounds. This assay is based on the measurement of the scavenging ability of antioxidant substances towards the stable radical. The free radical scavenging activity of the extracts were examined by in-vitro model using DPPH radical as described by **Shimada** *et al*[6]., with slight modification.

1ml of various concentration of extract (10-200 mg/ml) was mixed with 1ml of 0.8 mM DPPH solution. The mixture was shaken vigorously and left to stand for 30 min and the absorbance was measured at 517 nm against a blank reagent. Ascorbic acid was used as standard.

The percentage inhibition for scavenging DPPH radical was calculated according to the equation stated above.

3. SUPEROXIDE ANION RADICAL SCAVENGING ASSAY:

The superoxide anion radical scavenging activity of *A.spinosus* stem extracts were assessed using the method described by **Fontana** *et al*[7]., with slight modification.

To the various concentrations of the extracts (10-200 mg/ml), 1 ml of phosphate buffer (0.1 M, pH 7.2), 1 ml riboflavin (20 µg), 1 ml EDTA (12 mM) and NBT (0.1 mg/3ml) were added. After 5 minute of incubation at room temperature, the absorbance was measured at 590 nm against a blank reagent. Ascorbic acid was used as the standard.

The % inhibition was calculated as stated above.

4. NITRIC OXIDE RADICAL SCAVENGING ASSAY:

Nitric oxide generated from sodium nitroprusside interacts with oxygen to produce nitrite ions which was measured by the griess reaction. This assay was done by the procedure described by **Green** *et al*[8]., with minor modification.

The reaction mixture contained sodium nitroprusside (1 ml, 10 mM) in 2.5 ml phosphate buffer saline (pH 7.4) and various concentrations of (10-200 mg/ml) extracts were added to this mixture. The resulting solution was then incubated at room temperature for 3 hours. To the incubated solution 0.5 ml of griess reagent (0.5% sulphanilamide in 2.5% phosphoric acid and 0.05% N-napthyl ethylene diamine) was added and the absorbance of the chromophore formed was measured at 546 nm against a blank reagent. The standard ascorbic acid was used for comparison.

The free radical scavenging activity was determined by evaluating % inhibition as above.

5. REDUCING POWER ASSAY:

The reducing power ability of the extract was evaluated by the method described by **Oyaizu** *et al*[9].

The reaction mixture contained 1 ml of various concentration of extract (10-200 mg/ml), 2.5 ml of 1% potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 30 minute and the reaction was terminated by the addition of 2.5 ml of 10% trichloro acetic acid and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm against blank reagent. The reducing power ability of the sample is determined by the increase in absorbance of the sample. Ascorbic acid was used as standard for comparison.

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CHAPTER 5
RESULT AND DISCUSSION

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PERCENTAGE YIELD:

The crude powdered materials (360g) were extracted sequentially from most non-polar solvent i.e. Petroleum ether and the percentage yield collected are tabulated as follows. The yield of crude chloroform extract is the highest among the four samples whereas, yield of crude methanol extract is lowest compared to other extracts.

Formula to calculate the percentage yield:

Percentage yield= (weight of the extract/weight of the plant material taken) ×100

Extract	Percentage yield
Petroleum ether	0.36
Chloroform	0.79
Ethyl acetate	0.70
Methanol	0.15

Table 1: Percentage yield of *A.spinosus* extract

PHYTOCHEMICAL TEST:

During phytochemical test change of color was observed when the test reagent was added to the prepared sample. The result was recorded as present (+) or absent (-) depending on the outcome of the test.

	Petroleum ether	Ethyl acetate	Chloroform	Methanol
Alkaloid	+	+++	++++	++
Glycoside	+	+++	++++	++
Tannin	-	-	-	+
Flavonoid	+	++++	+++	++
Steroid	-	+	++	+++

Table 2: Phytochemical constituents of stem extract of *A.spinosus*

ANTIOXIDANT ACTIVITY OF *Amaranthus spinosus STEM EXTRACT:*

Activity of the antioxidants concerns with those compounds capable of protecting the organism system against the potential harmful effect of oxidative stress. In this study, the antioxidant capacity of extracts from *Amaranrthus spinosus* was accessed by five different assays. They are: Hydrogen peroxide scavenging activity, reducing power

assay, nitric oxide scavenging activity, superoxide radical scavenging activity and DPPH radical scavenging activity.

DPPH radical scavenging activity:

Free radical molecule DPPH accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The potential decrease in the absorbance of DPPH radical is due to the scavenging ability of *Amaranthus spinosus* Linn. Ascorbic acid and different extracts showed significant free radical scavenging activity and that was determined by calculating the percentage inhibition. The IC_{50} value of different extracts of the plant showed in Table 3. Ethyl acetate extract showed highest scavenging activity and petroleum ether showed lowest scavenging activity compared to ascorbic acid.

Extract	IC_{50} value (µg/ml)
Ascorbic acid	73.31
Petroleum ether	75.18
Chloroform	74.40
Ethyl acetate	73.31

Table 3: Antioxidant activity of *A.spinosus* extracts against DPPH method

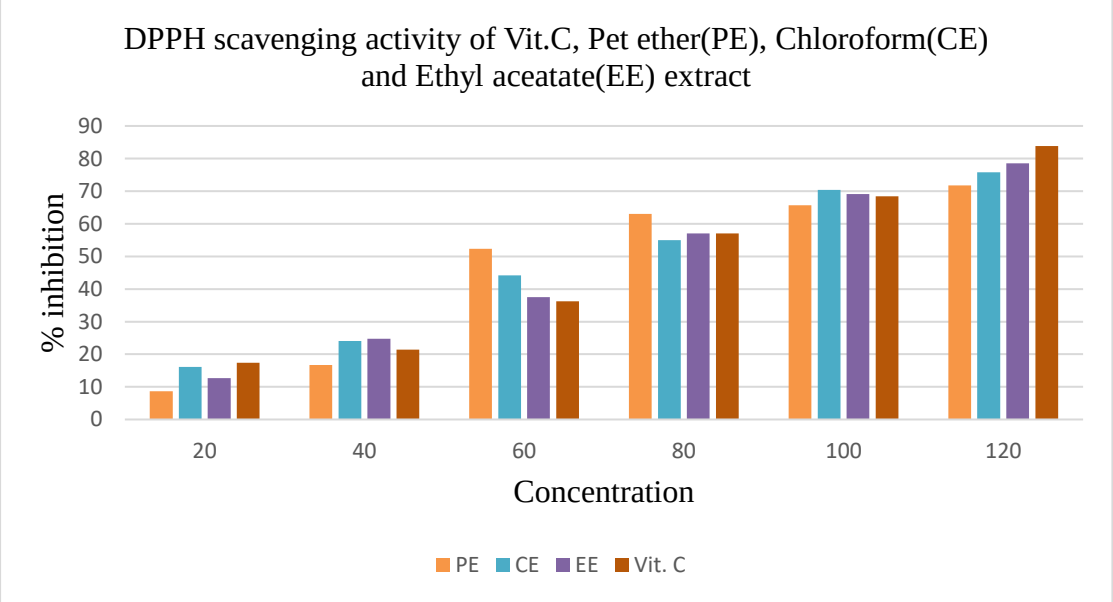

Figure 1: DPPH scavenging activity of *A.Spinosus* stem extract

Superoxide radical scavenging activity:

In this method, the potential decrease in the absorbance of superoxide radical is due to the scavenging activity of *Amaranthus spinosus* Linn. The percentage inhibition of ascorbic acid and different extracts were determined. Table 4 showed the IC_{50} values. Figure 2 showed superoxide radical scavenging activity where X-axis showed concentration of the extracts and Y-axis showed percentage inhibition. Chloroform extract showed highest scavenging activity compared to others.

Extract	IC_{50} Value (µg/ml)
Ascorbic acid	69.06
Petroleum ether	85.32
Chloroform	59.52
Ethyl acetate	83.61

Table 4: Antioxidant activity of *A.Spinosus* against superoxide radical scavenging activity

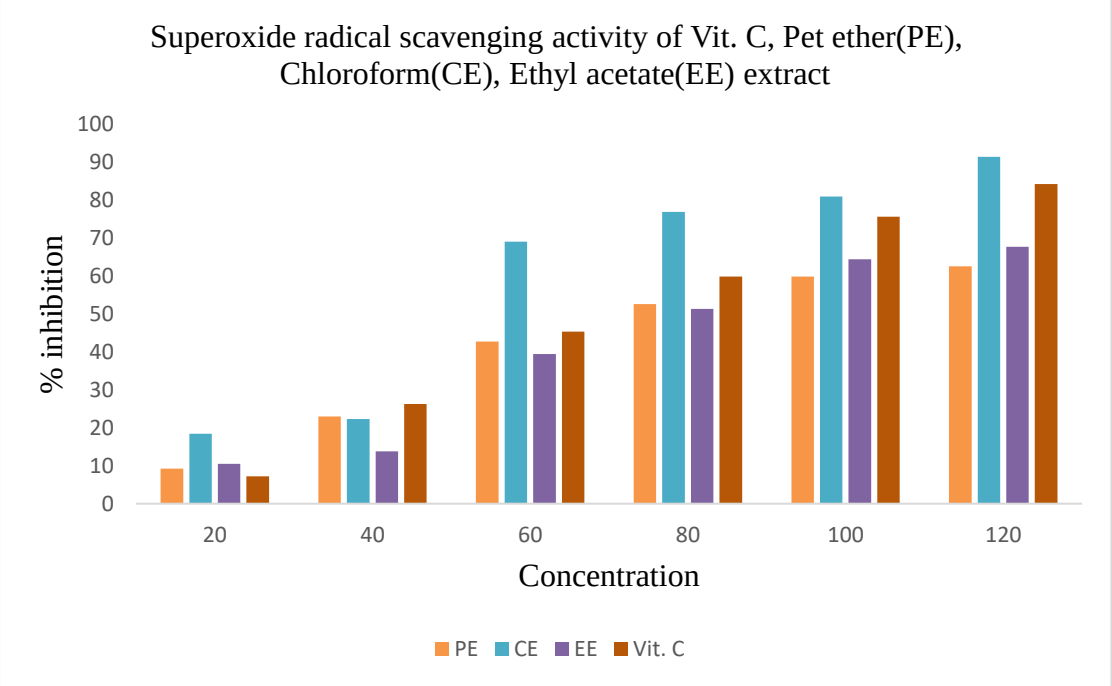

Figure 2: Superoxide radical scavenging activity of *A.Spinosus* stem extract

Nitric oxide scavenging activity:

In this method nitric oxide generated from sodium nitroprusside interacts with oxygen and produce nitrite ions. The scavenging activity is due to a decrease in the

concentration of nitrite ions. The highest scavenging activity was showed by petroleum ether extract and the lowest scavenging activity was showed by chloroform extract.

Extract	IC_{50} value (µg/ml)
Ascorbic acid	76.80
Petroleum ether	86.65
Chloroform	97.27
Ethyl acetate	88.02

Table 5: Antioxidant activity of *A.Spinosus* against nitric oxide scavenging method

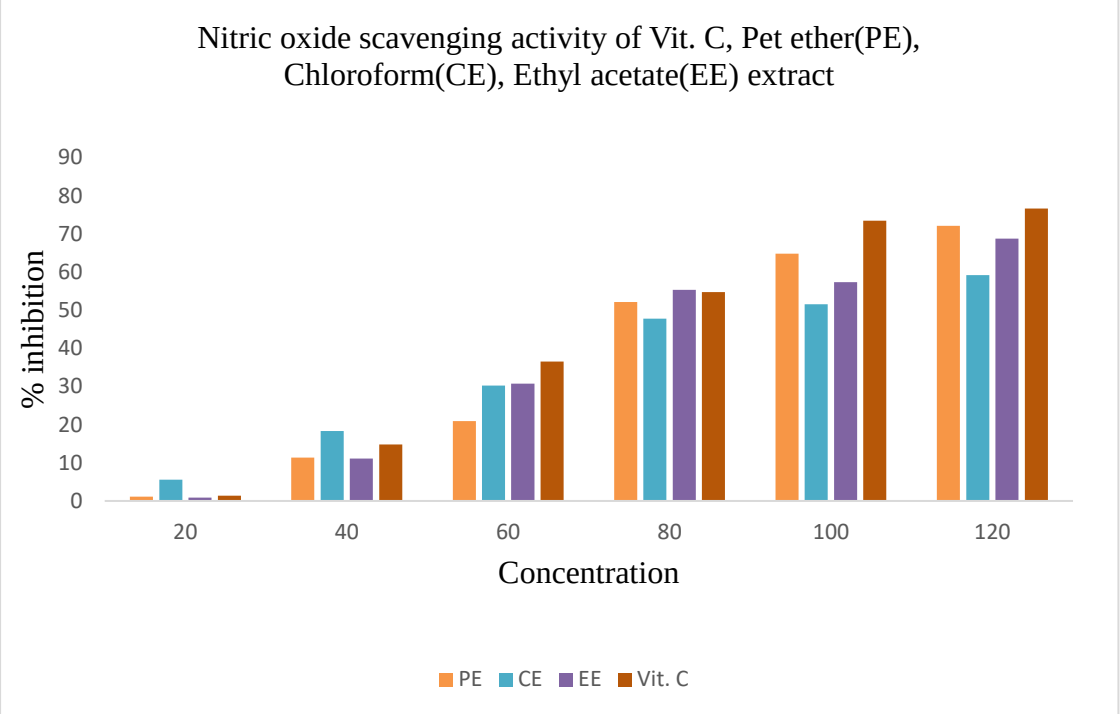

Figure 3: Nitric oxide scavenging activity of *A.Spinosus* stem extract

Hydrogen peroxide scavenging activity:

This method showed a dose-dependent inhibition of hydrogen peroxide. The chloroform extract showed good scavenging activity compared to the standard compound ascorbic acid. The IC_{50} values of the extracts were showed in Table 6 and the hydrogen peroxide scavenging activity was shown in figure 4 where the X-axis

showed the concentration of the extracts and Y-axis showed the percentage inhibitions. Ethyl acetate showed the lowest scavenging activity.

Extract	IC_{50} value (µg/ml)
Ascorbic acid	65.61
Petroleum ether	88.65
Chloroform	80.12
Ethyl acetate	119.04

Table 6: Antioxidant activity of *A.Spinosus* against hydrogen peroxide scavenging method

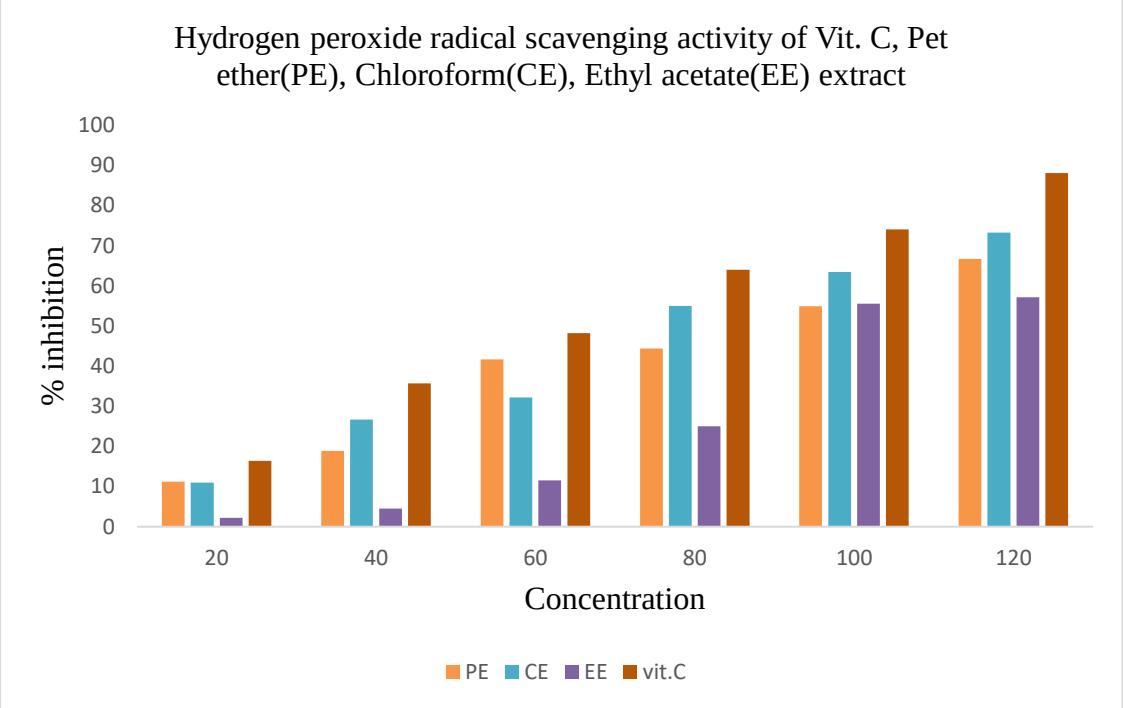

Figure 4: Hydrogen peroxide scavenging activity of *A.Spinosus* stem extract

Reducing power assay:

In this method the reductive ability and transformation of Fe^{3+} to Fe^{2+} was investigated. It is a convenient and rapid screening method. The reductive capabilities of the extracts were showed in figure 6 when compared to standard ascorbic acid. The reducing power increased significantly with increasing in the concentrations of the extracts. Ethyl acetate extract showed good scavenging activity at the concentration of 100 and 120mg/ml.

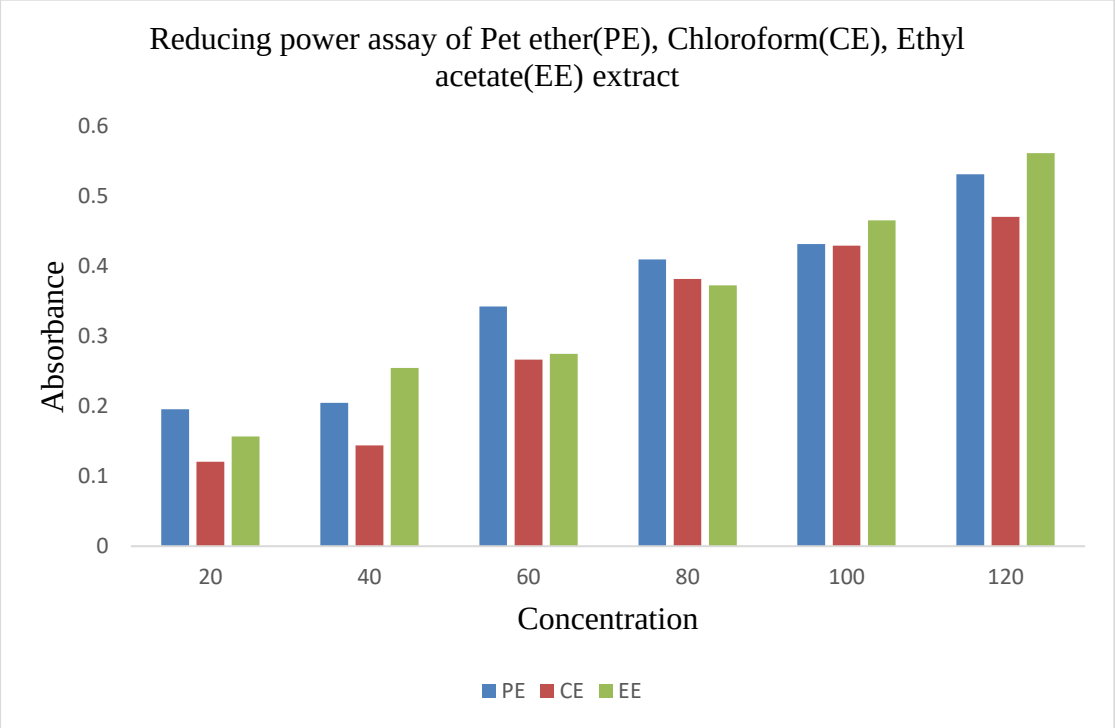

Figure 5: Reducing power assay of *A.spinosus* stem extract

DISCUSSION

From this study it can be demonstrated that the extracts of the stem of *A.spinosus* have significant in-vitro antioxidant and free radical scavenging activity. Antioxidant compounds cure wounds by removing the products that causes inflammation. Thus the extract of *Amaranthus spinosus* may have healing properties.

The plant extracts inhibited the DPPH radical in different manners. Thus the extracts are capable of donating an electron which reacts with DPPH radicals. The extract also showed various scavenging activities which causes an unequal distribution of the antioxidant molecules identified in the stem of the plant extract such as flavonoid.

The nitric oxide scavenging activity of all extracts demonstrated an ability to inhibit nitrite radical. Thus the extracts could serve as free radical inhibitors for their proton donating ability and could act as a primary antioxidant for the management of the diseases in which nitrite radicals are involved.

In the present study, all extracts exhibited superoxide scavenging activity which can be correlated with high content of flavonoids. Superoxide anions can produce other free radicals that cause cell damage. Thus the extracts of *A.spinosus* stem could be use to prevent cell damage.

The extracts could be use to penetrate biological membranes as they showed good hydrogen peroxide scavenging activity.

Reducing power of the extracts exerted antioxidant activity by breaking the free radical chain and donating a hydrogen atom.

The phytochemical screening of the extract showed the presence of alkaloid, flavonoid, glycoside and steroid. Thus some of the phytochemical constituents may be responsible for the antioxidant activity and may have therapeutic importance.

Vitamin C or ascorbic acid was used here because it is a water soluble free radical scavenger.

CHAPTER 6

CONCLUSION

CONCLUSION

For thousands of years, many traditional medicines are isolated from natural sources. In the present study, the evaluation of in-vitro antioxidant activity of the extracts of *Amaranthus spinosus* showed substantial and significant free radical scavenging activity, which may be due to the presence of flavonoids in them. The results would help to ascertain the potency of the crude extract from *Amaranthus spinosus* as potential source of natural antioxidants. It can be used to minimize or to prevent lipid oxidation in pharmaceutical products and to retard the formation of toxic oxidation in products. *Amaranthus spinosus* has been used for the treatment of a variety of diseases and it is an easily available plant for natural remedies. Further studies would be required for isolation and characterization of antioxidant compounds and also in-vivo studies are needed to know their mechanism of action as antioxidants.