



Daffodil *International* **University**

Project On

Antioxidant Activity of *Malva verticillata* (Lafa Shak)

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APPROVAL

This Project, Antioxidant Activity of the Plants of *Malva verticillata* submitted by Mohammad Habibullah Shovan to the Department of Pharmacy, Daffodil International University, has been accepted as satisfactory for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy and approved as to its style and contents.

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DECLARATION

I hereby declare that, this project report is done by me under the supervision of **Dr. Md. Nurul Islam**, Assistant Professor, Department of Pharmacy, Daffodil International University, impartial fulfillment of the requirements for the degree of Bachelor of Pharmacy. I am declaring that this Project is my original work. I also declare that neither this project nor any part thereof has been submitted elsewhere for the award of Bachelor or any degree.

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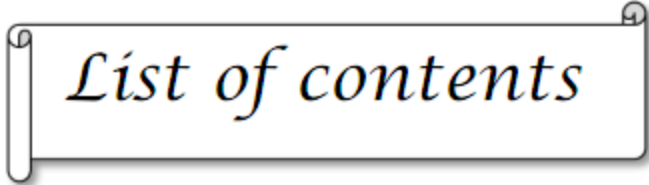
DEDICATION

*Dedicated to my
parents*

(Jebunnesa and Late Halim Uddin)

ABSTRACT

The present study has been designed to examine the antioxidant activity of the methanol extract of the whole plants of *Malva verticillata*. Antioxidant activity was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging and ferric reducing power assays. In addition, % yield, total phenolic, total flavonoid contents and total antioxidant activity were also determined. Total phenolic contents were expressed as gallic acid equivalent (GAE), total flavonoids contents were expressed as quercetin equivalent (QE) and total antioxidant activity was expressed as ascorbic acid equivalent (AAE) of the methanol extracts whole plants of *Malva verticillata*. The methanol extract showed strong DPPH free radical scavenging activity with an IC_{50} value of $202.55 \pm 5.2 \mu\text{g/ml}$ compared to the positive control ascorbic acid with an IC_{50} value of $173.83 \pm 3.98 \mu\text{g/ml}$. In addition, the methanol extract also showed strong ferric reducing activity which is comparable to the potent antioxidant ascorbic acid. Besides, the phenolic content, total flavonoid content, and total antioxidant capacity of the methanol extract were found to be $139.75 \pm 2.33 \text{ mg/g}$ of dried extract (GAE), $139.66 \pm 3.56 \text{ mg/g}$ of dried extract (QE), and $224.28 \pm 4.21 \text{ mg/g}$ of dried extract (AAE). Therefore, it is anticipated that the large amount of phenolic and flavonoids typed compounds contained in the methanol extract played a strong role in antioxidant action of this extract. Therefore, *Malva verticillata* could be used as a source of naturally occurring potent antioxidants. However, it is very important to find out the specific chemical constituents responsible for potent antioxidant activity of *Malva verticillata*.



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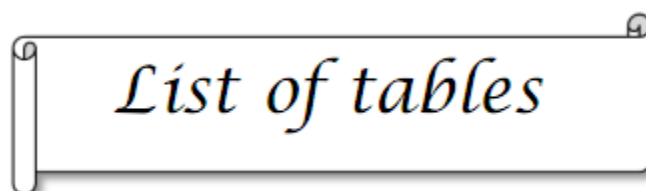
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Abbreviations

Abbreviate	Meaning
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
WHO	World Health Organization
IENIC	Interactive European Network for Industrial Crops
CAM	Complementary and Alternative Medicine
AYUSH	Ayurveda, Yoga & Naturopathy , Unani, Siddha and Homoeopathy
ORAC	Oxygen Radical Absorbance Capacity
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
GPx	Glutathione peroxidase
CAT	chloramphenicol Acetyl Transferase
ADP	Adenosine Di-nucleotide Phosphate
AIDS	<i>Acquired Immune Deficiency Syndrome</i>
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
GSH	Glutathione sulfhydryl group
ALS	Amyotrophic Lateral Sclerosis
UV	Ultra Violet
ARDS	Adult Respiratory Distress Syndrome
LDL	Low Density Lipoproteins
DNA	Deoxyribo Nucleic Acid
MCP	Monocyte Chemoattractant Protein
MPRKWHO	Medicinal Plants in the Republic of Korea World Health Organization
DPPH	1,1-diphenyl-2-picrylhydrazyl

FCR	Folin– Ciocalteu Reagent
GAE	Gallic Acid Equivalents
QE	Quercetin Equivalents

CHAPTER 1

INTRODUCTION

1.1 Phytochemistry

Phytochemistry is in the strict sense of the word the study of phytochemicals. These are chemicals derived from plants. In a narrower sense the terms are often used to describe the large number of secondary metabolic compounds found in plants. Many of these are known to provide protection against insect attacks and plant diseases. They also exhibit a number of protective functions for human consumers.

Phytochemistry can be considered sub-fields of Botany or Chemistry. Activities can be led in botanical gardens or in the wild with the aid of Ethno botany. The applications of the discipline can be for phytochemistry, or the discovery of new drugs, or as an aid for plant physiology studies.

Techniques commonly used in the field of phytochemistry are extraction, isolation and structural elucidation (MS, 1D and 2D NMR) of natural products.

Phytochemistry is widely used in the field of Chinese medicine especially in the field of herbal medicine.

Phytochemical technique mainly applies to the quality control of Chinese medicine, Ayurvedic medicine (Indian traditional medicine) or herbal medicine of various chemical components, such as saponins, alkaloids, volatile oils, flavonoids and anthraquinones.

(<http://en.wikipedia.org/wiki/Phytochemistry>)

1.2 Medicinal Plants

Medicinal plants are plant, plant parts, plant products, plant extracts and/or plant derived products that are employed in the treatment of diseases or used for their therapeutic properties. They are also used in the sense of improving the health status of human beings (NCCAM, 2005). Most of their effects were discovered through the folkloric medicine, in which the populations around the globe have developed their own strategies to remedy their illness (Lima et al., 2005).

The use of herbs as medicines has played an important role in nearly every culture on earth, including Asia, Africa, Europe and America (Wargovich et al., 2001). Several herbs provide some protection against cancer and stimulate the immune system. Furthermore, a diet in which culinary herbs are used generously to flavor food provides a variety of active phytochemicals that promote health and protect against chronic disease (Cheung and Tai, 2007). Additionally, several commonly used herbs have been identified by the National Cancer Institute as possessing cancer preventive properties (Al-Attar, 2006). Most of these plant-derived medicines were originally discovered

through the study of traditional cures and folkloric knowledge and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Gilani and Rahman, 2005).

Duarte et al., (2011) mainly interested in their research on the biosynthesis of the terpenoid indole alkaloids produced by the medicinal plant *Catharanthus roseus*, which include the anticancer drugs vinblastine and vincristine. Previous work involved the biochemical and molecular characterization of a key biosynthetic step leading to the production of the anticancer alkaloids.

1.3 Herbal medicine

Herbalism ("herbology" or "herbal medicine") is use of plants for medicinal purposes, and the study of such use. Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Modern medicine recognizes herbalism as a form of alternative medicine, as the practice of herbalism is not strictly based on evidence gathered using the scientific method. Modern medicine, does, however, make use of many plant-derived compounds as the basis for evidence-tested pharmaceutical drugs, and phytotherapy works to apply modern standards of effectiveness testing to herbs and medicines that are derived from natural sources. (<http://en.wikipedia.org/wiki/Herbalism>)

1.3.1 History of Herbal medicine

A number of ancient cultures wrote on plants and their medical uses. In ancient Egypt, herbs are mentioned in Egyptian medical papyri, depicted in tomb illustrations, or on rare occasions found in medical jars containing trace amounts of herbs (Nunn and John, 2002). The earliest known Greek herbals were those of Diocles of Carystus, written during the 3rd century B.C, and one by Krateuas from the 1st century B.C. Only a few fragments of these works have survived intact, but from what remains scholars have noted that there is a large amount of overlap with the Egyptian herbals (Robson et al., 2009). Seeds likely used for herbalism have been found in the archaeological sites of Bronze Age China dating from the Shang Dynasty (Hong and Francis, 2004). Over a hundred of the 224 drugs mentioned in the Huangdi Neijing, an early Chinese medical text, are herbs (Unschuld and Pual, 2003). Herbs were also common in the medicine of ancient India, where the principal treatment for diseases was diet (Ackerknecht and Erwin, 1982). *De Materia Medica* by Pedanius Dioscorides, a Roman physician, is a particularly important example of such writings (Harvard University Press, 2010). The documentation of herbs and their uses was a central part of both Western and Eastern medical scholarship through to the 1600s, and these works played an important role in the development of the science of botany.

1.3.2 Modern herbal medicine

The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care ("Traditional medicine"). Pharmaceuticals are prohibitively expensive for most of the world's population, half of which lived on less than \$2 U.S. per day in 2002 (Edgar J et al., 2002). In comparison, herbal medicines can be grown from seed or gathered from nature for little or no cost.

Many of the pharmaceuticals currently available to physicians have a long history of use as herbal remedies, including opium, aspirin, digitalis, and quinine. According to the World Health Organization, approximately 25% of modern drugs used in the United States have been derived from plants ("Traditional medicine."). At least 7,000 medical compounds in the modern pharmacopoeia are derived from plants (IENIC, 2000–2005). Among the 120 active compounds currently isolated from the higher plants and widely used in modern medicine today, 80% show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (Fabricant and Farnsworth, 2001).

1.3.3 Clinical test of Herbal medicine

In a 2010 survey of the most common 1000 plant-derived compounds, only 156 had clinical trials published. Preclinical studies (tissue-culture and animal studies) were reported for about one-half of the plant products, while 12% of the plants, although available in the Western market, had "no substantial studies" of their properties. Strong evidence was found that 5 were toxic or allergenic, so that their use ought to be discouraged or forbidden. Nine plants had considerable evidence of therapeutic effect (Cravotto et al., 2010).

The U.S. National Center for Complementary and Alternative Medicine of the National Institutes of Health funds clinical trials of the effectiveness of herbal medicines and provides “fact sheets” summarizing the effectiveness and side effects of many plant-derived preparations

(http://clinicaltrials.gov/ct2/results?recr=Closed&no_unk=Y&spons=NCCAM)

1.3.4 Prevalence of use of Herbal medicine

A survey released in May 2004 by the National Center for Complementary and Alternative Medicine focused on who used complementary and alternative medicines (CAM), what was used, and why it was used. The survey was limited to adults, aged 18 years and over during 2002, living in the United States. According to this survey, herbal therapy, or use of natural products other than vitamins and minerals, was the most commonly used CAM therapy (18.9%) when all use of prayer was excluded (Barnes et al., 2004). Herbal remedies are very common in Europe. In Germany, herbal medications are dispensed by apothecaries (e.g., Apotheke). Prescription drugs are sold alongside essential oils, herbal extracts, or herbal teas. Herbal remedies are seen by some as a treatment to be preferred to pure medical compounds that have been industrially produced (James and Duke, 2000). In India the herbal remedy is so popular that the government of India has created a separate department—AYUSH—under the Ministry of Health & Family Welfare. The National Medicinal Plants Board was also established in 2000 by the Indian government in order to deal with the herbal medical system (Kala et al., 2007).

1.3.5 Traditional herbal medicine systems

Native Americans medicinally used about 2,500 of the approximately 20,000 plant species that are native to North America (Moerman and Daniel, 1997).

Some researchers trained in both western and traditional Chinese medicine have attempted to deconstruct ancient medical texts in the light of modern science. One idea is that the yin-yang balance, at least with regard to herbs, corresponds to the pro-oxidant and anti-oxidant balance. This interpretation is supported by several investigations of the ORAC ratings of various yin and yang herbs (Boxin et al., 2003).

1.4 Antioxidant

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being

oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid (vitamin C), or polyphenols (Sies, 1997).

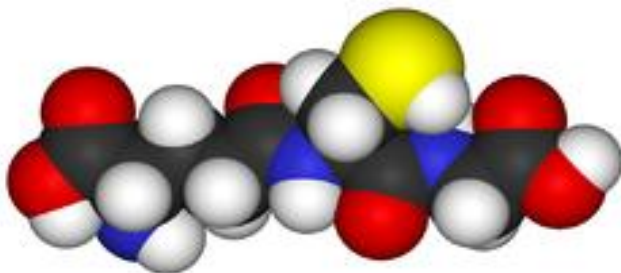


Fig 1.1: Model of the antioxidant metabolite glutathione. The yellow sphere is the redox-active sulfur atom that provides antioxidant activity, while the red, blue, white, and dark grey spheres represent oxygen, nitrogen, hydrogen, and carbon atoms, respectively.

Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is damage to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases.

Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness (Baillie JK et al., 2009). Although initial studies suggested that antioxidant supplements might promote health, later large clinical trials of antioxidant supplements including beta-carotene, vitamin A, and vitamin E singly or in different combinations suggest that supplementation has no effect on mortality or possibly increases it (Bjelakovic et al., 2007). Randomized clinical trials of antioxidants including beta carotene, vitamin E, vitamin C and selenium have shown no effect on cancer risk or have increased

cancer risk associated with supplementation (Pais and Dumitraşcu, 2013). Supplementation with selenium or vitamin E does not reduce the risk of cardiovascular disease (Rees et al., 2013).

Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German, 1999). Antioxidant activity could be measured simply by placing the fat in a closed container with oxygen and measuring the rate of oxygen consumption. However, it was the identification of vitamins A, C, and E as antioxidants that revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organisms (Knight, 1998). The possible mechanisms of action of antioxidants were first explored when it was recognized that a substance with anti-oxidative activity is likely to be one that is itself readily oxidized (Moureu and Dufraisse, 1922). Research into how vitamin E prevents the process of lipid peroxidation led to the identification of antioxidants as reducing agents that prevent oxidative reactions, often by scavenging reactive oxygen species before they can damage cells (Wolf, 2005).

1.4.1 Antioxidant enzyme

During normal metabolic functions, highly reactive compounds called free radicals are generated in the body; however, they may also be introduced from the environment. These molecules are inherently unstable as they possess lone pair of electrons and hence become highly reactive. They react with cellular molecules such as proteins, lipids and carbohydrates, and denature them. As a result of this, vital cellular structures and functions are lost and ultimately resulting in various pathological conditions. Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals. For the past decade, countless studies have been devoted to the beneficial effects of antioxidant enzymes. It has been found that a substantial link exists between free radicals and more than sixty different health conditions, including the aging process, cancer, diabetes, Alzheimer’s disease, strokes, heart attacks and atherosclerosis. By reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements, our body’s potential to reducing the risk of free radical related health problems is made more palpable. (Worthington, 2009).

Antioxidant enzymes are, therefore, absolutely critical for maintaining optimal cellular and systemic health and well being. This chapter reviews the pathophysiological role of some of the important enzymes involved in free radical scavenging with their clinical applications.

1.4.2 Free radicals and their scavengers

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction. The ability of the cell to utilize oxygen has provided humans with the benefit of metabolizing fats, proteins, and carbohydrates for energy; however, it does not come without cost. Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called free radical or reactive oxygen species (ROS). About 5% or more of the inhaled O₂ is converted to ROS such as superoxide, hydrogen peroxide and hydroxyl radicals by univalent reduction of O₂ (Uday Bandyopudya et al., 1999). Thus cells under aerobic condition are always threatened with the insult of ROS, which however are efficiently taken care of by the highly powerful antioxidant systems of the cell without any untoward effect. This antioxidant system includes, antioxidant enzymes (e.g., SOD, GPx and reductase, CAT, etc.), nutrient-derived antioxidants (e.g., ascorbic acid, tocopherols and tocotrienols, carotenoids, glutathione and lipoic acid), metal binding proteins (e.g., ferritin, lactoferrin, albumin, and ceruloplasmin) and numerous other antioxidant phytonutrients present in a wide variety of plant foods. Whenever the balance between ROS production and antioxidant defence is lost, ‘oxidative stress’ results which through a series of events deregulates the cellular functions leading to various pathological conditions (Chitra and Pillai, 2002).

1.4.3 Reactive Oxygen Species

Reactive oxygen species (ROS) is a term that encompasses all highly reactive, oxygen containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage. ROS are generated by a number of pathways. Most of the oxidants produced by cells occur as:

- A consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.
- Oxidative burst from phagocytes (white blood cells) as part of the mechanism by which bacteria and viruses are killed, and by which foreign proteins (antigens) are denatured.
- Xenobiotic metabolism, i.e., detoxification of toxic substances.

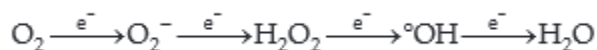
Consequently, things like vigorous exercise, which accelerates cellular metabolism; chronic inflammation, infections, and other illnesses; exposure to allergens and the presence of “leaky gut” syndrome; and exposure to drugs or toxins such as cigarette smoke, pollution, pesticides, and insecticides may all contribute to an increase in the body’s oxidant load.

1.4.4 Consequences of generation of ROS

Although O₂ can behave like a radical (a diradical) owing to presence of two unpaired electrons of parallel spin, it does not exhibit extreme reactivity due to quantum mechanical restrictions. Its electronic structure result in formation of water by reduction with four electrons, i.e.:



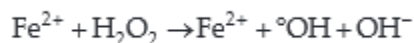
In the sequential univalent process by which O₂ undergoes reduction, several reactive intermediates are formed, such as superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and the extremely reactive hydroxy radical (°OH): collectively termed as the reactive oxygen species, the process can be represented as:



For the production of O₂, normally the tendency of univalent reduction of O₂ in respiring cells is restricted by cytochrome oxidase of the mitochondrial electron transport chain, which reduces O₂ by four electrons to H₂O without releasing either O₂-or H₂O₂. However, O₂-is invariably produced in respiring cells. This is due to the probable leak of single electron at the specific site of the mitochondrial electron transport chain, resulting in the appropriate single electron reduction of oxygen to O₂⁻. When the electron transport chain is highly reduced, and the respiratory rate is dependent on ADP availability; leakage of electrons at the ubisemiquinone and ubiquinone sites increases so as to result in production of O₂⁻and H₂O₂. For the production of H₂O₂, peroxisomal oxidases and flavoprotein, as well as D-amino acid oxidase, L-hydroxy acid oxidase, and fatty acyl oxidase participate. Cytochrome P-450, P-450 reductase and cytochrome b-5 reductase in the endoplasmic reticulum under certain conditions generate O₂⁻, and H₂O₂. During their catalytic cycles,

likewise, the catalytic cycle of xanthine oxidase has emerged as important source of O_2^- and H_2O_2 in a number of different tissue injuries.

Finally, for the production of OH^\bullet , except during abnormal exposure to ionization radiation, generation of OH^\bullet in vivo requires the presence of trace amount of H_2O_2 and Fe^{2+} salt forms OH^\bullet , as given following Fenton reaction:



Reactive oxygen species can attack vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids. To a lesser extent, carbohydrates are also the targets of ROS. These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein synthesis, DNA damage; ultimately resulting in cell death. O_2^- (Uday Bandyopudya et al., 1999) Damage to cells caused by free radicals is believed to play a central role in various human disorders like rheumatoid arthritis, hemorrhagic shock, cardiovascular disease, cystic fibrosis, metabolic disorders, neurodegenerative disease, gastrointestinal ulcer genesis, and AIDS. Some specific examples of ROS mediated disease are Alzheimer’s disease, Parkinson’s disease, oxidative modification of low-density lipoprotein in atherosclerosis, cancer, Down’s syndrome, and ischemic reperfusion injury in different tissues including heart, brain, kidney, liver, and gastrointestinal tract. Among these, role of ROS in atherosclerosis and ischemic injury in heart and brain studied extensively (Chitra and Pillai, 2002).

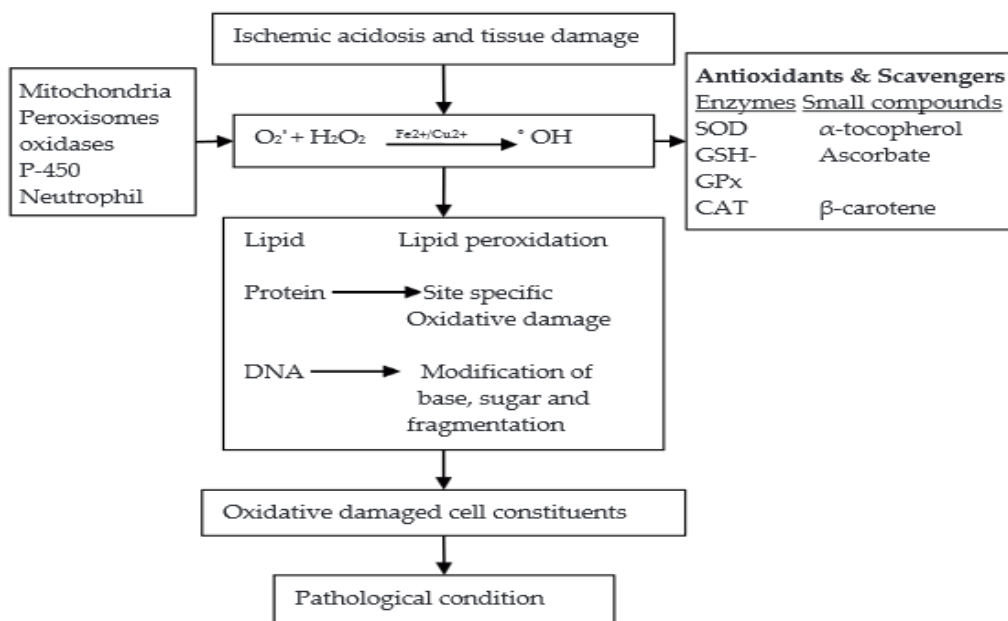


Figure 1.2: An overall picture of the metabolism of ROS and the mechanism of oxidative tissue damage leading to pathological conditions.

1.4.5 Antioxidant protection system

To protect the cells and organ systems of the body against reactive oxygen species (ROS), humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Table 1.1) (Mark Percival, 1998).

These components include:

a. Endogenous Antioxidants

- Bilirubin
- Thiols, e.g., glutathione, lipoic acid, N-acetyl cysteine
- NADPH and NADH
- Ubiquinone (coenzyme Q10)
- Uric acid
- Enzymes:
 - copper/zinc and manganese-dependent superoxide dismutase
 - iron-dependent catalase
 - selenium-dependent glutathione peroxidase

b. Dietary Antioxidants

- Vitamin C
- Vitamin E
- Beta carotene and other carotenoids and oxycarotenoids, e.g., lycopene and lutein
- Polyphenols, e.g., flavonoids, flavones, flavonol's, and Proanthocyanidins
-

c. Metal Binding Proteins

- Albumin (copper)
- Ceruloplasmin (copper)
- Metallothionein (copper)
- Ferritin (iron)
- Myoglobin (iron)

- Transferrin (iron)

ROS	NEUTRALIZING ANTIOXIDANTS
Hydroxyl radical	Vitamin C, Glutathione Flavonoids, Lipoic acid
Superoxide radical	Vitamin C, Glutathione, Flavonoids, SOD
Hydrogen peroxide	Vitamin C, Glutathione, beta carotene, Vitamin-E, flavonoids, lipoic acid
Lipid peroxides	Beta-carotene, Vitamin-E, Ubiquinone, flavonoids, Glutathione peroxidase

Table 1.1: Various ROS and corresponding neutralizing antioxidants

Defense mechanisms against free radical-induced oxidative damage include the following:

- i. catalytic removal of free radicals and reactive species by factors such as CAT, SOD, GPx and thiol-specific antioxidants;
- ii. binding of proteins (e.g., transferrin, metallothionein, haptoglobins, caeroplasmin) to pro-oxidant metal ions, such as iron and copper;
- iii. protection against macromolecular damage by proteins such as stress or heat shock proteins; and
- iv. reduction of free radicals by electron donors, such as GSH, vitamin E (α - tocopherol), vitamin C (ascorbic acid), bilirubin, and uric acid (Halliwell and Gutteridge, 1999).

Animal CAT is heme-containing enzymes that convert hydrogen peroxide (H_2O_2) to water and O_2 , and they are largely localized in sub cellular organelles such as peroxisomes. Mitochondria and the endoplasmic reticulum contain little CAT. Thus, intracellular H_2O_2 cannot be eliminated unless it diffuses to the peroxisomes (Halliwell and Gutteridge, 1999). GSH-Px removes H_2O_2 by coupling its reduction with the oxidation of GSH. GSH-Px can also reduce other peroxides, such as fatty acid hydro peroxides. These enzymes are present in the cytoplasm at millimolar concentrations and also present in the mitochondrial matrix. Most animal tissues contain both CAT and GSH-Px activity. SODs are metal-containing proteins that catalyze the removal of superoxide, generating water peroxide as a final product of the dismutation. Three isoforms have been identified, and they all are present in all eukaryotic cells. The copper-zinc SOD isoform is present in the cytoplasm, nucleus, and plasma. On the other hand, the manganese SOD isoform is primarily located in mitochondria.

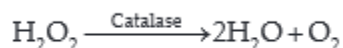
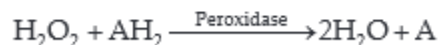
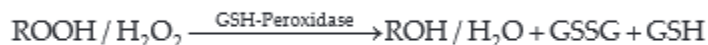
Dietary micronutrients also contribute to the antioxidant defense system. These include β - carotene, vitamin C, and vitamin E (the vitamin E family comprises both tocopherols and tocotrienols, with α -

tocopherol being the predominant and most active form). Water-soluble molecules, such as vitamin C, are potent radical scavenging agents in the aqueous phase of the cytoplasm, whereas lipid soluble forms, such as vitamin E and β - carotene, act as antioxidants within lipid environments. Selenium, copper, zinc, and manganese are also important elements, since they act as cofactors for antioxidant enzymes. Selenium is considered particularly important in protecting the lipid environment against oxidative injury, as it serves as a cofactor for GSH-Px.

The most abundant cellular antioxidant is the tripeptide, GSH (1-L- γ -glutamyl-L-cysteinyl glycine). GSH is synthesized in two steps. First, γ -glutamyl cysteine synthetase (γ -GCS) forms a γ -peptide bond between glutamic acid and cysteine, and then GSH synthetase adds glycine. GSH prevents the oxidation of protein thiol groups, either directly by reacting with reactive species or indirectly through glutathione transferases (Lauterburg et al., 1984)

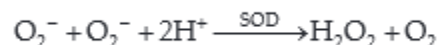
1.4.6 Antioxidant enzymes in health

Antioxidants are of different types so that they might be available for action when and where they are needed. They are natural (enzymes antioxidants and metal carrier proteins in the body), scavenging or chain breaking (like vitamin A, C, beta-carotene, etc.), pharmacologic antioxidants and others. Antioxidant compounds must be up” (converted) in the process of neutralizing free radicals. Therefore, one must continually produce more of the antioxidants in the body or ingest them either in diet or by supply mentation. The repair enzymes that can regrade some antioxidants are SOD, GPx, glutathione reductase (GR), CAT and the other metalloenzymes. SOD, CAT, and GPx constitute a mutually supportive team of defense against ROS. While SOD lowers the steady-state level of O_2^- , catalase and peroxidases do the same for H_2O_2 .



1.4.7 Superoxide dismutase

In 1967 biochemist Irwin Fridovitch of Duke University and Joe McCord discovered the antioxidant enzyme SOD, which provides an important means of cellular defense against free radical damage. This breakthrough caused medical scientists to begin to look seriously at free radicals (Chitra and Pillai, 2002). In most cases the process is automatically controlled and the number of free radicals does not become dangerously high. Fortunately, the body has, throughout the course of millions of years of evolution become accustomed to coping with free radicals and has evolved various schemes for doing this SOD (EC 1.15.1.1) is the antioxidant enzyme that catalyzed the dismutation of the highly reactive superoxide anion to O₂ and to the less reactive species H₂O₂. Peroxide can be destroyed by CAT or GPX reactions (Fridovich, 1995).



In humans, there are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD) (Sun E et al., 1995). SOD destroys O₂⁻ by successive oxidation reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates (Meier B et al., 1998). All types of SOD bind single charged anions such as azide and fluoride, but distinct differences have been noted in the susceptibilities of Fe-, Mn- or Cu/Zn-SODs. Cu/Zn-SOD is competitively inhibited by N₃⁻, CN⁻ (Leone M et al., 1998) and by F⁻ (Vance et al., 1998)

Mn-SOD is a homotetramer (96 kDa) containing one manganese atom per subunit those cycles from Mn (III) to Mn (II) and back to Mn (III) during the two step dismutation of superoxide. The respiratory chain in mitochondria is a major source of oxygen radicals. Mn-SOD has been shown to be greatly induced and depressed by cytokines, but is only moderately influenced by oxidants (MacMillan-Crow LA et al., 1998).

Inactivation of recombinant human mitochondrial Mn- SOD by peroxynitrite is caused by nitration of a specific tyrosine residue (Stralin and Marklund, 1994)

The biological importance of Mn-SOD is demonstrated among others by the following observations: (a) inactivation of Mn-SOD genes in *Escherichia coli* increases mutation frequency when grown under aerobic conditions (Yamakura et al., 1998); (b) elimination of the gene in *Saccharomyces cerevisiae* increases its sensitivity to oxygen (Farr et al., 1986), (c) lack of expression in Mn-SOD

knockout mice results in dilated cardiomyopathy and neonatal lethality (Van Loon APGM et al., 1986); (d) tumor necrosis factor (TNF) selectively induces Mn-SOD, but not Cu/Zn- SOD, CAT or GPX mRNA in various mouse tissues and cultured cells(Li et al., 1995); (e) transection of Mn- SOD cDNA into cultured cells rendered the cells resistant to paracetamol, TNF and Adriamycin-induced cytotoxicity, and radiation induced-neoplastic transformation (Kizaki et al., 1993); f) expression of human Mn-SOD genes in transgenic mice protects against oxygen induced pulmonary injury and Adriamycin-induced cardiac toxicity (St. Clair et al., 1991).

Cu/Zn-SOD (SOD-1) is another type of enzymes that has been conserved throughout evolution. These enzymes have two identical subunits of about 32 kDa, although a monomeric structure can be found in a high protein concentration from *E. coli* (Wispe' et al., 1992). Each subunit contains a metal cluster, the active site, constituted by a copper and a zinc atom bridged by a histamine residue (Battistoni et al., 1998). Cu/Zn-SOD is believed to play a major role in the first line of antioxidant defense. Calves that were fed milk supplemented with 25 ppm Cu and 100 ppm Zn showed a stronger immune response and a higher SOD activity (Stroppolo et al., 1998). Other recent reports involving SOD knock-outs have revealed that Mn- SOD is essential for life whereas Cu/Zn-SOD is not. Cu/Zn-SOD knock-out mice appear normal and exhibit differences only after traumatic injury, whereas Mn-SOD knockouts do not survive past 3 weeks of age .Among various human tissues Mn-SOD contents were roughly one-half as large as the Cu/Zn-SOD contents (Prasad et al., 1995).

Extracellular superoxide dismutase (EC-SOD) is a secretory, tetrameric, copper and zinc containing glycoprotein; with a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate. EC-SOD was found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph, and synovial fluid. EC-SOD is not induced by its substrate or by other oxidants and its regulation in mammalian tissues primarily occurs in a manner coordinated by cytokines, rather than as a response of individual cells to oxidants (Marklund, 1980).

1.4.7.1 Application

This enzyme has been known to promote the rejuvenation and repair of cells, while reducing the damages caused by free radicals. SOD is found in our skin and it is essential in order for our body to generate adequate amounts of skin-building cells called fibroblasts. Among the common natural sources of SOD are cabbage, Brussels sprouts, wheat grass, barley grass and broccoli. SOD plays a significant role in preventing the development of the Lou Gehrig's disease, also known as

Amyotrophic Lateral Sclerosis (ALS). This kind of illness can lead to death because it affects the nerve cells in the spinal cord and the brain. Apart from that, this enzyme is also used for treatment of inflammatory diseases, burn injuries, prostate problems, arthritis, corneal ulcer, and reversing the long term effects of radiation and smoke exposure. Additionally, if superoxide dismutase is made into a lotion and applied to the skin, it will prevent the formation of wrinkles. It will also heal wounds, reduce the appearance of scars, and lighten skin pigmentation that has been caused by UV rays. SOD is also known to help carry nitric oxide into our hair follicles. This is beneficial for people who are experiencing premature hair loss due to a genetic predisposition or free radicals. Because this enzyme is a very potent antioxidant, SOD combats the effects of free radicals that are causing hair follicles to die. Since nitric oxide relaxes the blood vessels and allows more blood to circulate to the hair follicles and SOD helps to remove the free radicals, hair loss can be prevented and even reversed. Taking dietary supplements that provide an adequate supply of Superoxide dismutase will be helpful in maintaining overall well being and health because it protects our entire body from the harmful effects of free radicals.

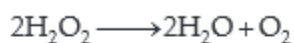
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1.4.8 Catalase

Catalase (CAT) is an enzyme responsible for the degradation of hydrogen peroxide. It is a protective enzyme present in nearly all animal cells.

1.4.8.1 Specificity

The reaction of CAT occurs in two steps. A molecule of hydrogen peroxide oxidizes the heme to an oxyferryl species. A porphyrin cation radical is generated when one oxidation equivalent is removed from iron and one from the porphyrin ring. A second hydrogen peroxide molecule acts as a reducing agent to regenerate the resting state enzyme, producing a molecule of oxygen and water.



CAT (EC 1.11.1.6) is a tetrameric enzyme consisting of four identical tetrahedrally arranged subunits of 60 kDa that contains a single ferriprotoporphyrin group per subunit, and has a molecular mass of

about 240 kDa (Buschfort et al., 1997). CAT reacts very efficiently with H₂O₂ to form water and molecular oxygen; and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase activity.

In animals, hydrogen peroxide is detoxified by CAT and by GPX. CAT protects cells from hydrogen peroxide generated within them. Eventhough CAT is not essential for some cell types under normal conditions; it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells. Survival of rats exposed to 100% oxygen was increased when liposome's containing SOD and CAT were injected intravenously before and during the exposure (Aebi, 1980). The increased sensitivity of transfected CAT-enriched cells to some drugs and oxidants is attributed to the property of CAT in cells to prevent the drug-induced consumption of O₂ either for destroying H₂O₂ to oxygen or for direct interaction with the drug (Turrens et al., 1984).

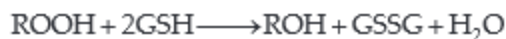
1.4.8.2 Application

CAT is used in the food industry for removing hydrogen peroxide from milk prior to cheese production. Another use is in food wrappers where it prevents food from oxidizing. CAT is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene - a few lens-cleaning products disinfect the lens using a hydrogen peroxide solution; a solution containing CAT is then used to decompose the hydrogen peroxide before the lens is used again. Recently, CAT has also begun to be used in the aesthetics industry. Several mask treatments combine the enzyme with hydrogen peroxide on the face with the intent of increasing cellular oxygenation in the upper layers of the epidermis.

(<http://library.umac.mo/ebooks/b28050174.pdf>)

1.4.9 Glutathione peroxidase

Glutathione peroxidase (GPx) is an enzyme that is responsible for protecting cells from damage due to free radicals like hydrogen and lipid peroxides. The GPx (EC 1.11.1.19) contains a single selenocysteine (Sec) residue in each of the four identical subunits, which is essential for enzyme activity (Speranza et al., 1993). GPX (80 kDa) catalyses the reduction of hydro peroxides using GSH, thereby protecting mammalian cells against oxidative damage. In fact, glutathione metabolism is one of the most essential antioxidative defense mechanisms.



1.4.9.1 Application

This is one of the most important enzymes in the body with antioxidant properties. Levels of GPx in the body are closely linked with that of glutathione, the master antioxidant. Glutathione (GHS for short) is a tripeptide that not only protects the cells against ill effects of pollution; it is also acts as body's immune system boosters. It is present in high concentrations in the cells and plays a pivotal role in maintaining them in reduced state lest they suffer damage by oxidation (from free radicals). The role as antioxidant is particularly important for brain as it is very sensitive to presence of free radicals. Combination of certain antioxidants like glutathione, vitamin C and E, selenium and glutathione peroxidase are very powerful in helping the body fight against the free radicals. GSH ensures that the red blood cells remain intact and protect the white blood cells (which are responsible for immunity). Glutathione is found in vegetables and fruit, but cooking will significantly reduce its potency. Taking it as a supplement is a good idea.

(<http://library.umac.mo/ebooks/b28050174.pdf>)

1.4.10 Clinical applications of antioxidant enzymes

- 1. Chronic Inflammation:** Chronic inflammatory diseases such as rheumatoid arthritis are self-perpetuated by the free radicals released by neutrophils. Both corticosteroids and non-steroids anti inflammatory drugs interfere with formation of free radicals and interrupt the disease process.
- 2. Acute Inflammation:** At the inflammatory site, activated macrophages produce free radicals. Respiratory burst and increased activity of NADPH oxidase are seen in macrophages and neutrophils.
- 3. Respiratory Diseases:** Breathing of 100 % oxygen for more than 24 hr produces destruction of endothelium and lung edema. This is due to the release of free radicals by activated neutrophils (Vasudevan et al., 2006). In premature newborn infants, prolonged exposure to high oxygen concentration is responsible for bronchopulmonary dysplasia. Adult respiratory distress syndrome (ARDS) is characterized by pulmonary edema. ARDS is produced when neutrophils are recruited to lungs which subsequently release free radicals. Cigarette smoking enhances the emphysema in alpha-1 protease inhibitor deficiency. Cigarette smoke contains

free radicals. Soot attracts neutrophils to the site which releases more free radicals. Thus, there is more elastase and less protease inhibitor, leading to lung damage.

4. **Diseases of the Eye:** Retrolental fibroplasia or retinopathy of prematurity is a condition seen in premature infants treated with pure oxygen for a long time. It is caused by free radicals, causing thromboxane release, sustained vascular contracture and cellular injury. Cataract formation is related with ageing process. Cataract is partly due to photochemical generation of free radicals. Tissues of the eye, including the lens, have high concentration of free radical scavenging enzymes.
5. **Shock Related Injury:** Release of free radicals from phagocytes damage membranes by lipid per oxidation. They release leucotrienes from platelets and proteases from macrophages. All these factors cause increased vascular permeability, resulting in tissue edema. Antioxidants have a protective effect.
6. **Arthrosclerosis and Myocardial Infarction:** Low density lipoproteins (LDL) promote atherosclerosis. They are deposited under the endothelial cells, which undergo oxidation by free radicals released from endothelial cells. This attracts macrophages. Macrophages are then converted into foam cells. This initiates the atherosclerotic plaque formation. Alpha tocopherol offers some protective effect.
7. **Peptic Ulcer:** Peptic ulcer is produced by erosion of gastric mucosa by hydrochloric acid. It is shown that superoxide anions are involved in the formation of ulcer. *Helicobacter pylori* infection perpetuates the disease. This infection potentiates the macrophage oxidative burst leading to tissue destruction.
8. **Skin Diseases:** due to inborn defects, porphyrins accumulate in the skin. Exposure of sunlight will lead to erythema and eruptions in the patients. Sunlight acting on porphyrins produces singlet oxygen, which trigger inflammatory reaction, leading to the above symptoms. Certain plant products, called psoralens are administered in the treatment of psoriasis and leukoderma. When the drugs is applied over the affected skin and then irradiated by UV light, singlet oxygen produced with clinical benefit.
9. **Cancer Treatment [39]:** Free radicals contribute to cancer development because of their mutagenic property. Free radicals produce DNA damage, and accumulated damages lead to somatic mutations and malignancy. Cancer is treated by radiotherapy. Irrational produces reactive oxygen species in the cells which trigger the cell death. To increase the therapeutic effect of radiation, radio-sensitizers are administered, which increase the production of ROS.

1.4.11 Other Antioxidants

1.4.11.1 Dietary Antioxidants

Vitamin C, vitamin E, and beta-carotene are among the most widely studied dietary antioxidants. Vitamin C is considered the most important water-soluble antioxidant in extracellular fluids. It is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated. Vitamin E, a major lipid-soluble antioxidant, is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation. Vitamin C has been cited as being capable of regenerating vitamin E. Beta-carotene and other carotenoids are also believed to provide antioxidant protection to lipid-rich tissues. Research suggests beta-carotene may work synergistically with vitamin E.

A diet that is excessively low in fat may negatively affect beta carotene and vitamin E absorption, as well as other fat-soluble nutrients. Fruits and vegetables are major sources of vitamin C and carotenoids, while whole grains and high quality, properly extracted and protected vegetable oils are major sources of vitamin E (Mark Percival, 1998).

1.4.11.2 Phytonutrients

A number of other dietary antioxidant substances exist beyond the traditional vitamins discussed above. Many plant-derived substances, collectively termed “phytonutrients,” or “phytochemicals,” are becoming increasingly known for their antioxidant activity. Phenolic compounds such as flavonoids are ubiquitous within the plant kingdom: approximately 3,000 flavonoid substances have been described. In plants, flavonoids serve as protectors against a wide variety of environmental stresses while, in humans, flavonoids appear to function as “biological response modifiers.” Flavonoids have been demonstrated to have anti-inflammatory, anti-allergenic, anti-viral, anti-aging, and anti-carcinogenic activity. The broad therapeutic effects of flavonoids can be largely attributed to their antioxidant properties. In addition to an antioxidant effect, flavonoid compounds may exert protection against heart disease through the inhibition of cyclooxygenase and lipoxygenase activities in platelets and macrophages (Mark Percival, 1998).

1.4.12 Antioxidant activity over disease

1.4.12.1 Activity over lipid peroxidation and skin diseases

Due to its interface function between the body and the environment, the skin is chronically exposed to both endogenous and environmental pro-oxidant agents, leading to the harmful generation of reactive oxygen species (ROS). There is compelling evidence that oxidative stress is involved in the damage of cellular constituents, such as DNA, cell membrane lipids or proteins. To protect the skin against the over-load of oxidant species, it contains a well-organized system of both chemical and enzymatic antioxidant which is able to work in a synergistic manner. Skin antioxidant network protects cells against oxidative injury and prevent the production of oxidation products, such as 4-hydroxy-2-nonenal or malonaldehyde, which are able to induce protein damage, apoptosis or release of pro-inflammatory mediators, such as cytokines. When oxidative stress overwhelms the skin antioxidant capacity the subsequent modification of cellular redox apparatus leads to an alteration of cell homeostasis and a generation of degenerative processes. Topical application or oral administration of antioxidants has been recently suggested as preventive therapy for skin photoaging and UV-induced cancer. The recognition that ROS can act as second messengers in the induction of several biological responses, such as the activation of NF- κ B or AP-1, the generation of cytokines, the modulation of signaling pathways, etc., has led many researchers to focus on the possible effects of antioxidants in many pathological processes. The recent demonstration that the peroxisome proliferators-activated receptors, whose natural ligands are polyunsaturated fatty acids and their oxidation products, have a central role in the induction of some skin diseases, such as psoriasis or acne, has indicated new links between free radicals and skin inflammation. Based on these findings, the review summarizes the possible correlations between antioxidant imbalance, lipid oxidative breakage and skin diseases, from both a pathological and therapeutic points of view (Venereol, 2003).

1.4.12.2 Activity over cardiovascular disease

Oxidative and inflammatory stresses are cardinal in the pathogenesis of hypertension and atherosclerosis. Oxidative stress also leads to the induction of inflammation through the activation of pro inflammatory transcription factors. Understanding the mechanisms leading to oxidative stress and the means of suppressing it are important in controlling complications related to atherogenesis, since oxidative and inflammatory stress are important in the pathogenesis of atherosclerosis. The

failure of chemical antioxidants [which scavenge reactive oxygen species (ROS)], such as vitamins E and C, has led to further exploration of the ROS-suppressive effects of drugs used in the treatment of cardiovascular disease (Hypertens, 2007).

1.4.12.3 Proposed Influence of Oxidants and Antioxidants on the Development of Atherosclerosis

Atherosclerosis is a complex process involving the deposition of plasma lipoproteins and the proliferation of cellular elements in the artery wall. This chronic condition advances through a series of stages beginning with fatty streak lesions composed largely of lipid-engorged macrophage foam cells and ultimately progressing to complex plaques consisting of a core of lipid and necrotic cell debris covered by a fibrous cap (Ross R, 1990). These plaques provide a barrier to arterial blood flow and may precipitate clinical events, particularly under conditions that favor plaque rupture and thrombus formation.

Over the past 2 decades, considerable evidence has been gathered in support of the hypothesis that free-radical-mediated oxidative processes and specific products arising therefrom play a key role in atherogenesis (Steinberg and Biol., 1997). At the center of this hypothesis are low-density lipoproteins (LDLs), which undergo multiple changes on oxidation, which are thought to be proatherogenic (see Figure↓). Oxidation of LDL lipids leads to the production of a diverse array of biologically active compounds, including some that influence the functional integrity of vascular cells. Among the well-characterized effects are increases in the expression of endothelial cell surface adhesion molecules that facilitate the mobilization and uptake of circulating inflammatory cells (Navab et al., 1996) and alterations in the chemotactic properties of monocytes and monocyte-derived macrophages (Quinn et al., 1987) in a manner expected to increase their residence within the artery wall. Oxidation of the apolipoprotein B component alters LDL receptor recognition properties, leading to avid internalization of LDLs by macrophages via scavenger receptors, a key step in the formation of macrophage-derived foam cells.

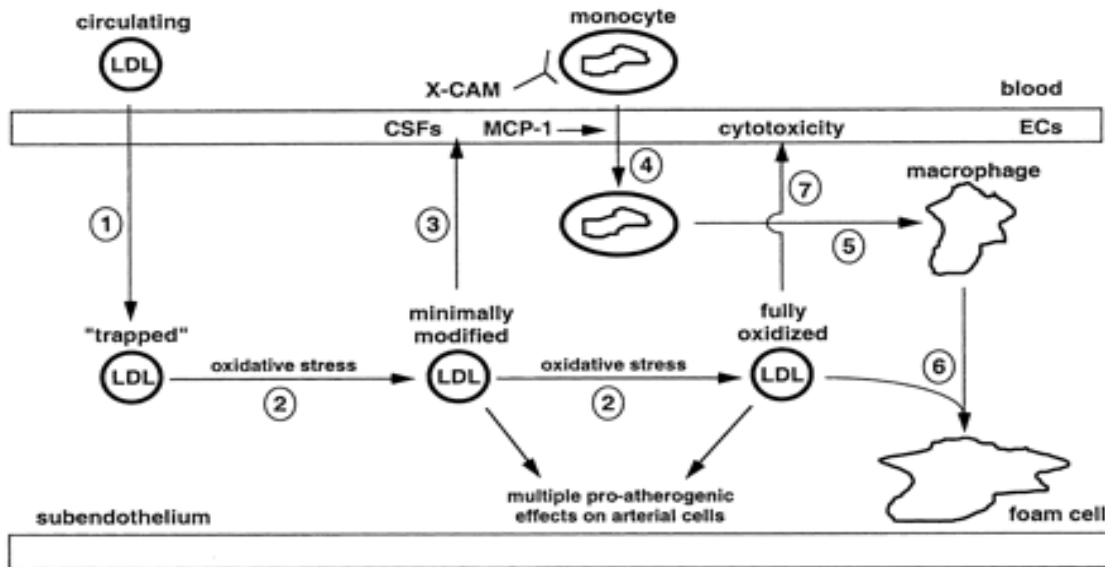


Figure 1.3: Proposed role of LDL oxidation in the initiation of fatty streak lesions. LDL crosses the endothelium in a concentration-dependent manner and can become trapped in the extracellular matrix (1). The sub endothelium is an oxidizing environment, and if the LDL remains trapped for a sufficiently long period of time, it undergoes oxidative changes (2). Mildly oxidized forms of LDL contain biologically active phospholipids oxidation products that affect the pattern of gene expression in endothelial cells (ECs), leading to, among other things, changes in the expression of monocyte binding molecules (designated X-CAM), monocyte chemoattractant protein (MCP-1), and macrophage colony stimulating factors (CSFs) (3). These factors in turn promote the recruitment of monocytes (4) and drive their phenotypic differentiation to macrophages (5). Further oxidation leads to alterations in apolipoprotein B such that LDL particles are recognized and internalized by macrophages (6), progenitors of the lipid-laden foam cells. Marked increases in lipid and cholesterol oxidation products render the LDL particles cytotoxic, leading to further endothelial injury and favoring further entry of LDL and circulating monocytes and thus a continuation of the disease process.

In addition to these effects, oxidative processes are proposed to play a role in lesion maturation and the precipitation of clinical events. This may involve effects on intimal proliferation, fibrosis, calcification, endothelial function and vaso reactivity, plaque rupture, and thrombosis (Gokce et al., 1996) to date; the role of oxidation in these processes has received less attention than that in the early stages of the disease.

Oxidants are products of normal aerobic metabolism and the inflammatory response. They constitute a chemically and compartmentally diverse group, and it is presently unknown which, if any, are

critical to the disease process. In addition to the different sources and types of oxidants, ambiguity in relating specific oxidants to the disease process arises from the multitude of pathophysiological events linked to oxidation, the paucity of methods for measuring these short-lived species within the sequestered environment of the artery wall, and the variable modulating effects of counteractive antioxidants. With regard to the latter, although oxidant formation is an inevitable feature of aerobic life, oxidant-mediated disease promotion is proposed to occur only under circumstances in which these agents overwhelm antioxidant defenses.

Like oxidants, antioxidants constitute a diverse group of compounds with different properties. They operate by inhibiting oxidant formation, intercepting oxidants once they have formed, and repairing oxidant-induced injury. In terms of the coronary heart disease process, several points of antioxidant intervention have been proposed, as recently reviewed in detail (Diaz et al., 1997) Inhibition of LDL oxidation is the most well characterized of these and includes effects on the concentration or reactivity of oxidants capable of modifying LDL and on the susceptibility or resistance of LDL to these oxidants. Better definition of these and other disease processes in which antioxidants may intervene will allow optimization of conditions for testing the importance of antioxidants in disease prevention and ultimately for intervening in the disease process should antioxidants prove to be effective in this regard.

1.5 The Plant Family

The Malvaceae, or the mallows, are a family of flowering plants estimated to contain 243 genera with 4225+ species ("Angiosperm Phylogeny Website", 2014). Well-known members of this family include okra, cotton, and cacao. The largest genera in terms of number of species include Hibiscus (300 species), Sterculia (250 species), Dombeya (250 species), Pavonia (200 species) and Sida (200 species) (Judd et al., 2008).

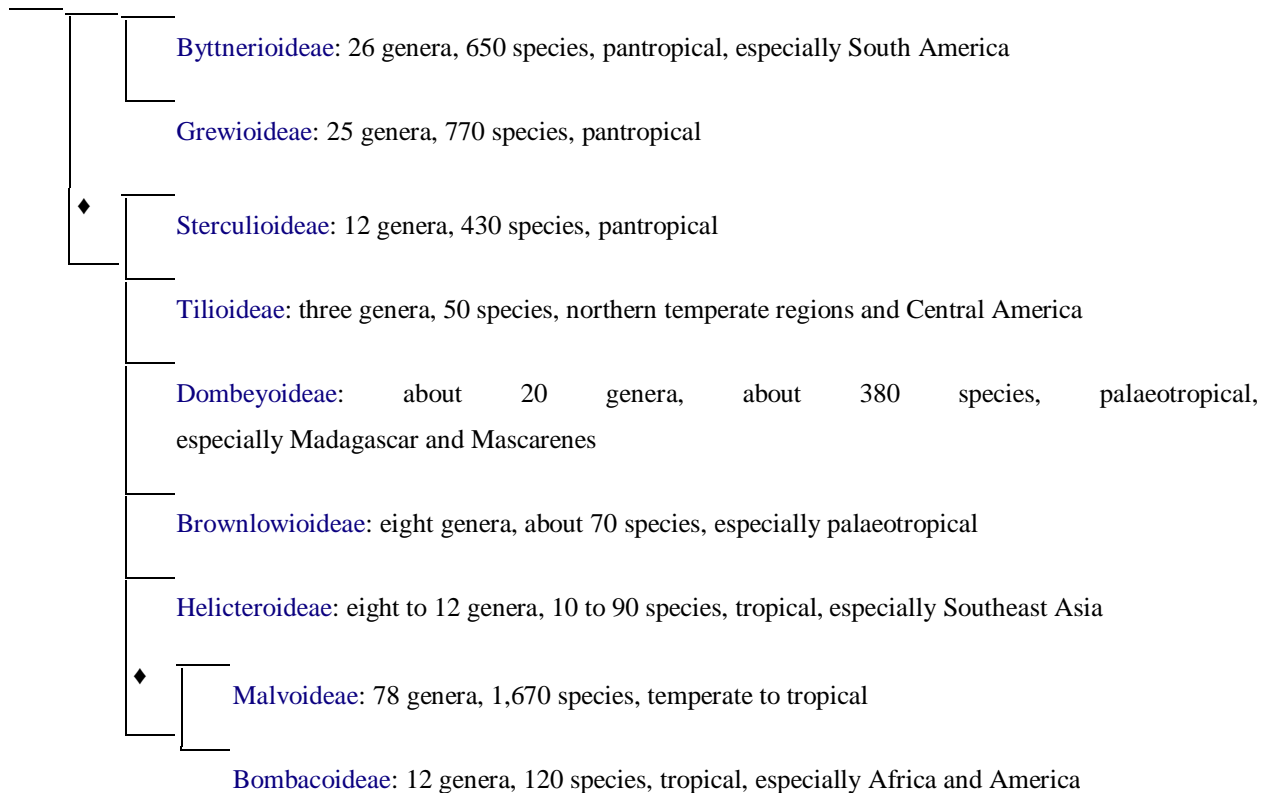
1.5.1 Taxonomy and nomenclature

The circumscription of the Malvaceae is controversial. The traditional Malvaceae *sensu stricto* comprise a very homogeneous and cladistically monophyletic group. Another major circumscription, Malvaceae *sensu lato*, has been more recently defined on the basis that molecular techniques have shown the commonly recognized families Bombacaceae, Tiliaceae, and Sterculiaceae, which have always been considered closely allied to Malvaceae *s.s.*, are not

monophyletic groups. Thus, the Malvaceae can be expanded to include all of these families so as to compose a monophyletic group. Adopting this circumscription, the Malvaceae incorporate a much larger number of genera.

1.5.2 Subfamilies

This article is based on the second circumscription, as presented by the Angiosperm Phylogeny Website. The Malvaceae *s.l.* (hereafter simply "Malvaceae") comprises nine subfamilies. A tentative cladogram of the family is shown below. The diamond denotes a poorly supported branching (<80%).



It is important to point out the relationships between these subfamilies are still either poorly supported or almost completely obscure, so the circumscription of the family may change dramatically as new studies are published.

If looking for information about the traditional Malvaceae *s.s.*, we recommend referring to Malvoideae, the subfamily that approximately corresponds to that group.

1.6 Introduction of *Malva verticillata*

Malva spp. is a genus of about 25–30 species of herbaceous annual, biennial, and perennial plants in the family Malvaceae (of which it is the type genus), one of several closely related genera in the family to bear the common English name mallow. The genus is widespread throughout the temperate, subtropical and tropical regions of Africa, Asia and Europe (Davis, 2010)The word "mallow" is derived from Old English "malwe", which was imported from Latin "malva", which originated in Ancient Greek (malakhē) meaning "yellow" or Hebrew (malúakh) meaning "salty"(Davis, 2010). A number of species, previously considered to belong to Lavatera, have been moved to Malva.

The leaves are alternate, palmately lobed. The flowers are from 0.5–5 cm diameter, with five pink or white petals.

The color mauve was in 1859 named after the French name for this plant.

1.6.1 Taxonomic Hierarchy

Kingdom	<u>Plantae</u> – plantes, Planta, Vegetal, plants
Subkingdom	<u>Viridiplantae</u>
Infrakingdom	<u>Streptophyta</u> – land plants
Superdivision	<u>Embryophyta</u>
Division	<u>Tracheophyta</u> – vascular plants, tracheophytes
Subdivision	<u>Spermatophytina</u> – spermatophytes, seed plants, phanérogames
Class	<u>Magnoliopsida</u>
Superorder	<u>Rosanae</u>
Order	<u>Malvales</u>
Family	<u>Malvaceae</u> – mallows, mauves
Genus	<u>Malva</u> L. – cheeseweed, mallow
Species	<i>Malva verticillata</i> L. – cluster mallow

1.6.2 Characteristics

- i) Flower petal color: pink to red, white
- ii) Leaf type: the leaves are simple (lobed or unlobed but not separated into leaflets)
- iii) Leaf arrangement alternate: there is one leaf per node along the stem.
- iv) Leaf blade edges: the edge of the leaf blade has lobes, or it has both teeth and lobes
- v) Flower symmetry: there are two or more ways to evenly divide the flower (the flower is radially symmetrical)
- vi) Number of sepals, petals or tepals: there are five petals, sepals, or tepals in the flower
- vii) Fusion of sepals and petals: both the petals and sepals are separate and not fused, the petals or the sepals are fused into a cup or tube
- viii) Stamen number: 13 or more
- ix) Fruit type (general): the fruit is dry but does not split open when ripe
- x) Fruit length: 8 mm

1.6.3 Morphological Description

Several species are widely grown as garden flowers, while some are invasive weeds, particularly in the Americas where they are not native.

Many species are edible as leaf vegetables and commonly foraged in the West. Known as ebegümeçi in Turkish, it is used as vegetable in Turkey in various forms such as stuffing the leaves with bulgur or rice or using the boiled leaves as side dish. *Malva verticillata* (Chinese: 冬寒菜; pinyin: dōngháncài, Korean: 아욱 auk) is grown on a limited commercial scale in China; when made as a herbal infusion, it is used for its colon cleansing properties and as a weight loss supplement.

Very easily grown, short-lived perennials often grown as ornamental plants. Mild tasting young mallow leaves can be a substitute for lettuce, whereas older leaves are better cooked as a leafy green vegetable. The buds and flowers can be used in salads.

Cultivation is by sowing the seeds directly outdoors in early spring. The seed is easy to collect, and they will often spread themselves by seed.

In Catalonia (Southern Europe) they use the leaves to cure stinging nettles sting.

Bodo tribals in Bodoland, Assam (Northeast India) cultivate a sub-species of malva and use it extensively in their traditional cuisine, although its use is not much known among other people of

India. Malva Leaves are a highly cherished vegetable dish in north Indian state of Kashmir. It is called "Soachal".

Malva sp. leaves have been used in the traditional Austrian medicine internally as tea or externally as baths for treatment of disorders of the skin, gastrointestinal tract and respiratory tract. (Vogl et al., 2013)

1.6.4 Uses

1. Toxic parts

When grown on nitrogen rich soils (and particularly when these are cultivated inorganically), the plant tends to concentrate high levels of nitrates in its leaves (Cooper and Johnson, 1984)

2. Edible uses

Leaves - raw or cooked (Facciola, 1990) the leaves of well-grown plants can be 15cm or more across. They have a mild and very pleasant flavor that makes an excellent addition to salads. We use them as a tasty alternative to the lettuce. Young seeds - raw or cooked. Used when green and immature (Harrington, 1967) pleasant nutty taste but the seed is too small and fiddly for most people to want to harvest.

3. Leaves

Unknown use

4. Seed

Unknown use

5. Material uses

Cream, yellow and green dyes can be obtained from the plant and the seed heads (Grae, 1974)

6. Unknown part

Dye

7. Medicinal uses

The seed contains mucilage, polysaccharides and flavonoids (MPRKWHO, 1998).

It is demulcent, diuretic, emollient, galactagogue and laxative (Duke and Ayensu, 1985). The seeds are used in Tibetan medicine, where they are considered to have a sweet and astringent taste plus a heating potency. They are used in the treatment of renal disorders, the retention of fluids, frequent thirst and diarrhea (Tsarong and Tsewang, 1994).

The root is used to cause vomiting in the treatment of whooping cough (Chopra et al., 1986).

The leaves and stems are said to be digestive. They are given to women in the advanced stages of pregnancy (Chopra et al., 1986).

1.6.5 Photograph of plant



Figure 1.4.1: *Malva verticillata*

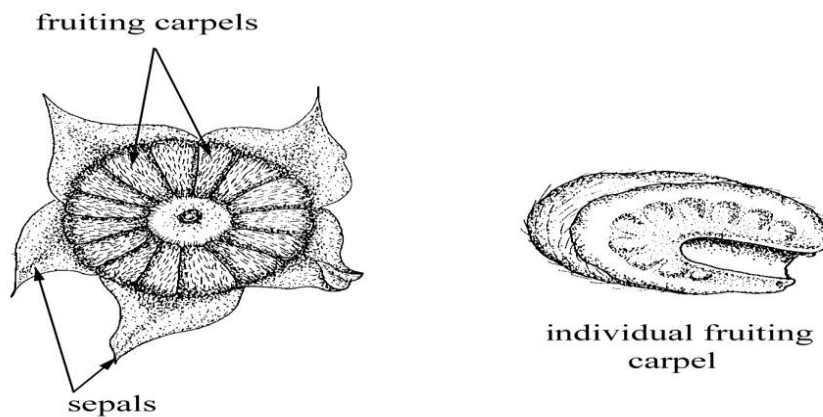


Figure 1.4.2: *Malva verticillata* fruits



Figure 1.4.3: Plant form of *Malva verticillata*

1.6.6 Species list of *Malva* plant:

They are different species belong to *Malva* as shown in table (2.1) (Davis, 2010):

Species name	Common name
<ul style="list-style-type: none">• <i>Malva aegyptia</i>• <i>Malva aethiopica</i>• <i>Malva alcea</i>	L.—Greater Musk-mallow, Vervain Mallow
<ul style="list-style-type: none">• <i>Malva assurgentiflora</i>• <i>Malva brasiliensis</i>	Desr.—Brazilian Mallow

<ul style="list-style-type: none"> • <i>Malva canariensis</i> • <i>Malva cathayensis</i> • <i>Malva cretica</i> • <i>Malva dendromorpha</i> 	Tree Mallow
<ul style="list-style-type: none"> • <i>Malva hispanica</i> • <i>Malva microcarpa</i> • <i>Malva microphylla</i> • <i>Malva mohileviensis</i> • <i>Malva moschata</i> 	L.—Musk-mallow
<ul style="list-style-type: none"> • <i>Malva pacifica</i> • <i>Malva parviflora</i> 	L.—Least Mallow, Cheese weed, Cheese weed Mallow, Small-whorl Mallow
<ul style="list-style-type: none"> • <i>Malva preissiana</i> 	Australian Hollyhock
<ul style="list-style-type: none"> • <i>Malva pseudolavatera</i> • <i>Malva pusilla</i> 	Small Mallow
<ul style="list-style-type: none"> • <i>Malva qaiseri</i> • <i>Malva rotundifolia</i> 	L.—Low Mallow

<ul style="list-style-type: none"> • <i>Malva stipulacea</i> • <i>Malva subovata</i> • <i>Malva transcaucasica</i> • <i>Malva tournefortiana</i> • <i>Malva neglecta</i> 	<p>L.—Common Mallow, High Mallow. Dwarf Mallow, Button weed, Cheese plant, Cheese weed, Common Mallow, Round leaf Mallow</p>
<ul style="list-style-type: none"> • <i>Malva nicaeensis</i> 	<p>All.—French Mallow, Bull Mallow</p>
<ul style="list-style-type: none"> • <i>Malva trifida</i> • <i>Malva verticillata</i> 	<p>L.—Chinese Mallow, Cluster Mallow</p>

Table 1.2: Species list of Malva plant

1.7 Aim of the Study

Malva sp. leaves have been used in the traditional medicine internally as tea or externally as baths for treatment of disorders of the skin, gastrointestinal tract and respiratory tract (Vogl et al., 2013). The seed contains mucilage, polysaccharides and flavonoids (MPRKWHO, 1998). It is demulcent, diuretic, emollient, galactogogue and laxative (Duke and Ayensu, 1985). They are used in the treatment of renal disorders, the retention of fluids, frequent thirst and diarrhea (Tsarong and Tsewang, 1994). The root is used to cause vomiting in the treatment of whooping cough (Chopra et al., 1986). The leaves and stems are said to be digestive. They are given to women in the advanced stages of pregnancy (Chopra et al., 1986). However, the antioxidant activity of *Malva verticillata* extract was not previously studied. Despite its importance, only a few studies have been conducted on the plant. Hence, the present investigation was aimed to investigate the antioxidant activity of the methanol extract of the plants using DPPH free radical scavenging assay, total antioxidant capacity and reducing power assessment. We have also investigated total phenolic contents and flavonoid contents of the methanol extract of *Malva verticillata*.

CHAPTER 2

EXPERIMENTAL

2.1 Experiment Plant

Malva verticillata included in Malvaceae was investigated in this study.

Plant Name	Family	Plant part used
<i>Malva verticillata</i>	Malvaceae	Whole plants

2.2 Preparation of the Plant Extracts for Experiments

2.2.1 Collection and Identification

For this present investigation the whole plants of *Malva verticillata* were collected from the local vegetable market. The whole plant parts were then sent to National Herbarium Mirpur, Dhaka. Expert of National herberium identified as *Malva verticillata* where a voucher specimen has been deposited (accession no.: DACB 37674).

2.2.2 Drying of the Samples

After collection of the plants all debris and adulterants were carefully removed to get fresh sample. Then the collected samples were dried for few days in the laboratory under room temperature until proper drying of the sample. After drying the plants were weighed (350g) and preserved in air tight container until their extraction.

2.2.3 Extraction of the dried plants

The whole plants of *Malva verticillata* (350g) were taken in an extraction vessel of the Soxhlet apparatus. About 800 ml methanol was added in the vessel and extracted by a Soxhlet apparatus at 60°C. Then the methanol containing extracted constituents were filtered through cotton. The process was repeated at least three times in order to maximum extraction of the chemical constituents from the sample. Finally, total filtrate was completely dried using a rotary evaporator in vacuum at a temperature of 45 °C and obtained dried crude extract which were used for investigation. The crude extract was preserved in the refrigerator until their experiment.

CHAPTER 3

EXPERIMENTAL DESIGN

3.1 Material and Methods

3.1.1 Materials

The general laboratory equipment is given in the following lists Table (3.1)

SN	Equipment
1	Electronic balance
2	Soxhlet apparatus
3	Rotary evaporator
4	Refrigerator
5	Heating Mantle
6	UV-Visible Spectrophotometer

Table (2.1): List of general laboratory equipment



Figure 2.1: Electronic balance



Figure 2.2: Soxhlet apparatus

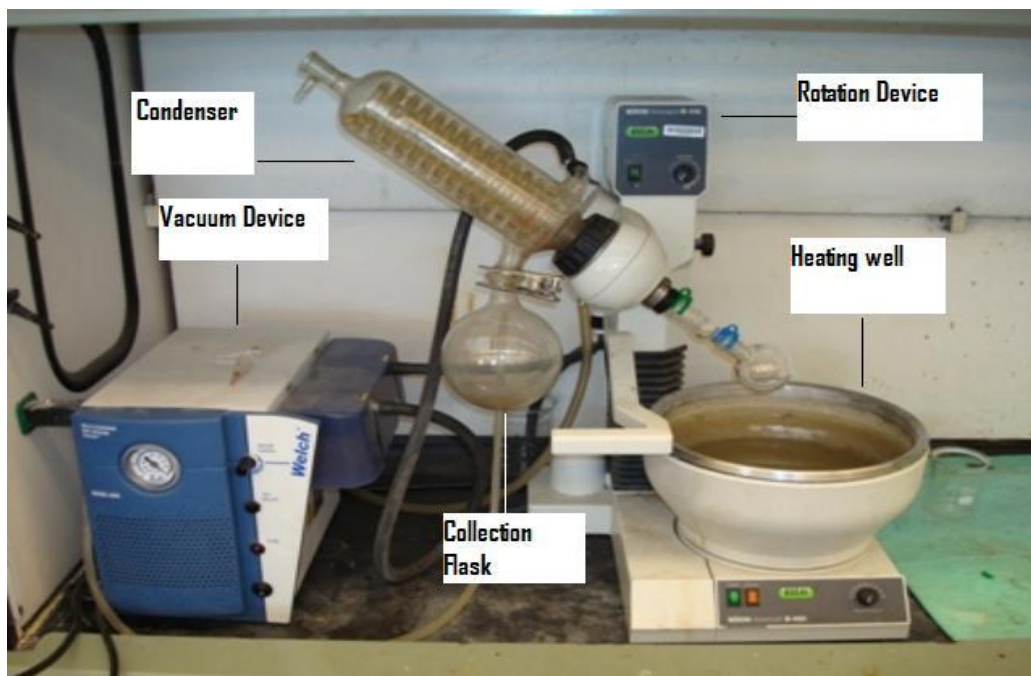


Figure 2.3: Rotary evaporator



Figure 2.4: Heating mantle



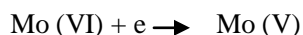
Figure 2.5: UV-Visible Spectrophotometer

3.1.2 Method

3.1.2.1 Determination of Total Phenolic Content

Principle:

The content of total phenolic compounds in plant methanolic extracts was determined by Folin–Ciocalteu Reagent (FCR) (Velioglu et al., 1998). The FCR actually measures a sample’s reducing capacity. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates - molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly (PMoW₁₁O₄₀)⁴⁻. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI):



Reagents:

Name of the Reagents	Source
Folin – ciocalteu reagent	Merck specialities private limited, India
Sodium carbonate (Na ₂ CO ₃)	E. Merck (India) limited
Ethanol or Methanol	Merck, Germany
Gallic acid (Analytical or Reagent grade)	Sigma Chemicals, USA

Table 2.2: List of the reagents used in the test and their source

Experimental procedure:

1. 0.5 ml of 1 mg/ml plant extract or standard of different concentration solution in a test tube was taken.
2. 0.5 ml of Folin – ciocalteu reagent solution into the test tube was taken.
3. After 5 minutes added 5 ml of Sodium carbonate (7% w/v) solution into the test tube followed by 6.5 ml deionized distilled water and mixed thoroughly.
4. Incubated the test tube for 90 minutes at 230 °C to complete the reaction.

5. Then the absorbance of the solution was measured at 765 nm using a spectrophotometer against blank.
6. A typical blank solution contained ethanol.
7. The Total content of phenolic compounds in plant methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula equation

$$C = (c \times V)/m$$

Where:

C = total content of phenolic compounds, mg/g plant extract, in GAE;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

3.1.2.2 Determination of Flavonoid Contents

Total flavonoids content was determined using the method described by Wang et al., 2000.

Reagents:

Name of the Reagents	Source
Aluminium Chloride (AlCl ₃)	Fine Chemicals, India
Potassium Acetate	E. Merck (India) limited
Ethanol or Methanol	Merck, Germany
Quercetin (Analytical or Reagent grade)	Sigma Chemicals, USA

Table 2.3: List of the reagents used in the test and their source

Experimental procedure:

1. 1 ml of plant extract or standard of different concentration solution in a test tube was taken.
2. 3 ml of methanol into the test tube was added.
3. 200 µl of 10% aluminium chloride solution into the test tube was added.

4. 200 µl of 1M potassium acetate solution into the test tube was added.
5. 5.6 ml of distilled water into the test tube was added.
6. Incubated the test tube for 30 minutes at room temperature to complete the reaction.
7. Then the absorbance of the solution was measured at 415 nm using a spectrophotometer against blank.
8. A typical blank solution contained methanol.
9. The Total content of flavonoid compounds in plant methanol extracts in quercetin equivalents was calculated by the following formula equation

$$C = (c \times V)/m$$

Where:

C = total content of flavonoid compounds, mg/g plant extract, in quercetin;

c = the concentration of quercetin established from the calibration curve, mg/ml;

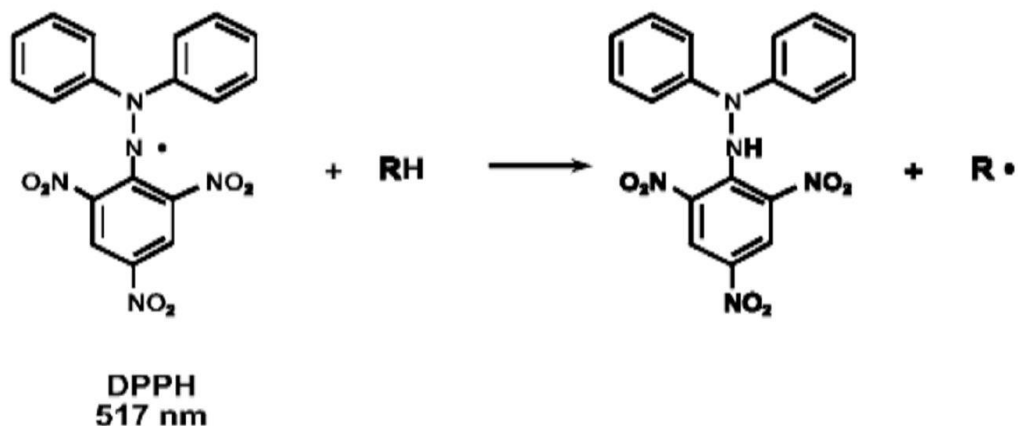
V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

3.1.2.3 DPPH free radical scavenging Assay

Principle:

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors. DPPH can make stable free radicals in aqueous or methanol solution. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. Resulting from a color change from purple to yellow the absorbance decreased when the DPPH was scavenged by an antioxidant, through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule (Brand-Willams et al., 1995).



Reagents:

Name of the Reagents	Source
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Sigma Chemicals, USA
Ethanol or Methanol	Merck, Germany
Ascorbic acid (Analytical or Reagent grade)	Merck, Germany

Table 2.4: List of the reagents used in the test and their source

Experimental procedure:

1. 400 µl of plant extract or standard of different concentration solution in a test tube was taken.
2. 1.6 ml of reagent solution into the test tube was added.
3. Incubated the test tube for 30 minutes to complete the reaction.
4. Then the absorbance of the solution was measured at 517 nm using a spectrophotometer against blank.
5. A typical blank solution contained ethanol.
6. The percentage (%) inhibition activity was calculated from the following equation

$$\{(A_0 - A_1)/A_0\} \times 100$$

Where,

A0 is the absorbance of the control, and

A1 is the absorbance of the extract/standard.

7. Then % inhibitions were plotted against log concentration and from the graph IC₅₀ was calculated.

3.1.2.4 Reducing Power Capacity Assessment

Principle:

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe³⁺/ferricyanide complex to the ferrous form. Therefore, Fe²⁺ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Makoto, 1986).

Reagents:

Name of the Reagents	Source
Potassium ferricyanide [K ₃ Fe(CN) ₆]	Merck (India) Limited
Trichloro Acetic acid	Fine Chemicals, India
Ferric Chloride (FeCl ₃)	Fine Chemicals, India
Ascorbic acid (Analytical or Reagent grade)	Merck, Germany

Table 2.5: List of the reagents used in the test and their source

Experimental procedure:

1. 400 µl of plant extract or standard of different concentration solution was taken in a test tube.
2. 500 µl of Potassium ferricyanide [K₃Fe(CN)₆], 1% solution added into the test tube.
3. Incubated the test tube for 10 minutes at 50°C to complete the reaction.

4. 500 µl of Trichloro Acetic acid, 10% solution added into the test tube.
5. Centrifuged the total mixture at 3000 rpm for 10 min.
6. 1ml supernatant solution was withdrawn from the mixture and mix with 2.5 ml of distilled water.
7. 200 µl of Ferric chloride (FeCl₃), 0.1% solution was added.
8. Then the absorbance of the solution was measured at 700 nm using a spectrophotometer against blank.
9. A typical blank solution contained the same solution mixture without plant extract or standard and it was incubated under the same conditions as the rest of the samples solution.
10. Also take the absorbance of the blank solution was measured at 700 nm against the solvent used in solution preparation.
11. Increased absorbance of the reaction mixture indicated increase reducing power. The percentage (%) Reducing capacity was calculated from the following equation.

$$\{(A_m - A_b)/A_b\} \times 100$$

Where,

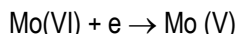
A_m is the absorbance of the reaction mixture, and

A_b is the absorbance of the blank.

3.1.2.5 Determination of Total Antioxidant Capacity

Principle:

The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol, and carotenoids. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acid pH. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI) and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm (Prieto et al., 1999).



Reagents used:

Name of the Reagents	Source
Concentrated H ₂ SO ₄ (98%)	E. Merck (India) limited
Sodium Phosphate (Na ₃ PO ₄)	Merck, Germany
Ammonium Molybdate	Merck, Germany
Ascorbic acid (Analytical or Reagent grade)	Merck, Germany

Table 2.6: List of the reagents used in the test and their source

Experimental procedure:

1. Take 300µl of plant extract or standard of different concentration solution in a test tube.
2. Add 3 ml of reagent solution into the test tube.
3. Incubate the test tube at 95⁰C for 90 minutes to complete the reaction.
4. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature.
5. A typical blank solution contained 3 ml of reagent solution and the appropriate volume (300µl) of the same solvent used for the sample, and it was incubated under the same conditions as the rest of the samples solution.
6. The antioxidant activity is expressed as the number of equivalents of ascorbic acid .and was calculated by the following formula equation

$$A = (c \times V)/m$$

Where:

A = total content of Antioxidant compounds, mg/g plant extract, in Ascorbic acid;

c = the concentration of Ascorbic acid established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant methanolic extract, g.

CHAPTER 4

RESULT

AND

DISCUSSION

4.1 Yield of extract

% Yield of the methanol extract of the plants of *Malva verticillata* is given bellow:

Extracts	Weight	% of the dried plants
Methanol extract	15 g	4.28

Table 3.1: % Yield of extract

4.2 Total Phenolic Content

Total phenolic contents was determined by using the Folin-Ciocalteu reagent and expressed as gallic acid equivalents (GAE) per gram of plant extract. The total phenolic contents of the methanol extracts of plants was calculated using the standard curve of gallic acid ($y = 0.004x - 0.013$; $R^2 = 0.986$). The total phenolic was found to be 139.75 ± 2.33 expressed as gallic acid equivalent (GAE).

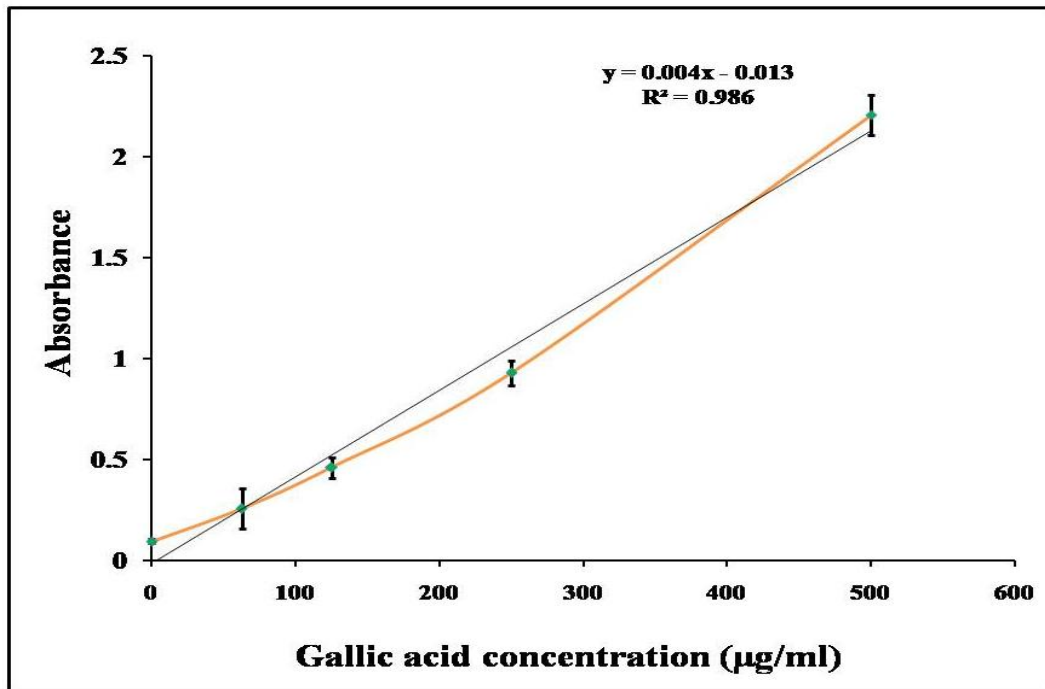


Figure 3.1: Standard curve using gallic acid for the measurement of total phenolic contents in the methanol extract of *Malva verticillata*.

Plant parts	Total phenol mg/g plant extract (in GAE)
Methanol extract	139.75 ± 2.33

Results are expressed as mean ± standard deviation of the duplicate experiments (n=2)

4.3 Total Flavonoid Content

Aluminium chloride colorimetric method was used to determine the total flavonoids content in the methanol extract of *Malva verticillata*. The total flavonoids contents of the methanol extract of *Malva verticillata* was calculated using the standard curve of quercetin ($y = 0.003x - 0.010$; $R^2 = 0.996$) and expressed as quercetin equivalents (QAE) per gram of the plant extract. The total flavonoids content was found to be 139.66 ± 3.56 mg/g expressed as quercetin equivalent (QE).

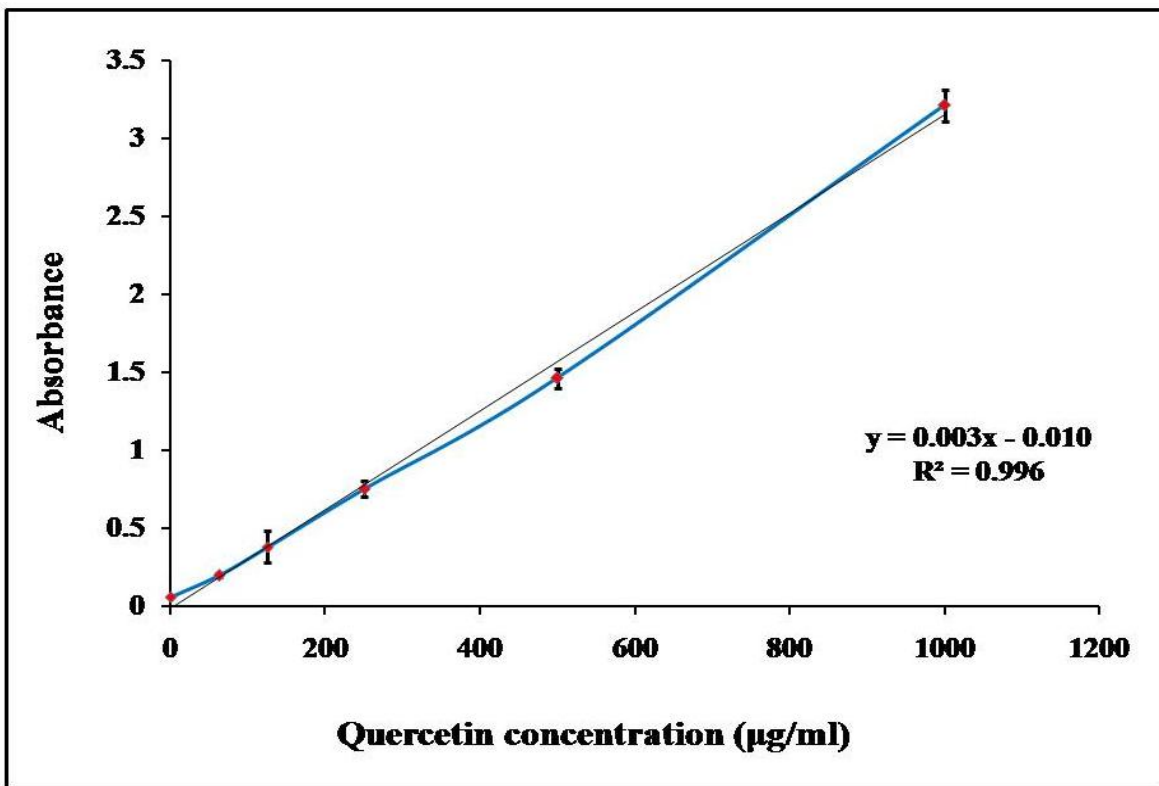


Figure 3.2: Standard curve using quercetin for the measurement of total flavonoid contents in the methanol extract of *Malva verticillata*.

Plant parts	Total flavonoids mg/g plant extract (in QE)
Methanol extract	139.66 ± 3.56

Results are expressed as mean ± standard deviation of the duplicate experiments (n=2)

4.4 DPPH Free Radical Scavenging Activity

The DPPH test is based on the exchange of hydrogen atoms between the antioxidant and the stable DPPH free radical. Practically, the reaction brings about the reduction of DPPH radicals to the corresponding hydrazine, which is manifested by a color change from violet to yellow, which is monitored spectrophotometrically. It is evident from the table that the % scavenging of DPPH radical was found to rise with increasing concentration of the samples. The positive control ascorbic acid of which IC₅₀ value is 173.83 ± 3.98 µg/ml. On the other hand, the methanol extract showed promising DPPH free radical scavenging activity with IC₅₀ value 202.55 ± 5.2 µg/ml.

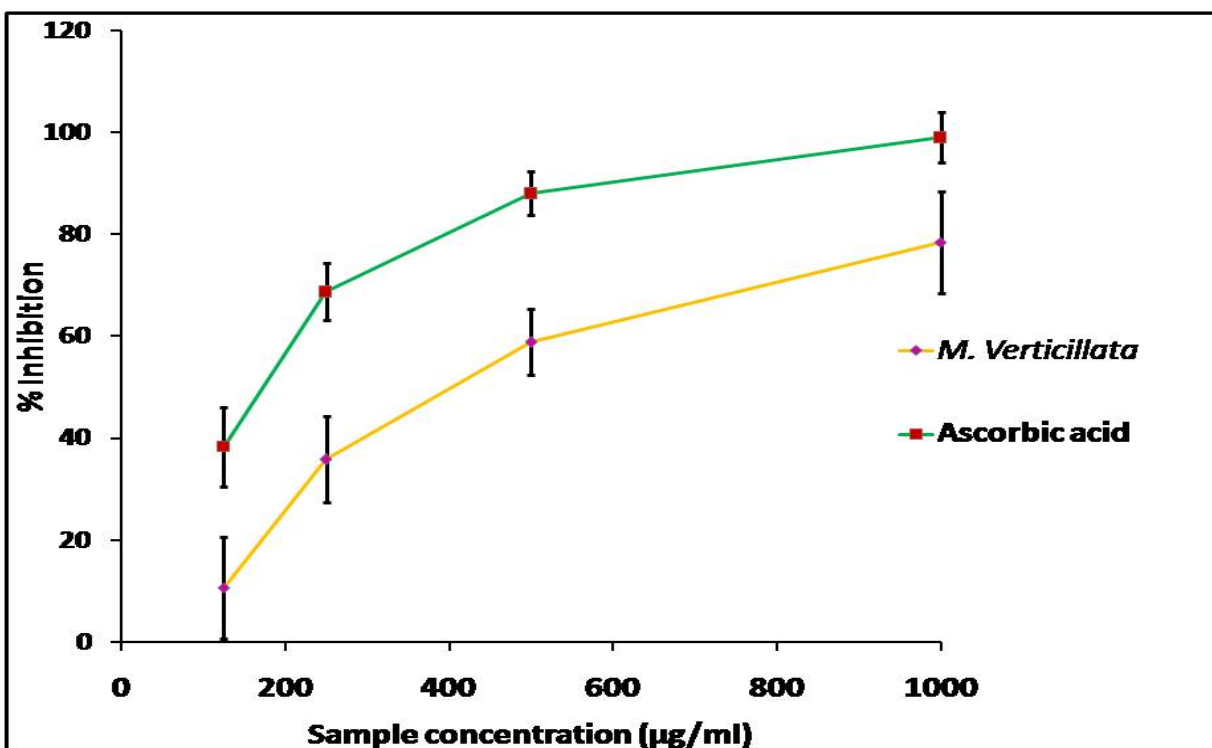


Figure 3.3: DPPH free radical scavenging activity of the methanol extract of *Malva verticillata* at different concentration. All values were expressed as the mean ± standard deviation of the duplicate experiments. Ascorbic acid was used as a positive control.

4.5 Reducing Power Assessment

The reductive capacity of the extracts were assessed using ferric to ferrous reductive activity as determined spectrophotometrically from the formation of Perl’s Prussian blue colored complex (Yildirim and Mavi, 2000). As shown in the figure 3.4, the absorbance value was increased with the increase of both sample and ascorbic acid concentration which implies the strength of ferric reducing power of the sample. Reducing power was found to increase with the increasing concentration of the methanol extract. Therefore, the methanol extract showed strong ferric reducing activity.

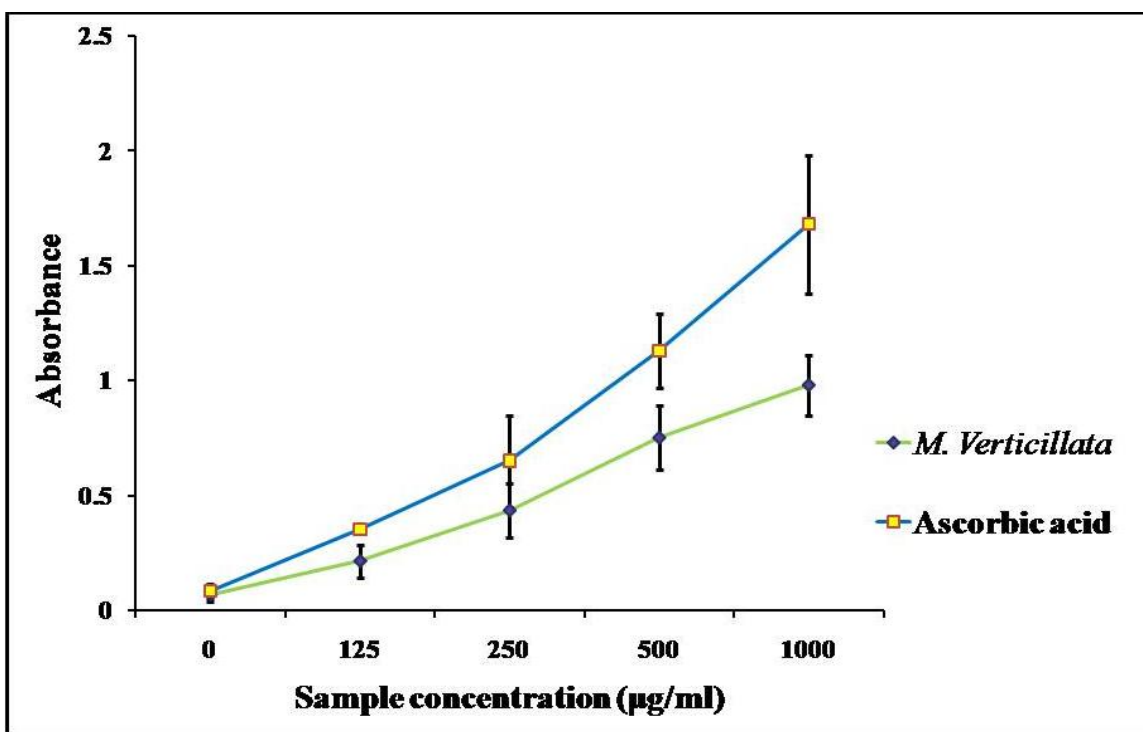


Figure 3.4: Reducing power of the methanol extract of *Malva verticillata* at different concentration. All values were expressed as the mean \pm standard deviation of the duplicate experiments. Ascorbic acid was used as positive control.

4.6 Total Antioxidant Capacity

Total antioxidant activity of the methanolic extracts of *Malva verticillata* was evaluated by the phosphomolybdenum method and expressed as ascorbic acid equivalent (AAE) per gram of plant extract. Total antioxidant contents were calculated using the standard curve of ascorbic acid ($y =$

$7 \times 10^{-5}x - 0.002$; $R^2 = 0.998$). The total antioxidant contents were found in the following order:
 224.28 ± 4.21 mg/g expressed as ascorbic acid equivalent (AAE).

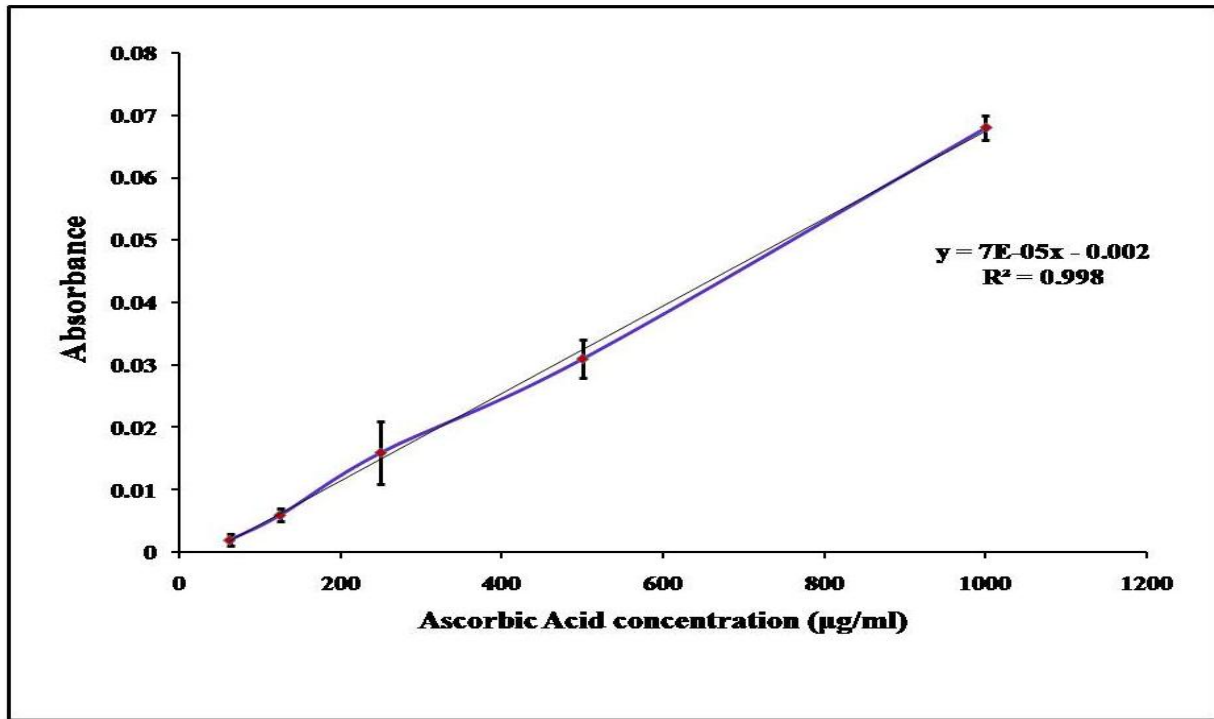


Figure 3.5: Standard curve using ascorbic acid for the measurement of total antioxidant in the methanol extract of *Malva verticillata*.

4.7 Discussion

It has been recognized that plant contains many natural substances. The phenolic compounds are widely distributed, sometimes present surprisingly high concentration, in plants and have an antioxidant activity. (Laporinic et al, 2005). The number of antioxidant compounds synthesized by plants as secondary products, mainly phenolics, serving in plant defense mechanisms to counteract ROS in order to survive, is currently estimated to be between 4000 and 6000 (Havsteen, 2002; Robards et al., 1999; Wollgast and Anklam, 2000). They have the ability to scavenge free radicals such reactive oxygen species (ROS) which are determined by their reactivity as hydrogen or electron donating agents (Fernandez Pachon et al, 2006). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Rice et al. 1995). It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Kessler et al., 2003, Cook and Samman, 1996).

Several techniques have been used to determine the antioxidant activity in vitro in order to allow rapid screening of substances since substances that have low anti oxidant activity in vitro, will probably show little activity in vivo (Nunes et al ., 2012) Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging there active oxygen species or protecting the antioxidant defense mechanisms (Umamaheswari et al., 2008).

Malva spp. is a genus of about 25–30 species of herbaceous annual, biennial, and perennial plants in the family Malvaceae (of which it is the type genus), one of several closely related genera in the family to bear the common English name mallow. The genus is widespread throughout the temperate, subtropical and tropical regions of Africa, Asia and Europe (Davis, 2010). It is demulcent, diuretic, emollient, galactagogue and laxative (Duke and Ayensu, 1985). *Malva verticillata* seeds are used in Tibetan medicine, where they are considered to have a sweet and astringent taste plus a heating potency. They are used in the treatment of renal disorders, the retention of fluids, frequent thirst and diarrhea (Tsarong and Tsewang, 1994).The root is used to cause vomiting in the treatment of whooping cough (Chopra et al., 1986). The leaves and stems are said to be digestive. They are given to women in the advanced stages of pregnancy (Chopra et al., 1986).However; the antioxidant activity of *Malva verticillata* extract was not previously studied. Despite its importance, only a few

studies have been conducted on the plant. Hence, the present investigation was aimed to investigate the antioxidant activity of the methanol extract of the whole plants using DPPH free radical scavenging assay and Ferric reducing assays. We have also investigated total phenolic contents, flavonoid contents and total antioxidant capacity of the methanol extract. In addition, % yield, total phenolic, total flavonoid contents and total antioxidant activity were also determined. The methanol extract showed strong DPPH free radical scavenging activity with an IC_{50} value of 202.55 ± 5.2 $\mu\text{g/ml}$ compared to the positive control ascorbic acid with an IC_{50} value of 173.83 ± 3.98 $\mu\text{g/ml}$. In addition the methanol extract also showed strong ferric reducing activity which is comparable to the potent antioxidant ascorbic acid. Besides, the phenolic content, total flavonoid content, and total antioxidant capacity of the methanol extract were found to be 139.75 ± 2.33 mg/g of dried extract (GAE), 139.66 ± 3.56 mg/g of dried extract (QE), and 224.28 ± 4.21 mg/g of dried extract (AAE). Therefore, it is anticipated that the large amount of phenolic and flavonoids typed compounds contained in the methanol extract played a strong role in antioxidant action of this extract.

Conclusion

In recent time natural antioxidants have attracted considerable attention to the nutritionist, food manufacturer and consumers due to their presumed safety and high therapeutic efficacy. In the present investigation, it can be concluded based on the results obtained from several experiments that the methanol extracts of *Malva verticillata* possesses antioxidant activity. All these activities may be attributed to the presence of polyphenolic compounds at high concentration in the plants. Therefore, *Malva verticillata* could be used as a source of naturally occurring potent antioxidants. However, it is very important to find out the specific chemical constituents responsible for potent antioxidant activity of *Malva verticillata*. The replacement of synthetic with natural antioxidants (because of implications for human health) may be advantageous. The results of in vivo studies suggest that methanolic extract of *Malva verticillata* may be useful in defense against renal disorders, the retention of fluids, frequent thirst and diarrhea (Tsarong and Tsewang, 1994), vomiting, whooping cough (Chopra et al., 1986), digestive due to its antioxidant properties.

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