

2017-11-16

Evaluation of Antioxidant Activity and Determination of Total Phenolic Content of leaves extract of *Heritiera littoralis* (Sundari tree)

Islam, Md. Aminul

Daffodil International University

<http://hdl.handle.net/20.500.11948/2255>

Downloaded from <http://dspace.library.daffodilvarsity.edu.bd>, Copyright Daffodil International University Library



Daffodil
International
University

Project On

Evaluation of Antioxidant Activity and Determination of Total Phenolic Content of leaves
extract of *Heritiera littoralis* (Sundari tree)

(This report presented in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy)

Supervised by

Md. Al-Faruk

Sr. Lecturer

Department of Pharmacy

Daffodil International University

Submitted by

Md. Aminul Islam

ID: 121-29-396

7th Batch, Section- B

Faculty of Allied Health Science

Department of Pharmacy

Daffodil International University

4/2 Sobhanbag, Dhanmondi, Mirpur Road

Dhaka, Bangladesh

Date of Submission:

12 May, 2016

APPROVAL

This Project, Evaluation of Antioxidant Activity and Determination of Total Phenolic Content of the leaves of *Heritiera littoralis* submitted by Md. Aminul Islam 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.

BOARD OF EXAMINERS

Head

Internal Examiner-1

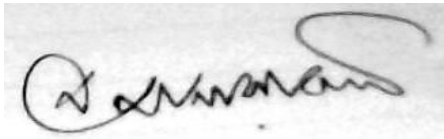
Internal Examiner-2

External Examiner

DECLARATION

I hereby declare that, this project report is done by me under the supervision of **Md. Al-Faruk**, Sr. Lecturer, 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.

Supervised By



Md. Al-Faruk

Sr. Lecturer

Department of Pharmacy

Faculty of Allied Health Science

Daffodil International University

Submitted By



Md. Aminul Islam

ID: 121-29-396

Department of Pharmacy

Daffodil International University

ACKNOWLEDGEMENT

At first I would like to thank the almighty Allah for giving me the opportunity and capability to complete this research. Then I would like to thank my parents for all the sacrifices that they have made on our behalf.

Then, I would like to express my deep thanks to our honorable supervisor, Md. Al-Faruk, Sr. Lecturer, Department of Pharmacy, Daffodil International University for his proper guidelines and suggestions to complete the research. I wish to convey my thanks and heartiest regard to him for providing important data and extended cooperation.

My Great thanks and appreciation goes for the Department Head Md. Ferdous Khan and all teachers of Pharmacy Department, Daffodil International University for their Kindness and great help whenever I needed.

I would like extend my thanks to the office staff of Department of Pharmacy, Daffodil International University. My special heartfelt thanks extend to all of my classmates and friends for their supportive help.

I would like to also extend my sincere gratitude to my parents and to all well-wisher for their wholehearted inspiration and open-ended support throughout the period of the project of the research work.

DEDICATION

***DEDICATED TO MY
PARENTS***

ABSTRACT

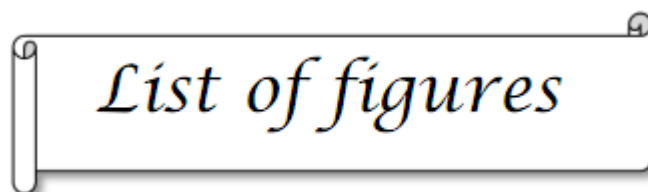
The present study has been designed to examine the antioxidant activity of the methanol extract of the Leaves of *Heritiera littoralis*. Antioxidant activity was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. In addition, total phenolic content was also determined. Total phenolic contents was expressed as gallic acid equivalent (GAE). The methanol extract showed moderate DPPH free radical scavenging activity with an IC_{50} value of 27.88 $\mu\text{g/ml}$ compared to the positive control ascorbic acid with an IC_{50} value of 7.27 $\mu\text{g/ml}$. Besides, the phenolic content of the methanol extract was found to be 68.21 mg/g of dried extract (GAE). Therefore, it is anticipated that the large amount of phenolic typed compounds contained in the methanol extract played a strong role in antioxidant action of this extract. Therefore, *Heritiera littoralis* 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 *Heritiera littoralis*.

List of contents

ABSTRACT		
CHAPTER: 01	INTRODUCTION	Page No.
1.1	Phytochemistry	2
1.2	Medicinal Plants	2
1.3	Herbal medicine	3
1.3.1	History of Herbal medicine	3
1.3.2	Modern herbal medicine	3-4
1.3.3	Clinical Test of Herbal medicine	4
1.3.4	Prevalence of use of Herbal medicine	4
1.3.5	Traditional herbal medicine systems	4
1.4	Antioxidant	5-6
1.4.1	Free radicals and their scavengers	6-7
1.4.2	Reactive Oxygen Species (ROS)	7
1.4.3	Antioxidant protection system	8-9
1.4.4	Benefit of Antioxidant	9-10
1.4.5	Clinical application of antioxidant	10-11
1.4.6	Other antioxidant	11
1.4.6.1	Dietary antioxidant	11

1.4.6.2	Phytonutrients	11
1.4.7	Antioxidant activity over diseases	12
1.4.7.1	Activity over lipid peroxidation and skin diseases	12
1.4.7.2	Activity over cardiovascular disease	13
1.5	The Plant Family	13
1.6	Introduction of <i>Heritiera littoralis</i>	13
1.6.1	Taxonomic Hierarchy	13
1.6.2	Characteristics	14
1.6.3	Distribution	14
1.6.4	Uses	15
1.6.5	Photograph of plant	16-17
1.7	Aim of the Study	18
CHAPTER 02: EXPERIMENTAL		
2.1	Experiment Plant	20
2.2	Preparation of the Plant Extracts for Experiments	20
2.2.1	Collection and Identification	20
2.2.2	Drying of the Samples	20
2.2.3	Extraction of the Dried plants	20
CHAPTER 03: EXPERIMENTAL DESIGN		
3.1	Material and Methods	22
3.1.1	Materials	22-23

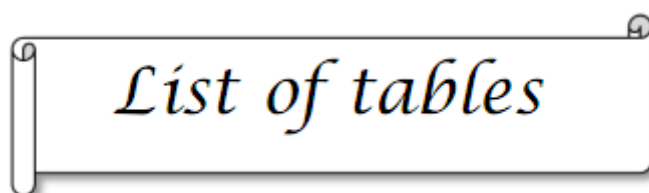
3.1.2	Method	24
3.1.2.1	Determination of Total Phenolic Content	24-25
3.1.2.2	DPPH free radical scavenging Assay	25-27
CHAPTER 4: RESULT AND DISCUSSION		
4.1	Total Phenolic Content	29
4.2	DPPH Free Radical Scavenging Activity	30-32
4.3	Discussion	32-33
	Conclusion	34
	References	35-38



List of figures

Figure No	Titles	Page No.
1.1	Model of the antioxidant metabolite glutathione	5
1.2.1	Heritiera littoralis	16
1.2.2	Heritiera littoralis leaves	16
1.2.3	Leaves (lower surface) of Heritiera littoralis	17
1.2.4	Flower of Heritiera littoralis	17
1.2.5	Fruits of Heritiera littoralis	17

2.1	Electronic balance	22
2.2	Soxhlet apparatus	22
2.3	Rotary evaporator	23
2.4	Heating mantle	23
2.5	UV-Visible Spectrophotometer	23
2.6	Schematic representation of the total phenolic content determination	25
2.7	Scavenging activity of DPPH	25
2.8	Schematic representation of the DPPH free radical scavenging assay	27
3.1	Standard curve using gallic acid for the measurement of total phenolic contents in the methanol extract of <i>Heritiera littoralis</i>	29
3.2	DPPH free radical scavenging activity of Ascorbic acid (ASA)	30
3.3	DPPH free radical scavenging activity of the methanol extract of <i>Heritiera littoralis</i> .	31
3.4	Comparative DPPH free radical scavenging activity between ascorbic acid and methanolic leaves extract of <i>Heritiera littoralis</i>	32



List of tables

Table No.	Titles	Page No.
1.1	Various ROS and corresponding neutralizing antioxidants	9

2.1	List of general laboratory equipment	22
2.2	List of the reagents used in the test and their source	24
2.3	List of the reagents used in the test and their source	26
3.1	Standard curve preparation by using absorbance of gallic acid	29
3.2	IC ₅₀ value of Ascorbic acid	30
3.3	IC ₅₀ value of Sample	31

Abbreviations

Abbreviate	Meaning
WHO	World Health Organization
CAM	Complementary and Alternative Medicine
ORAC	Oxygen Radical Absorbance Capacity
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
CAT	Chloramphenicol Acetyl Transferase
ADP	Adenosine Di-nucleotide Phosphate
NADPH	<u>Nicotinamide Adenine Dinucleotide Phosphate</u>
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
GSH	Glutathione sulfhydryl group
UV	Ultra Violet
LDL	Low Density Lipoproteins
DNA	Deoxyribo Nucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
FCR	Folin– Ciocalteu Reagent
GAE	Gallic Acid 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.

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.

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.

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.

1.4 Antioxidant

All aerobic organisms have antioxidant defense systems to offset harmful effects caused by free radicals. In the case of failure of the antioxidant defense system, antioxidants need to be supplemented from outside sources. Antioxidants can be found naturally in foods (Kedare and Singh, 2011). A majority of antioxidants naturally present in foods occur in phenolic structures and especially in flavonoid structures. In addition, antioxidants are added to nutrients to prevent deterioration in their taste, smell, and color. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) can be included in this group, which are known as synthetic antioxidants. The high cost of natural antioxidants has led to the use of synthetic antioxidants. However, studies conducted subsequently have demonstrated that synthetic antioxidants have toxic effects and, consequently, restrictions have been imposed on their use. Therefore, researchers have focused their studies on plant-derived natural antioxidants.

Many herbal plants contains antioxidant compounds which protects cells against degenerative effects of Reactive Oxygen Species (ROS) which is a free radical such as singlet oxygen, superoxide, peroxy, radicals, hydroxyl radicals. The concept of oxidative stress is that, when a balance between ROS production and antioxidant defenses is lost, 'oxidative stress' result which through a series of events deregulate the cellular function and leads to various diseases such as aging, arthritis, asthma, carcinogenesis, diabetes, rheumatism and various neuro degenerative disease.

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).

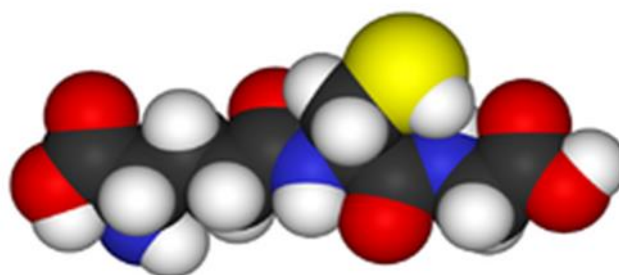


Figure (1.1): Model of the antioxidant metabolite glutathione. The yellow sphere is the redox-active sulfur atom that provides antioxidant activity, while the red, blue, white, and dark grey spheres represent oxygen, nitrogen, hydrogen, and carbon atoms, respectively.

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 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).

The natural antioxidants may have free-radical scavengers, reducing agents, quenches of singlet oxygen etc. The antioxidants can interfere with the oxidation process by reacting with free radicals. Recently interest has increased considerably in finding natural occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity. Antioxidants principles from natural resources possess multi affectedness in their multitude and magnitude of activity and provide enormous scope in correcting imbalance. Food industry uses natural antioxidants as a replacement of conventional synthetic antioxidants.

1.4.2 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.

1.4.3 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:

- i. 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).

1.4.4 Benefits of Antioxidants

- i. In different parts of the body
There are a wide range of antioxidants found in nature, and because they are so varied, different antioxidants provide benefits to different parts of the body. For example, beta-carotene (and other carotenoids) is very beneficial to eye health; lycopene is beneficial for helping maintain prostate health; flavonoids are especially beneficial for heart health; and proanthocyanidins are beneficial for urinary tract health.
- ii. In skin health
When skin is exposed to high levels of ultraviolet light, photo-oxidative damage is induced by the formation of different types of reactive species of oxygen, including singlet oxygen, superoxide radicals, and peroxide radicals. These forms of reactive oxygen damage cellular lipids, proteins, and DNA, and they are considered to be the primary contributors to erythema (sunburn), premature aging of the skin, photodermatoses, and skin cancers. Astaxanthin, followed by beta-carotene combined with vitamin E has been shown to be one

of the most powerful antioxidant combinations for helping protect the skin from reactive species of oxygen.

iii. Immune system support

Singlet oxygen can compromise the immune system, because it has the ability to catalyze production of free radicals. Astaxanthin and Spirulina have been shown to enhance both the non-specific and specific immune system, and to protect cell membranes and cellular DNA from mutation. Astaxanthin is the single most powerful quencher of singlet oxygen, and is up to ten times stronger than other carotenoids (including beta-carotene), and up to 500 times stronger than alpha tocopherol (Vitamin E), while Spirulina has a variety of antioxidants and other substances that are beneficial in boosting immunity.

1.4.5 Clinical applications of antioxidant

- i. 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.
- ii. 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.
- iii. Respiratory Diseases: Breathing of 100 % oxygen for more than 24 hour 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.
- iv. 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.

- v. **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.
- vi. **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.
- vii. **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 triggers inflammatory reaction, leading to the above symptoms. Certain plant products, called psoralens are administered in the treatment of psoriasis and leukoderma. When the drug is applied over the affected skin and then irradiated by UV light, singlet oxygen produced with clinical benefit.
- viii. **Cancer Treatment:** 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. Irradiation 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.

1.4.6 Other Antioxidants

1.4.6.1 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).

1.4.6.2 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).

1.4.7 Antioxidant activity over disease

1.4.7.1 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).

1.4.7.2 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).

1.5 The plant family

The plant under investigation- *Heritiera littoralis* includes to the family Malvaceae. The Malvaceae is a family of flowering plants estimated to contain 243 genera with 4225+ species. 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).

1.6 Introduction of *Heritiera littoralis*

Coastal people utilized mangrove plants for medicinal and other purpose over the years. Mangrove plants are specialized woody plants growing in the swamps of tidal-coastal areas and river deltas of tropical and subtropical parts of the world. *Heritiera littoralis* (family: Malvaceae) commonly known as Sundari (Bengali) is a preeminent mangrove plant occurring in the Sundarbans forest located in the southern part of Bangladesh and adjoining West Bengal province of India. This plant is also found in coastal regions of countries like Myanmar, Thailand, and Northern Malaysia. It is an evergreen medium to tall size tree, height ranges from 15 to 25 m. It has a buttressed stem and ancient, longitudinally splintered bark. The dark green leaves are grouped near the ends of the twigs and have short petioles. The species instigates producing pneumatophores at the age of 3 years. Regions with heavy annual rainfall of 1600 mm to 5334 mm and a warm equable climate of 7.22 °C to 37.78 °C are required to grow this species. It flowers in the month of March and April. Unisexual flowers are arranged in panicles. The fruit carpels are extended to 3.81 to 5.08 cm and fall to the land when they ripen in the month of July and August. The seeds ripen in June and July. The tree plays an important role as a source of timber and fuel wood from the Sundarbans. The plant (Leaves, roots and bark) also has applications in traditional folk medicine as evidenced by its extensive use for treating diabetes, hepatic disorders, gastrointestinal disorders, goiter, and skin diseases by the local people and traditional health practitioners. A number of investigations indicated that the plant possesses significant antioxidant, antinociceptive, antihyperglycemic, antimicrobial, and anticancer activities.

1.6.1 Taxonomic Hierarchy

Domain	:Eukaryota
Kingdom	:Plantae
Subkingdom	:Viridiplantae
Phylum	:Tracheophyta
Subphylum	:Euphyllophytina
Class	:Magnoliopsida
Subclass	:Dilleniidae
Superorder	:Malvanae
Order	:Malvales
Family	:Malvaceae
Genus	:Heritiera
Species	:Heritiera littoralis

1.6.2 Characteristics

- i) Stem: A tree with conspicuous, branched, sinuous, plank buttresses.
- ii) Leaves: Leaves 5-12 x 3-6 cm, elliptic, upper surface green, lower surface shining with silvery scales, tapering at both ends.
- iii) Flower: Flowers are unisexual, in which each flower only has either the male or female sexual organ, dull purple, bell-shaped with 4–6 teeth, 6.3 mm wide, and in 5–18 cm.
- iv) Flower color: Pink, purple.
- v) Fruit: Fruiting carpel ellipsoid, about 5-10 x 3-6 cm, surface smooth, keeled on one side, but not winged. Seed surrounded by a fibrous pericarp about 5-10 mm thick.
- vi) Fruit color: Brown, Purple.
- vii) Fruit type: Indehiscent dry fruit.
- viii) Seeds: seeds are oblong-ellipsoid, flattened, brown, and about 3 cm long.
- ix) Root: Roots with numerous woody peg-like pneumatophores.

1.6.3 Distribution

Heritiera littoralis is native to coastal regions of the Indo-Pacific, its range extending from the east coast of India through Bangladesh and Malaysia to Myanmar and Thailand. Compared to other species of mangrove, it grows in less saline environments and on drier ground that gets inundated by the tide only infrequently. It thrives on clayey soils and is the dominant species in these habitats, typically growing on the low banks that form around the edges of saucer-shaped, newly-emerged islands. It is the dominant mangrove species in the area and its local name 'Sundari' gives the Sundarbans region its name.

1.6.4 Uses

i. Edible uses

The seed is occasionally eaten and has been used as a substitute for cola nuts. The seed oil is characterized by high contents of the cyclopropenoic acids malvalic acid (54%) and sterculic acid (12.5%).

ii. Medicinal uses

A root decoction is used to treat mouth infection and toothache. Fruits and seed extract is used to treat diarrhea and dysentery. The bark of *H. littoralis* is rich in procyanidins. The ethanol extract has been shown to have antioxidant properties. It also shows antimicrobial activities against *Kocuria rhizophila*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* and is non-toxic in brine shrimp toxicity tests.

iii. Other uses

The bark contains 12 - 15% tannin on a dry weight basis. It is used for toughening nets. The seed also contains tannins. The twigs are used as toothbrushes. The poisonous activity of the roots to fish is due to the presence of sesquiterpenoids such as heritonin and vallapin. The latter compound has also shown activity against boll weevils.

The heartwood is reddish brown or dark brown, often with a chocolate or purple tinge. The wood is hard, strong and durable, the grain interlocked, texture fine and even. It often smells like leather. The timber is difficult to season, being subject to considerable end splitting and surface checking. It rapidly blunts edged tools due to the presence of silica, but turns fairly well and takes a good finish. The wood is moderately durable when exposed to the weather or in contact with the ground; a life of 3 years in contact with the ground under tropical conditions is probably as much as can be expected. In durability tests in Tanzania, fungi showed a particularly high affinity for *Heritiera littoralis* wood. The wood is not susceptible to powder-post beetles, and is reported to be resistant to marine borers, but not always to termites. It is probably difficult to impregnate with preservative because gum-like deposits are present. It is used for ships' masts when sufficiently straight and long, as well as for house posts, joists, wheel hubs, boat ribs, furniture, rice pounders and other domestic articles. It is recommended for steamed bent work. The wood pulp is suitable for the production of wrapping, writing and printing paper. It is an excellent firewood, having a high energy value.

1.6.5 Photograph of plant



Figure (1.2.1): *Heritiera littoralis*



Figure (1.2.2): *Heritiera littoralis* leaves



Figure (1.2.3): Leaves (lower surface) of *Heritiera littoralis*



Figure (1.2.4): Flower of *Heritiera littoralis*



Figure (1.2.5): Fruits of *Heritiera littoralis*

1.7 Aim of the study

Heritiera littoralis is used in gastrointestinal disorders including diarrhea, dysentery, constipation, indigestion, and stomachache. It is also recommended for skin diseases including dermatitis, rash, eczema, boils, itch, scabies, sores, infections, and hepatic disorders including jaundice, hepatitis. It is also useful for treating diabetes and goiter. It is a good insect repellent and has wound healing activity. However, the antioxidant activity of leaves extract of *Heritiera littoralis* was not previously studied. Hence, the present investigation was aimed to investigate the antioxidant activity of the methanol extract of the plants using DPPH free radical scavenging assay. We have also investigated total phenolic contents of the methanol extract of leaves of *Heritiera littoralis*.

CHAPTER 2: EXPERIMENTAL

2.1 Experimental plant

Heritiera littoralis included in malvaceae was investigated in the study.

Plant Name	Family	Plant part used
<i>Heritiera littoralis</i>	Malvaceae	Leaves

2.2 Preparation of the Plant Extracts for Experiments

2.2.1 Collection and Identification

For this present investigation the leaves of *Heritiera littoralis* were collected from the Sundarban. The leaves plant parts were then sent to National Herbarium Mirpur, Dhaka. Expert of National herberium identified as *Heritiera littoralis* where a voucher specimen has been deposited.

2.2.2 Drying of the Samples

After collection of the plants leaves 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 leaves were weighed and preserved in air tight container until their extraction.

2.2.3 Extraction of the dried plants

The leaves of *Heritiera littoralis* 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 (2.1)

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

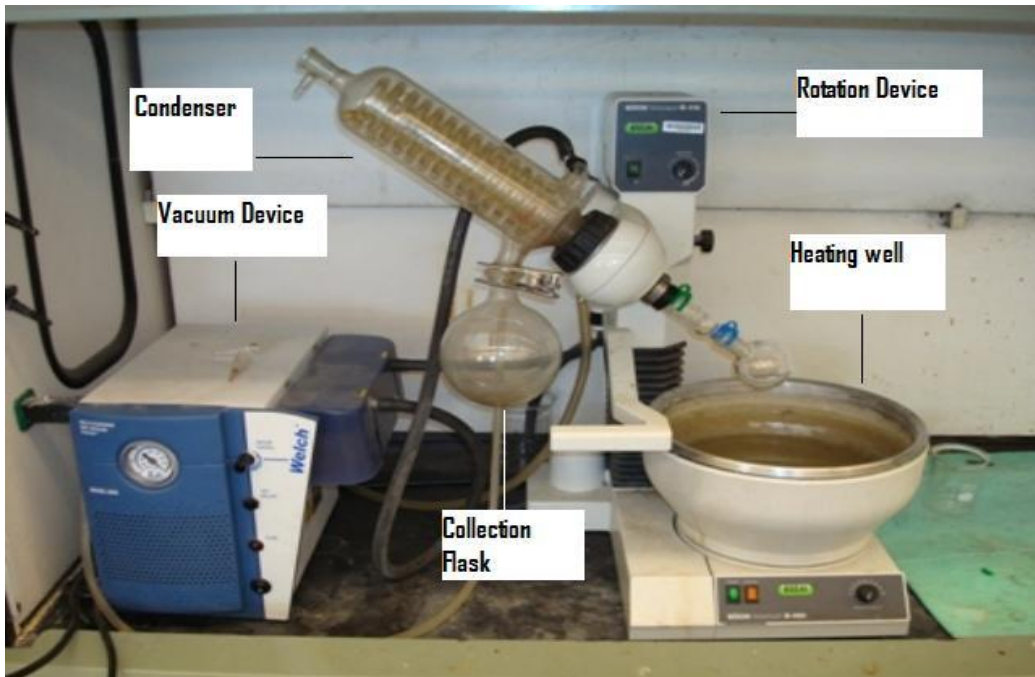


Figure (2.3): Rotary evaporator



Figure (2.4): Heating mantle



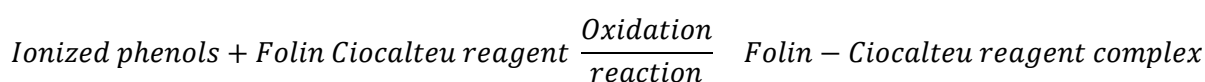
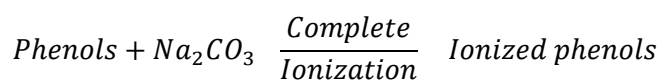
Figure (2.5): UV-Visible Spectrophotometer

3.1.2 Methods

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. When Folin-Ciocalteu reagent is used in this ionized phenolic solution the reagent will readily oxidize the phenols. Usual color of Folin-Ciocalteu reagent is yellow and after the oxidation process the solution becomes blue. The intensity of the color change is measured in a spectrophotometer at 760 nm. The absorbance value will reflect the total phenolic content of the compound (Harbertson J and Spayd S, 2006).



Reagent

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

Total Phenolic Compound Analysis

To 0.5 ml of extract solution (conc. 2 mg/ml), 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of Na₂CO₃ (7.5 % w/v) solution were added. The mixture was incubated for 20 minutes at room temperature. After 20 minutes the absorbance was measured at 760 nm by UV-spectrophotometer and using the standard curve prepared from gallic acid solution with different concentration, the total phenols content of the sample was measured. The phenolic contents of the sample were expressed as mg of GAE (gallic acid equivalent) / gm of the extract.

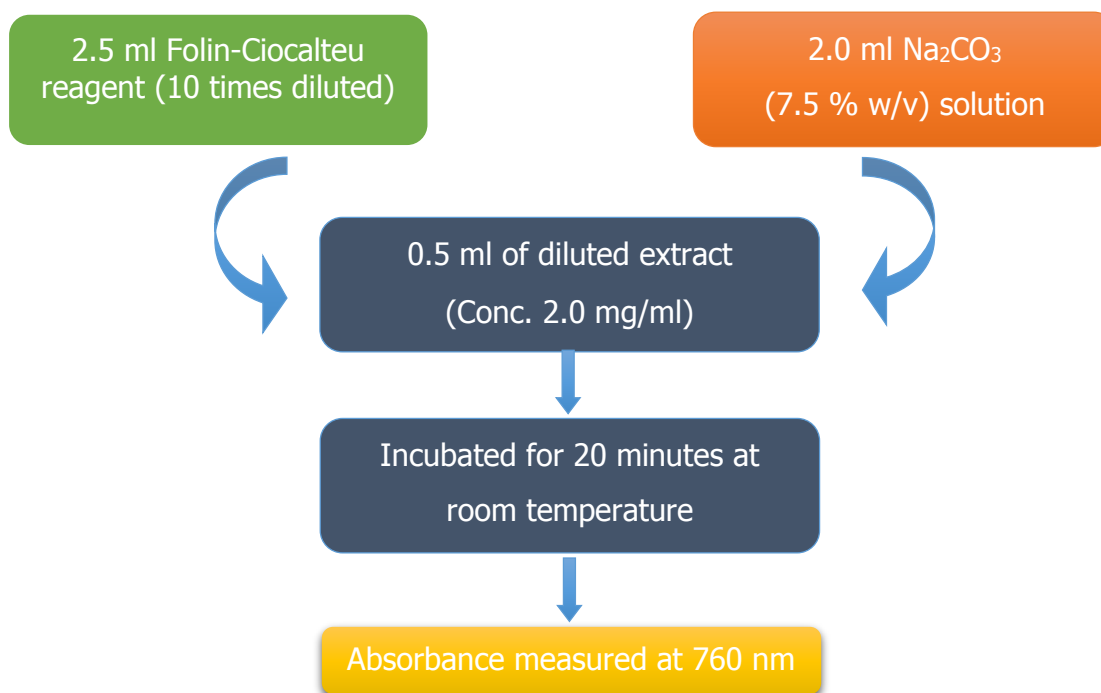


Figure (2.6): Schematic representation of the total phenolic content determination

3.1.2.2 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-Williams et al., 1995).

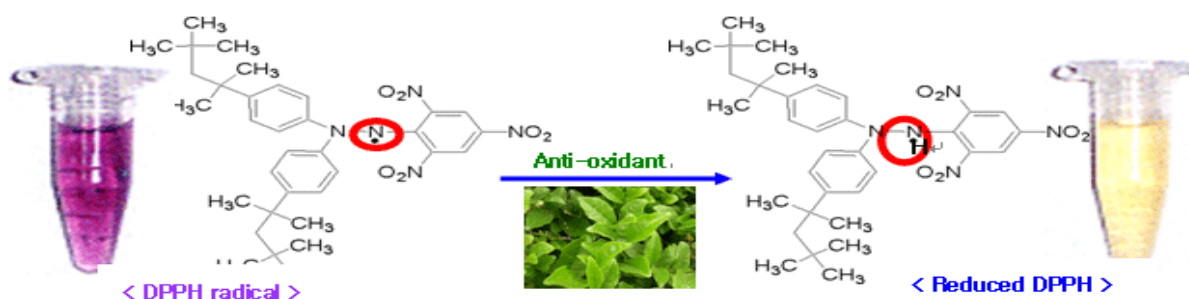


Figure (2.7): Scavenging activity of DPPH

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

Preparation of solution

At first 10 mg extract of *H. littoralis* was measured by electronic balance and mixed with 10 ml of methanol (99-100%) to prepare 1000 µg/ml solution of extract as stock solution.

Another ten different concentrations of solutions were prepared by proper dilution method. These concentrations were 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml, 3.90 µg/ml, 1.95 µg/ml, 0.97 µg/ml. The following technique was followed to prepare different concentrations of solutions from stock solution:

$$\begin{aligned} & \text{Volume which we have to take from stock solution} \\ &= \frac{\text{desired concentration}}{\text{supplied concentration}} * \text{desired volume} \end{aligned}$$

In the same way, various concentrations (500 µg/ml – 0.97 µg/ml) of ascorbic acid solutions were prepared.

2 mg DPPH powder was measured by electronic balance and mixed with 100 ml of methanol (99-100%) to prepare 20 µg/ml DPPH solution. It should be kept in cool, dry and dark place.

Assay of Free Radical Scavenging Activity

2.0 ml of a methanol solution of the sample (Control / extractives) at different concentration from 500.0 to 0.977 µg/ml were mixed with 3.0 ml of a DPPH methanol solution (20 µg/ml). After 30 minutes reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer.

Inhibition of free radical DPPH in percent (I %) was calculated as follows-

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

Where, A_0 is the absorbance of the control reaction (containing all reagents except the test material), and A_1 is the absorbance of the extract/standard. Extract/standard concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

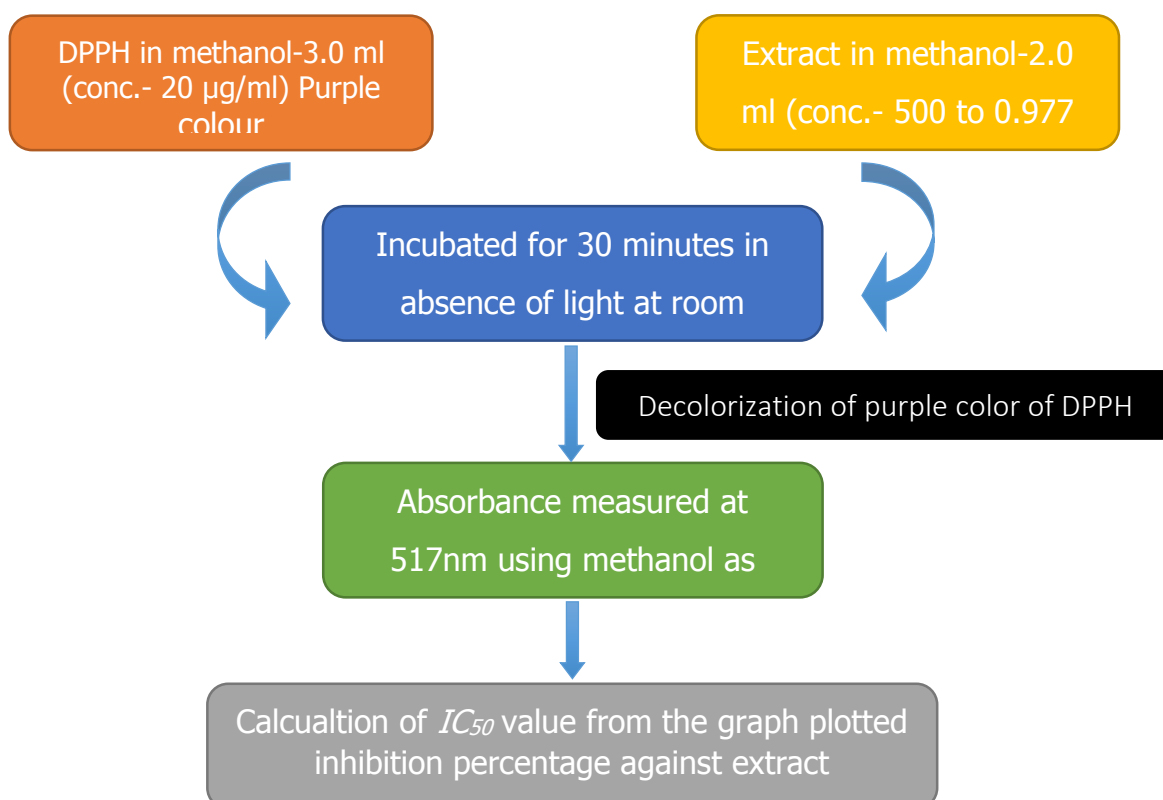


Figure 2.8: Schematic representation of the method of assaying free radical scavenging activity.

CHAPTER 4:
RESULT
AND
DISCUSSION

4.1 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 ($y = 0.0076x - 0.0064$; $R^2 = 0.9993$). The total phenolic was found to be 68.21 expressed as gallic acid equivalent (GAE).

SL. No.	Conc. of the standard ($\mu\text{g} / \text{ml}$)	Absorbance	Regression line	R^2
1	100	0.754	$y = 0.0076x - 0.0064$	0.9993
2	50	0.368		
3	25	0.172		
4	12.5	0.081		
5	6.25	0.035		
6	3.125	0.017		
7	1.5625	0.008		
8	0.78125	0.005		
9	0.3906	0.003		
10	0	0		

Table (3.1): Standard curve preparation by using absorbance of gallic acid

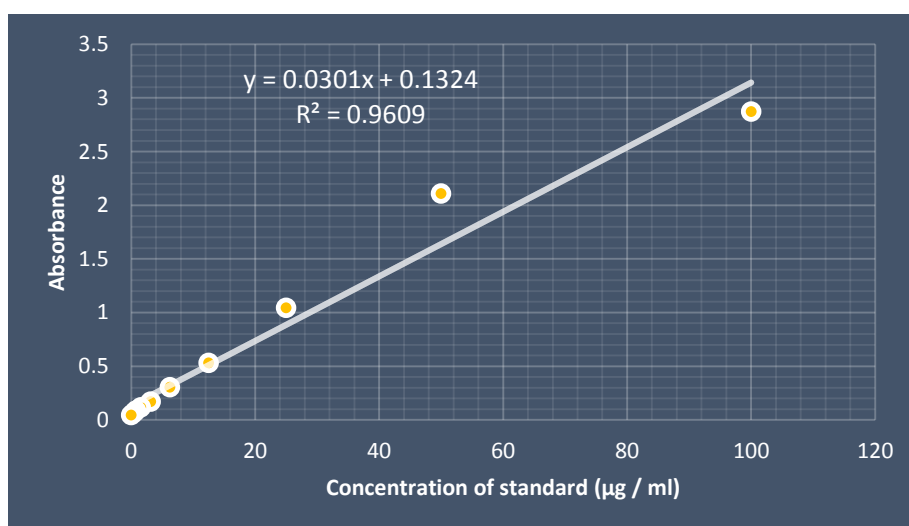


Figure (3.1): Standard curve of Gallic acid for total phenolic determination

Plant parts	Absorbance	Total phenol mg/g plant extract (in GAE)
Leaves of <i>Heritiera littoralis</i>	0.512	68.21

4.2 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 7.27 µg/ml. On the other hand, the methanol extract showed promising DPPH free radical scavenging activity with IC₅₀ value 27.88 µg/ml.

Absorbance of control	Conc. (µg/ml)	Absorbance of Ascorbic acid	Inhibition (%)	IC ₅₀ (µg/ml)
0.587	500	0.021	96.42	7.27
	250	0.039	93.35	
	125	0.054	90.80	
	62.5	0.098	83.30	
	31.25	0.125	78.70	
	15.625	0.194	66.95	
	7.813	0.282	51.95	
	3.906	0.365	37.81	
	1.953	0.445	24.19	
	0.977	0.570	2.89	

Table (3.2): IC₅₀ value of Ascorbic acid

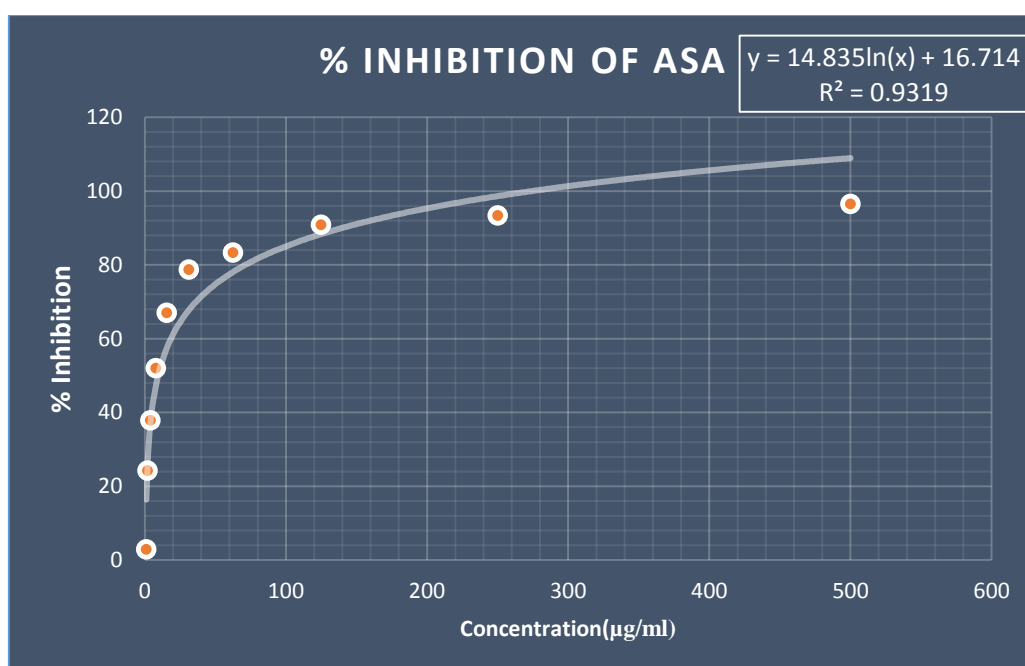


Figure (3.2) DPPH free radical scavenging activity of Ascorbic acid (ASA)

Absorbance of control	Conc. ($\mu\text{g/ml}$)	Absorbance of sample	Inhibition (%)	IC ₅₀ ($\mu\text{g/ml}$)
0.587	500	0.052	91.14	27.88
	250	0.098	83.30	
	125	0.147	74.95	
	62.5	0.210	64.22	
	31.25	0.275	53.15	
	15.625	0.361	38.50	
	7.813	0.419	28.62	
	3.906	0.478	18.56	
	1.953	0.534	9.02	
	0.977	0.585	0.34	

Table (3.3): IC₅₀ value of methanolic leaves extract of *Heritiera littoralis*

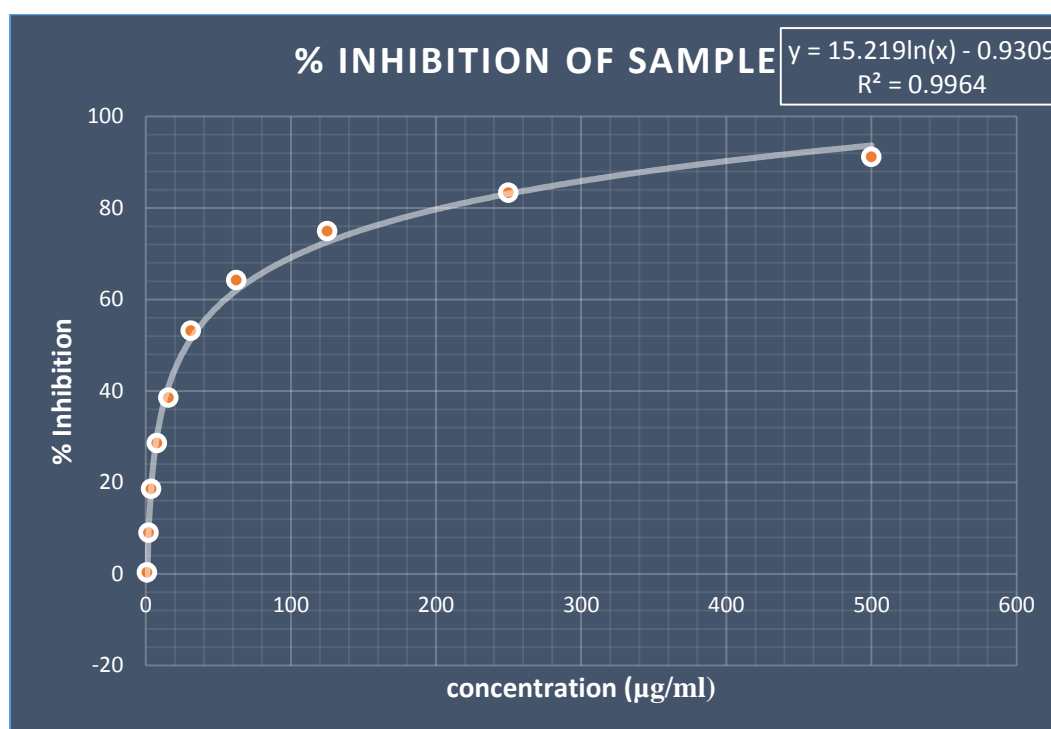


Figure (3.3): DPPH free radical scavenging activity of methanolic leaves extract of *Heritiera littoralis* at different concentration



Figure (3.4): Comparative DPPH free radical scavenging activity between ascorbic acid and methanolic leaves extract of *Heritiera littoralis*

4.3 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).

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 antioxidant 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).

Heritiera littoralis is an evergreen moderate size tree growing abundantly in Sundarbans. The trees attain up to 25 m in height; trunk is about 50 cm in diameter at the base is prominently buttressed. The young branches of the trees are covered with shining golden-brown scales. *Heritiera littoralis* is an important mangrove species having ethnomedicinal uses in traditional medicines. The people living beside the Sundarbans use this plant extensively for treating various ailments. It is used in gastrointestinal disorders including diarrhea, dysentery, constipation, indigestion, and stomachache. It is also recommended for skin diseases including dermatitis, rash, eczema, boils, itch, scabies, sores, infections, and hepatic disorders including jaundice, hepatitis. It is also useful for treating diabetes and goiter. It is a good insect repellent and has wound healing activity.

However; the antioxidant activity of *Heritiera littoralis* leaves 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 plant leaves using DPPH free radical scavenging assay. We have also investigated total phenolic contents of the methanol extract.

The present study has been designed to examine the antioxidant activity of the methanol extract of the leaves part of *Heritiera littoralis*. Antioxidant activity was determined by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. In addition, total phenolic contents was also determined. The methanol extract showed moderate DPPH free radical scavenging activity with an IC₅₀ value of 27.88 µg/ml compared to the positive control ascorbic acid with an IC₅₀ value of 7.27 µg/ml. In addition, the total phenolic content of the methanol extract were found to be 68.21 mg/g of dried extract (GAE). Therefore, it is anticipated that the large amount of phenolic typed compounds contained in the methanol extract played a strong role in antioxidant action of this extract.

Conclusion

Antioxidants have been widely used in the food industry to prolong shelf life. However, there is a widespread agreement that some synthetic antioxidants such as butyl hydroxyl anisole and butyl hydroxyl toluene (BHA and BHT, respectively) need to be replaced with natural antioxidants because of their potential health risks and toxicity. Thus, the search for antioxidants from natural resources has received much attention, and efforts have been made to identify new natural resources for active antioxidant compounds. Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals and inhibiting peroxidation. Antioxidants that scavenge free radicals play an important role in prevention of cardiovascular disease, aging, cancer, and inflammatory disorders. Recent researches have shown that the antioxidants of plant origin with free-radical scavenging properties could have great importance as therapeutic agents in several diseases caused due to oxidative stress. Plant extracts and phytoconstituents found effective as radical scavengers and inhibitors of lipid peroxidation. Many synthetic antioxidant compounds have shown toxic and/or mutagenic effects, which have stimulated the interest of many investigators to search natural antioxidant.

In the present investigation, it can be concluded based on the results obtained from several experiments that the methanol extracts of *Heritiera littoralis* leaves displayed a very free radical scavenging activity in the DPPH assay ($IC_{50} = 27.88$ approx. $\mu\text{g/ml}$) which is comparable to that of ascorbic acid ($IC_{50} = 7.27$ approx. $\mu\text{g/ml}$), a well-known standard antioxidant. All these activities may be attributed to the presence of polyphenolic compounds at high concentration in the plants. Therefore, *Heritiera littoralis* leaves 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 *Heritiera littoralis*. The replacement of synthetic with natural antioxidants (because of implications for human health) may be advantageous.

REFERENCES

- ❖ Ackerknecht, Erwin (1982). *A Short History of Medicine*. JHU Press, 39. ISBN 978-0-8018-2726-6.
- ❖ Hong, Francis (2004). "History of Medicine in China" (PDF). *McGill Journal of Medicine* 8 (1): 7984.
- ❖ Jacob R.A., (1995). The integrated antioxidant system, *Nutrition Research*, 15: 755-766.
- ❖ Prakash A., (2001), Antioxidant Activity, Analytical progress report of Medallion Laboratories, 19 (2): 1-2.
- ❖ Badami S., Gupta M. K., Suresh B., (2003), Antioxidant activity of the ethanolic extract of *Striga orobanchioides*. *Journal of Ethnopharmacology* Volume 85, Issues 2-3, 227-230.
- ❖ Helle Wangenstein, Huong Cam Thi Dang, Shaikh Jamal Uddin, Mahiuddin Alamgir and Karl Egil Malterud. (2009), Antioxidant and Antimicrobial Effects of the Mangrove Tree *Heritiera fomes*.
- ❖ Chitra K.P., K.S.Pillai. (2002) "Antioxidants in Health". *Ind. J. Physiol. Pharmacol.*, 46 (1): 01-05).
- ❖ Ariga T. (2004). The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors*, 21, 197-201.
- ❖ B. Halliwell, J. Gutteridge (Eds.), (1999) *Free Radicals in Biology and Medicine*, Oxford University Press, New York, 105–245.
- ❖ Knight JA (1998). "Free radicals: their history and current status in aging and disease". *Annals of Clinical and Laboratory Science* 28 (6): 331–46. PMID 9846200.
- ❖ Tomlinson PB. (1986). *The Botany of Mangroves*. Cambridge University Press, Cambridge.
- ❖ Bandaranayake WM. (1998) Traditional and medicinal uses of mangroves. *Mangroves and Salt Marshes*, 2, 133-148.
- ❖ Rahman LM. (2000) The Sundarbans: A unique wilderness of the world. *USDA Forest Service Proceedings RMRS*, 2, 143-148.
- ❖ Majumdar SG, Patra G. (1978) Chemical investigation on some Mangrove species. Part IV. *Heritiera minor*, a typical Mangrove species. *Journal of the Indian Chemical Society*, 55, 414-415.
- ❖ Uday Bandyopudya, et al. 1999 "ROS: oxidative damage and pathogenesis". *Curr. Sci.*, 77: 658-666.

- ❖ "Sundari". Banglapedia. (24 February 2015), retrieved from <http://en.banglapedia.org/index.php?title=Sundari>
- ❖ "Angiosperm Phylogeny Website". Retrieved 15 July 2014.
- ❖ Vanden Berghe, Edward (2014). "Heritiera fomes Buch.-Ham.". World Register of Marine Species. Retrieved 2015-02-22.
- ❖ Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995) Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft and Technologie*, 28:25–30.
- ❖ D.M.Vasudevan et.al. Free radicals and antioxidants. Text book of Biochemistry, 24. 212-215 Jaypee Brothers Publications pvt. Ltd. New Delhi.
- ❖ "Herbalism." Wikipedia: The Free Encyclopedia. Wikimedia Foundation, Inc., (26 April 2016). Web. < <https://en.wikipedia.org/wiki/Herbalism> >
- ❖ "Phytochemistry." Wikipedia: The Free Encyclopedia. Wikimedia Foundation, Inc., (11 January 2016), Web. <<http://en.wikipedia.org/wiki/Phytochemistry>>
- ❖ "*Heritiera littoralis*" Wikipedia: The Free Encyclopedia. Wikimedia Foundation, Inc., (13 April 2016, Web). <https://en.wikipedia.org/wiki/Heritiera_littoralis>
- ❖ Dungun, *Heritiera littoralis* Family Malvaceae (13 January 2016) retrieved from <http://www.wildsingapore.com/wildfacts/plants/mangrove/heritiera/heritiera.htm>
- ❖ *Heritiera littoralis* Aiton, retrieved from <https://florafaunaweb.nparks.gov.sg/special-pages/plant-detail.aspx?id=2952>
- ❖ *Heritiera littoralis* , (1 april 2016), retrieved from <http://tropical.theferns.info/viewtropical.php?id=Heritiera+littoralis>
- ❖ Aurelia Magdalena Pisoschi and Gheorghe Petre Negulescu, Methods for Total Antioxidant Activity Determination: A Review
- ❖ Biju John, Sulaiman, SateeshGeorge AND V R K Reddy. (2013), Total Phenolics and Flavonoids in selected medicinal plants from kerela.
- ❖ Md. Aslam Hossain, Sandesh Panthi, Md. Asadujjaman, Shams Ara Khan, Farzana Ferdous, Samir Kumar Sadhu. (2013), Phytochemical and Pharmacological Assessment of the Ethanol Leaves Extract of *Heritiera fomes* Buch. Ham. (Family- Sterculiaceae)
- ❖ Nirjhar Dasgupta, Pallavi Chowdhury, Sauren Das. (2015), Comparative Adaptability Assessment of Two Mangroves from Indian Sundarbans: Some Biochemical Appearances.
- ❖ Shahidi F., Janitha P. K., Wanasumandra P.D. 1992 Phenolic Antioxidant.

- ❖ Hong, Francis (2004). "History of Medicine in China" (PDF). *McGill Journal of Medicine* 8 (1): 7984.
- ❖ Harbertson, J. and Spayd, S. (2006). "Measuring phenolics in the winery". *Am. J. Enol. Vitic.* 57, 280-288.
- ❖ Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 1995; 64: 97–112.
- ❖ Fernandez-Pachon M., Villano S. D., Troncoso A. M. and Garcya –Parilla M. C. (2006) Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. *Anal Chim. Acta*, 563, 101-108.
- ❖ Mark Percival. 1998 "Antioxidants". *Clinical Nutrition Insights*, 31: 01-04.
- ❖ Sies H (Mar 1997). "Oxidative stress: oxidants and antioxidants". *Experimental Physiology* 82 (2): 291–5. doi:10.1113/expphysiol.1997.sp004024. PMID 9129943.
- ❖ Uday Bandyopudya, et al. 1999 "ROS: oxidative damage and pathogenesis". *Curr. Sci.*, 77: 658-666.
- ❖ Wolf G (Mar 2005). "The discovery of the antioxidant function of vitamin E: the contribution of Henry A. Mattill". *The Journal of Nutrition* 135 (3): 363–6. PMID 15735064.
- ❖ Wollgast, J. and Anklam E (2000) *Food Research International*, 33, 423–447.

----THE END----