INVESTIGATION OF *IN VITRO* ANTIOXIDANT PROPERTIES OF *MARSILEA MINUTA* PLANT EXTRACT

By

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A thesis submitted to the School of Pharmacy, in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The project titled "Investigation of *in vitro* antioxidant properties of *Marsilea minuta* plant extract" submitted by Nehal Mahbub (ID: 20346036) of Summer 2020 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy (Hons) in June 2025.

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Ethics Statement

The study does not involve any animal or human trial.

Abstract

This study aimed to evaluate the antioxidant potential of the ethanol extract of *Marsilea minuta*, commonly referred to as dwarf waterclover, an aquatic fern belonging to the Marsileaceae family. Antioxidant activity was assessed using the hydrogen peroxide (H_2O_2) scavenging activity and total antioxidant capacity (TAC) tests. The results indicated that *Marsilea minuta* had a higher IC_{50} value (305.42 µg/mL) compared to the standard, ascorbic acid (229.64 µg/mL), suggesting that a higher concentration of the extract was required to achieve comparable antioxidant activity. In the TAC assay, the extract demonstrated a strong antioxidant capacity of 105.67 mg ascorbic acid equivalents (AAE) per gram of dry extract at the concentration of 1200 µg/mL. Furthermore, the concentration-dependent absorbance analysis revealed a steeper calibration curve for *Marsilea minuta* than for the ascorbic acid standard, indicating a stronger dose-response relationship. These findings highlight the considerable antioxidant potential of *Marsilea minuta*, supporting its prospective use in the treatment of oxidative stress-induced diseases such as neurodegenerative disorders, cancer, cardiovascular diseases, diabetes, etc.

Keywords: *Marsilea minuta*, Ethanol extract. Antioxidant activity, H_2O_2, TAC.

Dedication

To my beloved parents, whose unwavering and unconditional love, endless sacrifices and steadfast support with unshakable belief in me have been my greatest source of strength and inspiration.

Acknowledgement

I would like to express my deepest gratitude to my supervisor, Acting Chairperson and Professor Dr. Raushanara Akter, for her guidance, encouragement, and support throughout this research. I extend sincere thanks to the faculty and staff of the School of Pharmacy at Brac University for providing the necessary resources and academic environment. Lastly, heartfelt thanks to my family and friends for their continuous support and motivation.

Table of Contents

List of Tables

List of Figures

List of Acronyms

MOA	Mechanism Of Action
ROS	Reactive Oxygen Species
TAC	Total Antioxidant Capacity

Chapter 1

Introduction

1.1 A brief history of Medicinal Plant

Medicinal plants serve prominent medicinal functions for humans and animals due to their healing capabilities. Roots, bark, leaves, seeds, and flowers all have curing, purgative, and other high-value health-enhancing benefits which are derived from these plants.

Approximately 60,000 years of archaeological evidence suggests the usage of medicinal plants and herbs (Solecki, 1975). It has also been documented that ancient civilizations including India, China, Egypt, Greece, and Central Asia documented medicinal applications of several herbs 2,500 to 5,000 years ago (Ang-Lee, 2001). A Sumerian clay tablet unearthed in Nagpur estimated to be 5,000 years old is recognized to have one of the earliest inscriptions of herbal medicine (Qiu, 2007).

In centuries past, for an extensive range of cultures, the use of herbs was considered a reliable and easy approach of application for treatment. Many developed countries put together extensive references—or Materia Medica—that documents the healing qualities and the uses of the plants in a certain civilization. As noted by Firenzuoli and Gori (2007), substances in their crude form—vegetable, animal, or mineral—have always played an important part in the treatment of diseases.

The scientific investigation of therapeutic and medicinal plants is one of the earliest forms of knowledge, dating back to people from several ancient civilizations like China, India, Egypt, and Greece. Halberstein (2005) pointed out that the quest for remedies became a search for natural substances that could offer relief from several ailments, which is how the basis of phytotherapeutic practices came into being.

One of the most important reasons why medicinal plants are still so widely used nowadays is because of their synergistic potential—briefly speaking, the undiscovered power that lies in the relationship between various components found in the plant which may act together to increase therapeutic outcomes, lessen harmful reactions or counteract side effects. This property proves valuable in the case of difficult-to-treat diseases like cancer. Moreover, some compounds derived from plants have the unique ability to halt or slow down the progress of a disease, which makes them highly sought after in modern and traditional drug discovery.

1.2 Medicinal Plants in Bangladesh and their usage

Bangladesh, nestled in the Indian subcontinent, is rich in various species of plants. This biodiversity allows the people to access the traditional medicine of Bangladesh to use plants as fundamental constituents to treat different health ailments. Medicinal plants are the part and parcel of ancestral heritage of Bangladesh. Apart from biodiversity, the country is also blessed with other natural resources like forests, minerals, and agricultural crops. The exploration and utilization of these resources has aided the development of Ayurveda, Unani, and Homeopathy system of traditional medicine (Ghani & Asiatic Society of Bangladesh, 2003).

The evolution of Ayurvedic medicine, homeopathy, and Unani therapies, along with other folk remedies spans decades in Bangladesh, and as such is deeply woven in the country's culture (Haque et al., 2018; Ocvirk et al., 2013). Bangladesh has a wide variety of medicinal plants and as Mollik et al. (2010) stated, the traditional healers in Bangladesh have recognized around 1,000 plants that can be used to treat various health complications.

Folk medicine is still widely practiced in Bangladesh, and traditional healers or local doctors called kavirajes assume the primary role. Likewise, medical practitioners exist in the indigenous tribal communities. These tribal healers also utilize medicinal plants in their healing procedures, very much in line with the folk medicine systems operated by the kavirajes.

Writing families keep a sort of therapeutic knowledge in history, which is passed on over generations of baidyas and tribal healers. Consequently, indigenous baidyas and tribal healers have had the opportunity to evolve immense knowledge over the last few millennia regarding medicinal plants growing in their regions.

Bangladesh supports approximately 6,500 species of plants, with about 5,700 species of angiosperms, some 68 species of woody legumes, 130 species of fiber plants, over 500 species of medicinal plants, 29 species of orchids, 3 species of gymnosperms, and around 1,700 species of pteridophytes (Islam, 2003). Among them, medicinal plants have great therapeutic potentials and are traditionally used in their crude forms such as extracts, pastes, decoctions, juices, and powders to cure or to manage several ailments (Rahmatullah et al., 2011).

In Bangladesh, a huge majority uses the plant medicine for various reasons, namely: (1) lack of access to the system of routine healthcare, particularly in the rural and remote areas; (2) medicinal plants are cheap and easy to collect; (3) age-old cultural confidence in herbalism of the common people and passing it from one generation to another; (4) the belief that side effects due to herbal drugs are less if compared to synthetic drugs; and (5) support of family tradition through which the use of herbalism is encouraged. Various ethnic tribes like Chakma, Marma, Manipuri, and Garo are some of the indigenous groups of Bangladesh, which regularly use medicinal plants in treating patients.

Table 1: Commonly used medicinal plant in Bangladesh (Bardhan S, et al., 2018)

Plant species	Family	Local name	Traditional uses
Abutilon indicum L.	*Malvaceae*	Potari	They exert antifungal, antimycotic and antidiarrhoeal activity. Leaf extracts are used to treat gonorrhea and vaginal infections.
Adina sessilifolia L.	*Rubiaceae*	Kam gass	Fungal infections, impetigo, minor cellulitis and folliculitis are treated by the leaf paste of this plant.
Bridelia retusa L.	*Euphorbiaceae*	Kantakui/kantakhashi	Fungal infections, impetigo, minor cellulitis and folliculitis are treated by the ripe fruits, bark and leaf paste of this plant.
Caesalpinia bondue L.	*Fabaceae*	Nata, natai, kokoi, dahara	Aqueous extract along with prepared root, leaves of this plant is used to treat urinary tract infections
Nyctanthes arbor-tristis	*Oleaceae*	Night jasmine, Shefali	Flower extracts consist of antifilarial qualities. Seed extracts are used to treat fungus infections.
Holarrhena antidysentrica L.	*Apocynaceae*	Bitter oleander	Used to treat dysentery.
Phyllanthus niruri L.	*Phyllanthaceae*	Bhui Amla or Bhui Amloki	It possesses anti-malarial, anti-viral activity against hepatitis B virus.

1.3 Reactive oxygen species and the body's production of free radicals

Free radicals are unstable chemical species possessing one or more unpaired valence electrons in their outer orbital, while reactive oxygen species (ROS) contain oxygen-derived molecules exhibiting intensified chemical reactivity that predominantly originate from cellular aerobic respiration (Halliwell & Gutteridge, 2015). These reactive species are mostly produced as natural by-products of cellular metabolic processes. They include superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) (Sato et al., 2013; Navarro-Yepes et al., 2014).

If maintained in balance, then ROS show important physiological functions such as in apoptosis, immune responses, cell differentiation, phosphorylation modifications, and transcription factor modulation (Rajendran et al., 2014).

Free radicals in the human body may be formed from internal and external causes. Internal causes include different biological processes such as ischemia, inflammation, activation of the immune system, infections, cancer development, psychological or physical stress, heavy exercise, and the normal aging process. Meanwhile, external factors include environmental pollutants, radiation, heavy metals, and other toxic substances.

1.3.1 Incidence of oxidative stress in various disorders

Oxidative stress arises when an imbalance develops between the production of reactive oxygen species and the organism's capacity to neutralize these compounds or reverse their harmful effects (Pizzino et al., 2017). While ROS are routinely produced during normal oxygen metabolism and serve essential physiological roles, including cellular signaling regulation, external stressors—such as ultraviolet (UV) radiation, ionizing radiation, environmental toxins, heavy metals, and certain pharmaceuticals—can significantly increase ROS levels. This

excessive ROS accumulation overwhelms cellular antioxidant defenses, leading to oxidative damage in tissues and cells (Pizzino et al., 2017).

Extensive research indicates that oxidative stress plays a significant role in the onset and progression of various diseases, including but not limited to cancer, diabetes, cardiovascular disorders, metabolic syndromes, and atherosclerosis (Taniyama & Griendling, 2003).

1.3.2 Free radical and oxidative stress-induced harm to the body and its tissues

Free radicals and reactive oxygen species (ROS) interact with lipids in cellular membranes, leading to structural alterations that subsequently affect the function of proteins and DNA, ultimately contributing to cellular damage. Polyunsaturated fatty acids, which possess conjugated double bonds, are particularly prone to free radical attacks due to the ease with which bisallylic hydrogen atoms can be abstracted—a result of their low homolytic bond dissociation energy. This initial reaction produces lipid radicals that rapidly interact with molecular oxygen, further intensifying lipid peroxidation. The consequence of this chain reaction includes impairment of ion channels, inactivation of membrane-associated enzymes or transport proteins, and changes to the membrane structure, such as increased rigidity or permeability. Proteins are also a major target of free radical-induced damage (Davies, 2001). Oxidative modifications can result in the loss of sulfhydryl (-SH) groups through thiol oxidation, the formation of mixed disulfides, or the conversion of methionine residues to their sulfoxide forms (Fuehrer & Klotz, 2015).

The structural and functional integrity of proteins can be disrupted by oxidative modifications, and can lead to the formation of aldehydes or ketones from amino acids, or the generation of hydroperoxides and ring cleavage in residues like histidine and tryptophan. These alterations interfere with cellular signaling pathways (Spickett & Pitt, 2010) and may compromise protein

functionality and stability (Dean et al., 1997), thereby contributing to the development of various diseases.

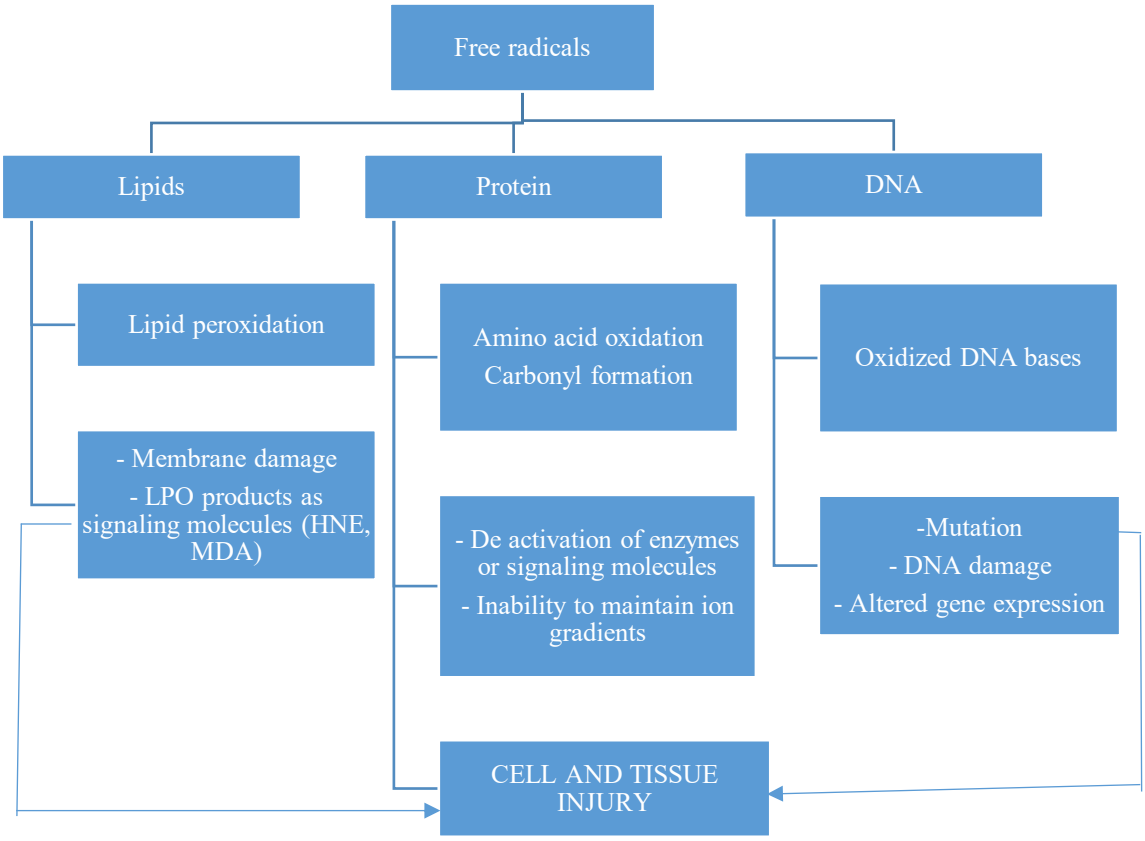


Figure 1: MOA through which free radicals cause cell damage (kehrer & Klotz, 2015)

Free radicals interacting with DNA can compromise genetic integrity and disrupt gene regulation, causing harmful alterations within cells. Reactive oxygen species (ROS) can induce structural changes to both the nucleotide bases and the sugar backbone of DNA (Cooke et al., 2003). When the cellular mechanisms for DNA repair are inadequate, this damage may become irreversible, potentially resulting in cell death. Such persistent DNA alterations can lead to mutations and errors in gene transcription.

1.3.3 Diseases caused by free radicals and oxidative stress

According to Sachdev et al. (2023), reactive oxygen species (ROS) are highly reactive molecules that can damage crucial cellular components like lipids, proteins, carbohydrates, and DNA, potentially causing cell injury or even death in extreme cases. Although excessive ROS

levels are known to be harmful, recent studies suggest that moderate ROS levels may actually contribute positively to plant growth and biological functions. However, when ROS production becomes excessive, it can trigger oxidative stress, which is a key factor in the development of chronic and degenerative diseases, as well as acute conditions such as stroke and trauma. Additionally, oxidative stress contributes to accelerated tissue aging.

Table 2: Diseases/tissue injuries with free radical component (Kehrer & Klotz, 2015)

Lung	**Nervous system**	**Skin**	**Heart and cardiovascular r system**	**Eye**	**Immune disorders**
Normobaric hypertoxic injury	Alzheimer's disease	Radiation: solar or ionizing	Ischemia/reperfusion: after infarction or transplant	Retinopathy of prematurity (oxygen)	Rheumatoid arthritis
Bronchopulmonary dysplasia	Amyotrophic lateral sclerosis	Thermal injury	Atherosclerosis	Photic retinopathy	Autoimmune diseases such as Lupus
Asbestosis, Cigarette smoke	Down's syndrome	Contact dermatitis	Selenium deficiency (Keshan disease)	Cataracts	Inflammation
Idiopathic pulmonary fibrosis	Myasthenia gravis, Parkinson's disease	Porphyria, Chemicals: Photosensitizers e.g., Tetracyclines	Hemochromatosis		

1.4 Antioxidants

Antioxidants represent biologically active molecules capable of delaying or preventing oxidative damage to cellular components at relatively low concentrations compared to susceptible substrates (Halliwell & Gutteridge, 2015). These protective agents function as essential components of the endogenous defense system, mitigating harmful effects induced by reactive oxygen species through radical neutralization and redox balance maintenance (Sies, 2017). The pathological consequences of uncontrolled oxidative stress encompass various chronic conditions, including atherosclerosis, malignant transformation, and neurological degeneration (Lushchak, 2021). These protective compounds are systematically classified into two major groups: naturally occurring and synthetic. Natural antioxidants include compounds such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), carotenoids, and various phenolic compounds. These are primarily obtained from plant-based foods and play a crucial role in neutralizing free radicals within biological systems (Pham-Huy et al., 2008). In contrast, synthetic antioxidants like butylated hydroxytoluene (BHT; E321) and butylated hydroxyanisole (BHA; E320) are chemically manufactured and commonly used as additives in food products to prevent oxidation and prolong shelf life (Shahidi & Ambigaipalan, 2015). In addition to preserving food, antioxidants have therapeutic applications, particularly in clinical settings where they contribute to anti-aging by cellular protection from oxidative damage, oncological prevention via DNA damage reduction, cardiovascular protection through lipoprotein oxidation inhibition and neuroprotection against oxidative neurodegeneration (Forman & Zhang, 2021). They are also used in developing antioxidant-based therapies and supplements. Antioxidants are also important in formulating skincare products that mitigate oxidative damage to the skin.

1.4.1 Types of antioxidants

Table 3: Classification of antioxidants based on origin and solubility (Maity et al., 2022)

Origin		**Solubility**	
Natural antioxidants	Synthetic antioxidants	Water soluble	Lipid soluble
Ascorbic acid	BHA	Glutathione	Carotenes
Citric acid	BHT	Vitamin C	Alpha tocopherol (vitE)
Glutathione	EDTA salts	Lipoic acid	Ubiquinol (coenzyme Q)
Carotene	H_3PO_4	Uric acid	

Table 4: Classification of antioxidants based on mechanism of actions (Maity et al., 2022

Antioxidants based on the mechanism of actions			
By preferentially oxidized	**By blocking oxidative chain reaction**	**By synergistic action**	**By chelating the trace amount of heavy metals**
Sodium bisulphate	BHA	H_3PO_4	EDTA salts
Thiourea	BHT	Citric acid	
Sodium meta bisulphite	Alpha tocopherol		
Vitamin C			

1.4.2 A description of the plant *Marsilea minuta*

Marsilea minuta L., a small fern species from the *Marsileaceae* family, typically grows in wetland areas such as rice paddies, pond margins, and marshes. It has a characteristic clover-like appearance due to its four-parted leaflets, which display nyctinastic movement (closing during low-light conditions). Traditionally, this plant has been extensively utilized in various folk medicinal practices to address ailments like epilepsy, sleep disorders, and mental health issues. Investigations into its phytochemical composition have identified compounds such as flavonoids, alkaloids, tannins, and phenolic acids, which are considered to be responsible for its antioxidant and antimicrobial activities (Mondal et al., 2019). Recent investigations into *Marsilea minuta* extracts have demonstrated promising antioxidant potential, highlighting its possible role in mitigating oxidative stress-related disorders.

1.4.3 Morphology

Marsilea minuta L., commonly referred to as dwarf water clover, is a small fern typically found in moist and shallow water environments. It belongs to the family *Marsileaceae* and is known for its ability to grow in both aquatic and terrestrial conditions, adjusting its physical traits accordingly (Rashid, 1999; Ghosh & Banerjee, 2021).

The plant features thin, creeping rhizomes that branch extensively and support the growth of roots from both the nodes and internodes. These rhizomes typically measure between 0.4 and 0.8 mm in thickness.

Its leaves arise on long, round stalks (petioles) that can extend up to 13 cm. Each leaf is divided into four fan-shaped leaflets that are smooth and hairless, forming a clover-like structure. The size of the leaflets varies, typically ranging from 0.8 to 1.7 cm in length and 1.2 to 2.0 cm in width. In aquatic settings, the plant tends to grow longer petioles to support floating leaves, while in drier areas, it develops shorter, sturdier leaves (Rashid, 1999).

Reproductive structures known as sporocarps form near the base of the fertile leaves. These are small, curved, bean-shaped structures, about 2.6 to 4.1 mm long, with a visible line (raphe) that runs most of their length and small teeth on both ends (Ghosh & Banerjee, 2021).

1.4.4 Fruits and Flowering period

Marsilea minuta does not produce traditional flowers and fruits like flowering plants as it is a heterosporous fern. As an alternative, it reproduces via sporocarps, which are specialized reproductive structures. The sporocarps are bean-shaped and form near the base of fertile leaves, containing both microspores and megaspores.

Sporocarp production (flowering/fruiting equivalent) usually occurs during monsoon and post-monsoon seasons in tropical and subtropical regions, typically from June to October, depending on the local climate (Ghosh & Banerjee, 2021; Rashid, 1999).

1.4.5 Taxonomic classification of *Marsilea minuta*

Table 5: Taxonomic hierarchy of Marsilea minuta (World Flora Online, 2024)

Kingdom	**Plantae**
Clade	*Tracheophyta*
Division	*Polypodiophyta (Pteridophytes)*
Class	*Polypodiopsida*
Order	*Salviniales*
Family	*Marsileaceae*
Genus	*Marsilea*
Species	*Marsilea minuta L.*

1.6 Information on chemical constituents of other species of *Marsilea minuta*

There are several species of the genus *Marsilea* that are known to have a variety of bioactive chemical components, including *Marsilea minuta*. Flavonoids, alkaloids, tannins, saponins, phenolic compounds, triterpenoids, and glycosides have all been detected by phytochemical screens. Particularly prevalent flavonoids with anti-inflammatory and antioxidant qualities are kaempferol and quercetin.

The plant's alkaloids are well-known for their sedative and neuroprotective properties, while its tannins and phenolic components support its antioxidant and antibacterial properties. Triterpenoids are frequently associated with anti-inflammatory reactions, while saponins may help decrease cholesterol. Methanolic extracts of *Marsilea minuta* have shown significant phytochemical activity, as reported by Kumar et al. (2010).

Further, Nathiya et al. (2014) highlighted the plant's neuropharmacological properties, which may be attributed to triterpenoids and alkaloids.

Similar compounds and antioxidant effects have also been reported in related species such as *Marsilea quadrifolia* (Sinha et al., 2015), suggesting the therapeutic potential of this genus.

1.7 Information on the pharmacological properties of other species of *Marsilea minuta*

Numerous pharmacological properties have been shown by several species of the *Marsilea* genus, including *Marsilea minuta*. Antioxidant, neuropharmacological, anti-inflammatory, analgesic, antipyretic, antibacterial, hepatoprotective, and wound-healing effects are among them. Extracts from *Marsilea minuta* and *Marsilea quadrifolia* exhibit strong antioxidant properties due to their rich content of polyphenols and flavonoids, which help in neutralizing free radicals (Sinha et al., 2015; Nathiya et al., 2014).

In addition, extracts from *Marsilea minuta* has shown significant sedative and anxiolytic effects in animal models, supporting its traditional use for neurological disorders (Nathiya et al.,2014).

Some studies also suggested that various extracts from *Marsilea* species have been found to reduce inflammation and pain in experimental studies, likely due to the presence of triterpenoids and flavonoids (Kumar et al., 2010).

Furthermore, methanolic extracts of *Marsilea minuta* have also been shown to reduce fever, making it potentially useful for treating febrile conditions. Moreover, Leaf and stem extracts of *Marsilea minuta* have exhibited antibacterial and antifungal activities, attributed to the presence of alkaloids and phenolic compounds (Ghosh et al., 2011).

Although not widely studied, some related species like *Marsilea quadrifolia* have shown promise in promoting wound healing and protecting liver tissues from damage.

1.8 Project Rationale

The plant species *Marsilea minuta* is a member of the Marsileaceae family, and other Marsilea species often have good antioxidant, neuropharmacological, anti-inflammatory, analgesic, antipyretic and antimicrobial activities. For instance, *Marsilea quadrifolia* has antioxidant, anti-inflammatory, antidiabetic, hepatoprotective and neuroprotective activities. *Marsilea crenata* has antioxidant, anxiolytic and anticonvulsant effects. *Marsilea aegyptiaca has* significant antibacterial, antifungal and wound healing effect.

Marsilea minuta is anticipated to possess the similar biological property as *Marsileaceae* because both species are members of the same genus and share similar chemical components, such as flavonoids, alkaloids, tannins, saponins, phenolic compounds, triterpenoids, and glycosides. Therefore, *Marsilea minuta* was chosen to differentiate its antioxidant characteristics because there haven't been sufficient investigations done on it.

1.8.1 Aim

The aim of the study was to characterize and assess the antioxidant properties of *Marsilea minuta* plant extract.

1.8.2 Objective of the project

1. To determine the H_2O_2 scavenging capacity of ethanolic extracts of *Marsilea minuta*.
2. To quantify its total antioxidant capacity using the phosphomolybdenum method.
3. To compare the antioxidant efficacy of *Marilea minuta* with the standard antioxidant, L-ascorbic acid.

Chapter 2

Methodology

2.1 Plant material collection and preparation

The plant species *Marsilea minuta* was selected after cursory analysis about different bioactive properties of the plant. Around 10 kg of this plant were collected from "Bangladesh National Herbarium" located at Mirpur, Dhaka, Bangladesh. The specimen was given the accession number DACB-121316. This organization has collected these plants from various places of Bangladesh, mainly from wetland ecosystems, ponds and rice fields- as *Marsilea* species are semi-aquatic plants. They have done the preliminary observation and identification by looking for the morphological features such as creeping rhizomes, four-lobed leaflets and small sporocarps. Moreover, a field manual or flora (e.g., *Flora of Bangladesh*) is used by the organization to preliminarily identify the species before collection. They collected multiple samples to ensure the inclusion of different plant parts like leaves, stems, roots and reproductive structures (sporocarps).

After collection from the Bangladesh National Herbarium (BNH), the plants were placed in clean newspaper to avoid contamination and dried under continuous airflow at room temperature to prevent photodegradation of bioactive compounds that may occur with direct sunlight exposure. More than one month drying period was required to achieve optimal moisture removal. The dried plant material was then ground to a fine powder using an electric blender. Then, these fine powders of *Marsilea minuta* were immersed in 4 solvents in an order from less to more polarity: n-hexane, chloroform, ethanol, and methanol. And the order of these solvents based on increasing polarity:

n-Hexane < Chloroform < Ethanol < Methanol.

The powdered *Marsilea minuta* material was initially immersed in n-hexane for a period of 72 hours under static conditions. The resulting mixture was filtered through Whatman No. 1 filter paper (11µm pore size) to separate the n-hexane filtrate from the solid residue. The retained residue was subsequently subjected to chloroform extraction under identical temporal and physical conditions.

This sequential extraction protocol was systematically repeated using solvents of increasing polarity: anhydrous ethanol (96% purity) followed by methanol (HPLC grade). Each solvent cycle maintained consistent parameters (3-day duration, 1:10 w/v plant-to-solvent ratio, ambient temperature of 25±2°C).

All filtrates were collected in amber glass vessels to prevent photochemical degradation and concentrated (dried) using a rotary evaporator. The dried extracts were then stored in air tight containers in the refrigerator and they were ready for subsequent phytochemical analysis.

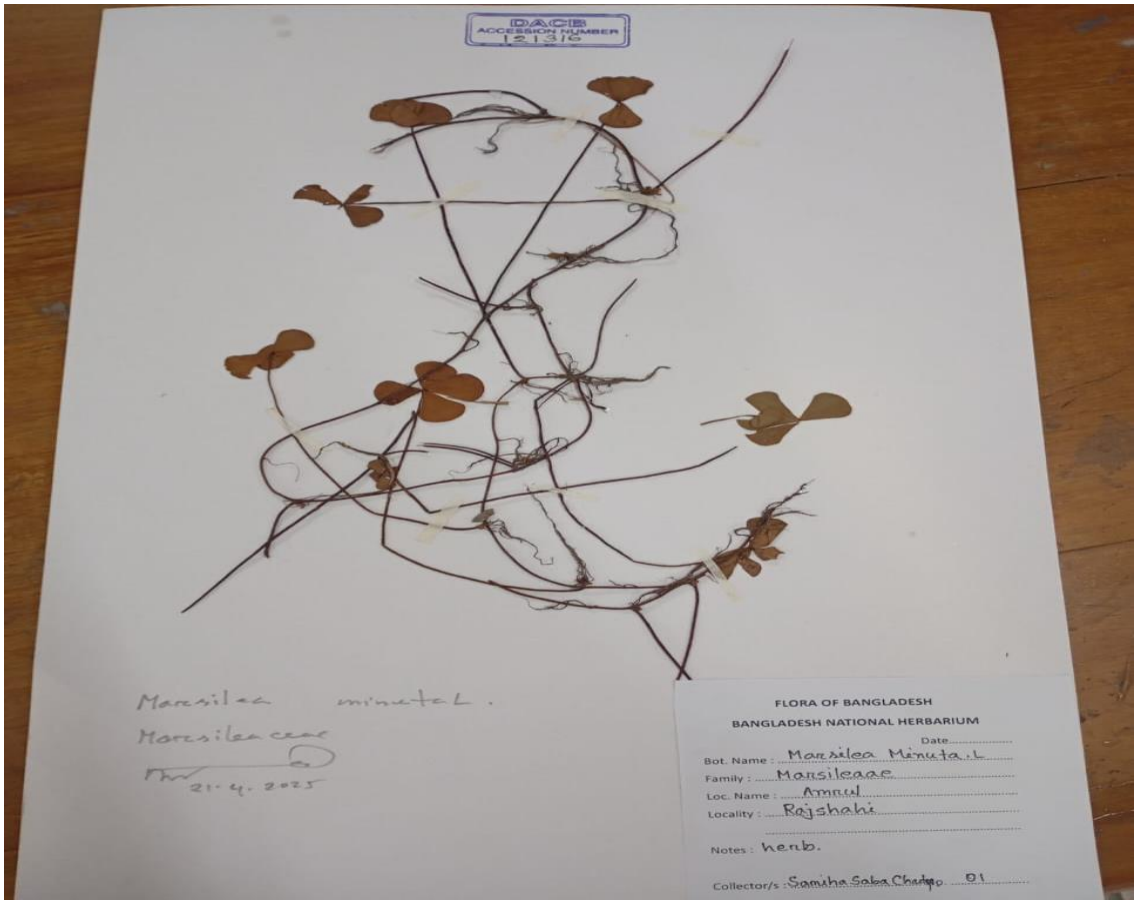


Figure 2: Marsilea minuta plants

2.2 The fundamentals of antioxidant study

The antioxidant potential of the medicinal plant extract was evaluated using both the hydrogen peroxide (H_2O_2) scavenging assay and the total antioxidant capacity (TAC) test.

2.3 Hydrogen Peroxide (H_2O_2) scavenging activity

Hydrogen peroxide (H_2O_2) is found naturally in various sources, including the human body, environmental settings, water, plant tissues, and microbial organisms. It can enter the body through skin contact, eye exposure, or by inhaling its mist or vapor. Upon decomposition into oxygen and water, H_2O_2 generates hydroxyl radicals (•OH), which are highly reactive and can lead to lipid peroxidation and damage to DNA (Haida & Hakiman, 2019).

In order to assess the H_2O_2 scavenging potential, the Nabavi et al. (2008) approach was used. This process involved making a 40 mM hydrogen peroxide solution with a 50 mM phosphate

buffer at pH 7.4 and measuring the absorbance at 230 nm. Two millilitres of the hydrogen peroxide solution were combined with one millilitre of the sample extract or standard to generate a reaction mixture. Ten minutes after the combination was let to stand, the absorbance was measured and contrasted with a control. Phosphate buffer was used exclusively to create the blank solution; hydrogen peroxide was not used. In order to ascertain the H_2O_2 scavenging activity, the following formula was employed:

H_2O_2 scavenge (%) = [(A230 Control−A230 Sample)⁄A230 Control] ×100

Apparatus and Reagents

Table 6: *Apparatus and reagents needed in the H_2O_2 scavenging activity*

Apparatus	**Reagents**
UV-Vis spectrophotometre	H_2O_2 solution 40mM
Test tubes	Phosphate buffer (7.4)
Volumetric flask	Ethanol
Pipettes and pipette pumpers	Ascorbic acid
Weighing machine	Marsilea Minuta plant's ethanolic extracts
Vortex machine	Distilled water

2.3.1 H_2O_2 preparation

The stock hydrogen peroxide that was available in our lab is 30% w/w, which is approximately 9.8 M or 9800 mM. The desired concentration was 40mM. and the desired volume was 70 mL for our experiment.

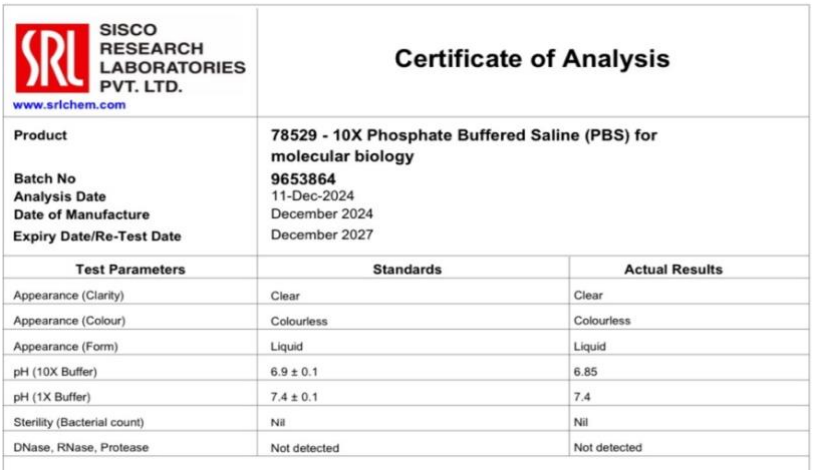
Figure 3: Stock H_2O_2 30% w/w (9.8M)

To create 70 mL of 40 mM concentrated H_2O_2 preparation, 0.28572 mL of 30% H_2O_2 stock was mixed in 69.71428 mL of phosphate buffer (pH7.4) Before the experiment, the H_2O_2 was made and stored in a volumetric flask. Every time the experiment was conducted, a new solution was made.

2.3.2 Phosphate buffer preparation (pH 7.4)

The stock phosphate buffer that was bought for the experiment was 10X Phosphate buffered saline (PBS) and its pH was 6.85. The stock 10X buffer needed to be diluted into 1X buffer to make the pH 7.4. To make 1X buffer from 10X buffer, 10X buffer was needed to dilute by adding distilled water in a ratio of 1:9.

For preparation of 100mL 1X buffer (pH 7.4), 10mL stock 1X buffer was taken and 90mL distilled water was added to make 100mL 1X buffer.



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Certificate of Analysis

Product	**78529 - 10X Phosphate Buffered Saline (PBS) for molecular biology**
Batch No	**9653864**
Analysis Date	11-Dec-2024
Date of Manufacture	December 2024
Expiry Date/Re-Test Date	December 2027

Test Parameters	Standards	Actual Results
Appearance (Clarity)	Clear	Clear
Appearance (Colour)	Colourless	Colourless
Appearance (Form)	Liquid	Liquid
pH (10X Buffer)	6.9 ± 0.1	6.85
pH (1X Buffer)	7.4 ± 0.1	7.4
Sterility (Bacterial count)	Nil	Nil
DNase, RNase, Protease	Not detected	Not detected

Figure 4: Certificate of Analysis of 10x PBS

2.3.3 Sample preparation for H_2O_2 activity of *Marsilea minuta*

The ethanolic extract of Marsilea minuta was prepared as a 250 µg/mL stock solution by dissolving 16 mg of plant material in 64 mL of ethanol. Subsequent serial dilutions of this stock produced a concentration series spanning 250 µg/mL to 15.63 µg/mL for experimental analysis.

Table 7: Serial dilution of sample solution to achieve a range of concentrations from 250 µg/mL down to 15.63 µg/mL.

Target concentration of sample solution (µg/mL)	Volume taken (mL)	Initial concentration (µg/mL)	Added ethanol (mL)	Final volume (mL)
125	4	250	4	8
62.5	4	125	4	8
31.25	4	62.5	4	8
15.63	4	31.25	4	8

Each sample dilution was accurately labeled and stored in separate test tubes and the test tubes are stored in a cool, dark place until further use

2.3.4 Standard preparation for H_2O_2 activity of *Marsilea minuta*

A stock solution of ascorbic acid was prepared by dissolving 16 mg in 64 mL of absolute ethanol, yielding an initial concentration of 250 µg/mL. Serial dilutions were then performed on this stock solution to generate a concentration gradient spanning from 250 µg/mL to 15.63 µg/mL for experimental use.

Table 8: Serial dilution of standard solution to achieve a range of concentrations from 250 µg/mL down to 15.63 µg/mL.

Target concentration of standard solution (µg/mL)	Volume taken (mL)	Initial concentration (µg/mL)	Added ethanol (mL)	Final volume (mL)
125	4	250	4	8
62.5	4	125	4	8
31.25	4	62.5	4	8
15.63	4	31.25	4	8

After that, each standard dilution was appropriately marked and labeled in separate test tubes and then placed in a dry, dark environment.

2.3.5 Blank Preparation

To make the blank solution, 9 mL of phosphate buffer and 1.5 mL of ethanol were added in a test tube and mixed them through a vortex machine and added the appropriate labeling.

2.3.6 Experimental procedure

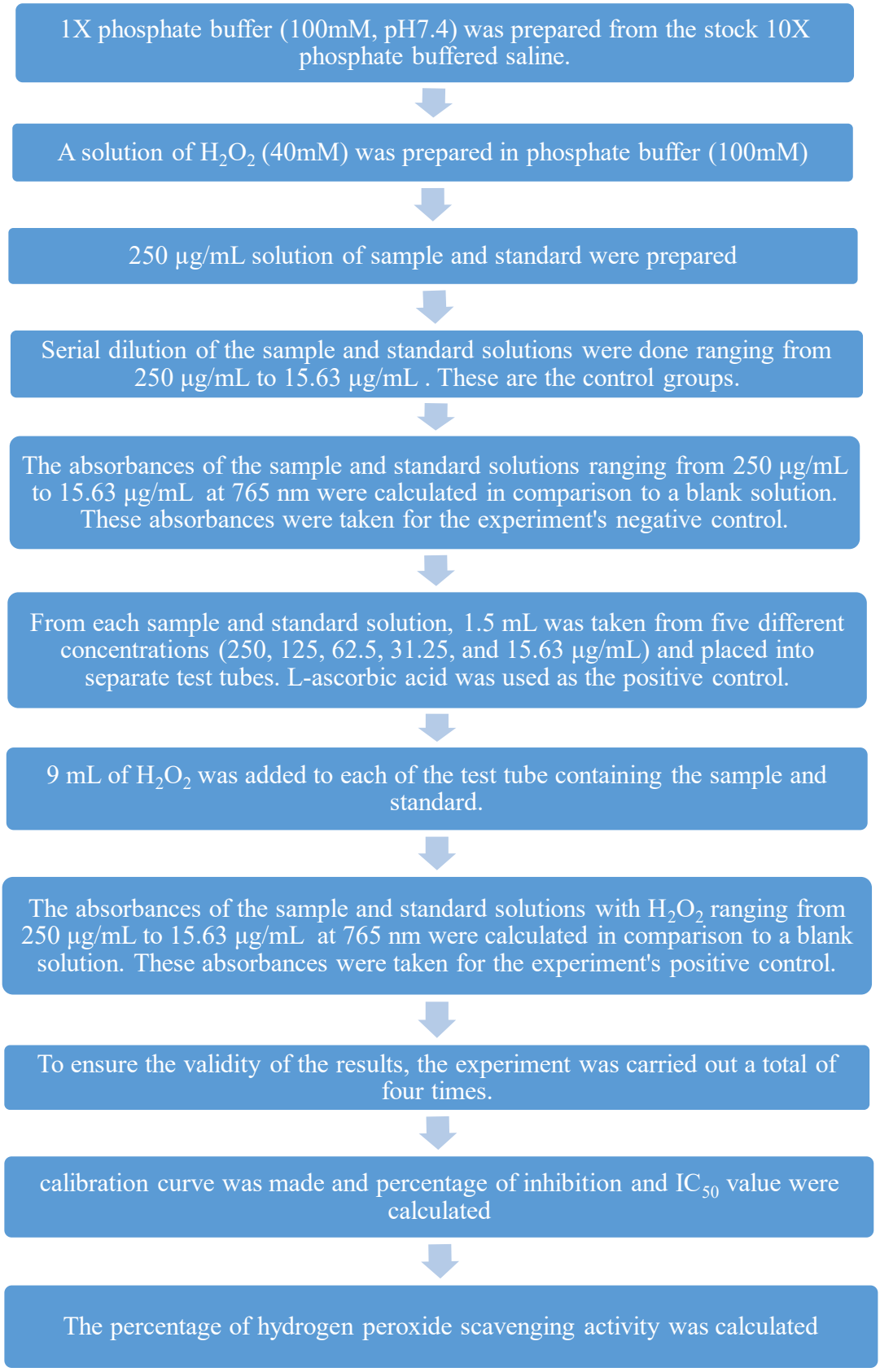

Figure 5: Experimental procedure for the determination of H_2O_2 scavenging activity

2.4 Total antioxidant capacity study

The phosphomolybdenum reduction assay (Prieto et al., 1999) was utilized to quantify total antioxidant capacity (TAC). This spectrophotometric method operates through the redox conversion of hexavalent molybdenum (Mo^{6+}) to pentavalent molybdenum (Mo^{5+}) when reacting with electron-donating antioxidant compounds. Under controlled acidic conditions, this reduction generates a characteristic green-colored phosphomolybdate(V) complex. Quantitative analysis was performed by measuring the chromophore's absorbance at $\lambda = 695$ nm using UV-Vis's spectroscopy. A reference calibration curve was constructed using serial dilutions of L-ascorbic acid (75-1200 µg/mL) as the primary antioxidant standard.

The antioxidant activity of the samples was expressed in terms of ascorbic acid equivalent (AAE) using the formula:

$$TAC\ (mg\ AAE/g) = (c \times V) / m$$

Where:

- c = concentration of ascorbic acid from the calibration curve (mg/mL)
- V = volume of extract used (mL)
- m = mass of extract used (g)

This method was used to determine the antioxidant capacity of *Marsilea minuta* as demonstrated in the study by Chavan et al. (2019).

Apparatus, reagents and chemicals:

Table 9: *Reagents, chemicals and apparatus need in the total antioxidant capacity study experiment*

Reagents and chemicals	Apparatus
Ethanol	Test tubes
Ammonium Molybdate	Volumetric flask
Trisodium Phosphate	Pipettes and pipette pumpers
L-ascorbic acid	UV-Vi's spectrophotometer
Sulfuric acid	Weighing machine

2.4.1 Preparation of Sulfuric acid solution

To prepare a 0.6M H_2SO_4 solution from a concentrated 98% stock solution, the known properties of sulfuric acid were used. 98% H_2SO_4 has a density of 1.84 g/mL and a molar mass of 98.08 g/mol; this means 1 mL of the 98% concentrated acid contains about 1.84 g of H_2SO_4, and therefore 1000 mL contains 1840 g. Since its 98% pure, the actual amount of pure acid is 1803.2 g. Dividing this by the molar mass gives approximately 18.385 moles per liter (M). So, the concentration of the stock 98% H_2SO_4 is 18.385M. And for this experiment purpose, a solution of concentration 0.6M is needed to prepare.

Therefore, 3.2635 mL of concentrated 98% sulfuric acid was precisely measured and placed into a 100 mL volumetric flask in order to produce 100 mL of 0.6 M sulfuric acid solution. After then, distilled water was added little by little until the content reached 100 milliliters.

2.4.2 Preparation of Ammonium Molybdate solution

The stock ammonium molybdate that was bought for the experiment had the molecular weight of 1235.86 g/mol. It means, to make 1M solution, in 1000mL total volume, 1235.86 g $(NH_4)_6Mo_7O_{24}$ had to dissolve. Therefore, to make 0.004M solution, in 100 mL total volume, 0.494344 g ammonium molybdate was dissolved.

So, 0.494344 grams of ammonium molybdate were first placed into a 100 mL volumetric flask to prepare 100 mL of a 0.004 M ammonium molybdate solution Then, distilled water was added gradually until the total volume reached the 100 mL mark.

During the experiment, sometimes ammonium molybdates would not dissolve easily to the distilled water; there could be many reasons behind this problem. This problem was also encountered while it was being done. One of the many solutions is to increase the pH of the distilled water from 7.4 to close to 9. This slightly alkaline condition of the distilled water helped to dissolve the ammonium molybdate powders.

2.4.3 Trisodium Phosphate solution

The stock trisodium phosphate that was bought for the experiment had the molecular weight of 380.12 g/mol. It means, to make 1M solution, in 1000mL total volume, 380.12 g Na_3PO_4 had to dissolve. Therefore, to make 0.028M solution, in 100 mL total volume, 1.064336 g ammonium molybdate is dissolved.

So, 1.064336 grams of trisodium phosphate were measured and transferred into a 100 mL volumetric flask to prepare a 0.028 M trisodium phosphate solution. The flask was then filled with distilled water up to the 100 mL mark to achieve the desired molarity.

2.4.4 Sample Preparation

After precisely weighing 120 mg of *Marsilea minuta* extract, it was mixed in 10 mL of ethanol in order to produce a stock solution with a concentration of 12,000 µg/mL. The original stock solution was then serially diluted to create a series of working solutions with concentrations of 1200 µg/mL, 600 µg/mL, 300 µg/mL, 150 µg/mL, and 75 µg/mL.

Table 10: Serial dilution of sample solution ranging the concentrations from 1200 µg/mL to 75 µg/mL

Target (final) concentration (µg/mL)	Initial concentration (µg/mL)	Target (final) volume (mL)	Initial volume needed to be taken (mL)	Ethanol added (mL)
1200	12000	10	1	9
600	1200	10	5	5
300	600	10	5	5
150	300	10	5	5
75	150	10	5	5

Each dilution was accurately labeled and stored in separate test tubes and the test tubes are stored in a cool, dark place until further use.

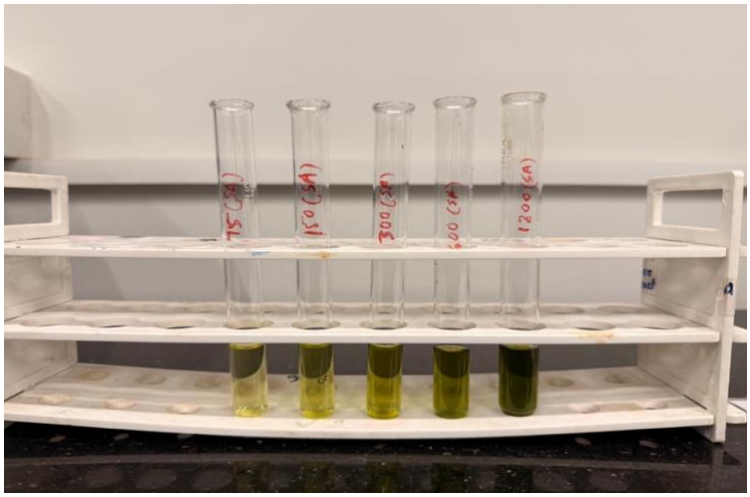

Figure 6: Sample solution at 5 different concentrations (1200 µg/mL, 600 µg/mL, 300 µg/mL, 150 µg/mL, and 75 µg/mL) in 5 different test tubes

2.4.5 Standard Preparation

The reference for calculating the total antioxidant capacity was L-ascorbic acid. After accurately weighing 120 mg of ascorbic acid, it was diluted in 10 mL of ethanol to create a stock solution with a concentration of 12,000 µg/mL. The initially prepared stock solution was then serially diluted to create a series of working solutions with concentrations of 1200 µg/mL, 600 µg/mL, 300 µg/mL, 150 µg/mL, and 75 µg/mL.

Table 11: Serial dilution of standard solution ranging the concentrations from 1200 µg/mL to 75 µg/mL

Target (final) concentration (µg/mL)	Initial concentration (µg/mL)	Target (final) volume (mL)	Initial volume needed to be taken (mL)	Ethanol added (mL)
1200	12000	10	1	9
600	1200	10	5	5
300	600	10	5	5
150	300	10	5	5
75	150	10	5	5

Each dilution was accurately labeled and stored in separate test tubes and the test tubes are stored in a cool, dark place until further use.

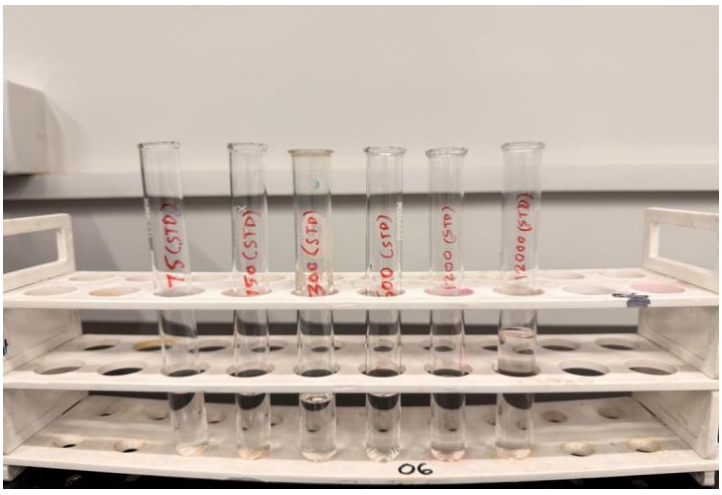

Figure 7: Standard solution at 5 different concentrations (1200 µg/mL, 600 µg/mL, 300 µg/mL, 150 µg/mL, and 75 µg/mL) in 5 different test tube

2.4.6 Blank Preparation

A blank solution was created using 1.5 mL ethanol and 15 mL reagent solution.

There are 3 reagents; sulfuric acid, ammonium molybdate and trisodium phosphate. So, we added 5 mL of each reagent to make 15 mL reagent solution and added 1.5 mL ethanol.

2.4.7 Experimental procedure

Figure 8: Experimental procedure for the determination of Total Antioxidant Capacity

Chapter 3

Results

3.1 Evaluation of H_2O_2 scavenging activity of *Marsilea minuta*

By using the UV-Vis spectrophotometer from the Shimadzu corporation, I found out the absorbances of the control groups of sample and standard solutions (without H_2O_2) and the absorbances of sample and standard solutions after adding hydrogen peroxide.

Then by using the H_2O_2 scavenging activity or % of inhibition determination formula, I found out the values of % of inhibition or the H_2O_2 scavenging activity.

H_2O_2 scavenging (%) = [(A230 Control−A230 Sample)⁄A230 Control] ×100

Table 12: Marsilea minuta % of inhibiion Value Calculation (µg/mL)

Sample concentration (µg/mL)	Absorbance of control (sample without H_2O_2)	Absorbance of sample	% of inhibition
250	0.698	0.415	40.545
120	0.413	0.267	35.352
62.5	0.218	0.153	29.816
31.25	0.131	0.104	20.610
15.63	0.109	0.086	11.34

Then, by plotting the sample concentrations in the x-axis and % of inhibition in the y-axis in a graph, we get the “y=mx+c” line. By solving the value of “x” from this equation, the value of IC_{50} (Inhibitory Concentration 50%) was calculated. IC_{50} is the concentration of extract required to inhibit 50% of the H_2O_2.

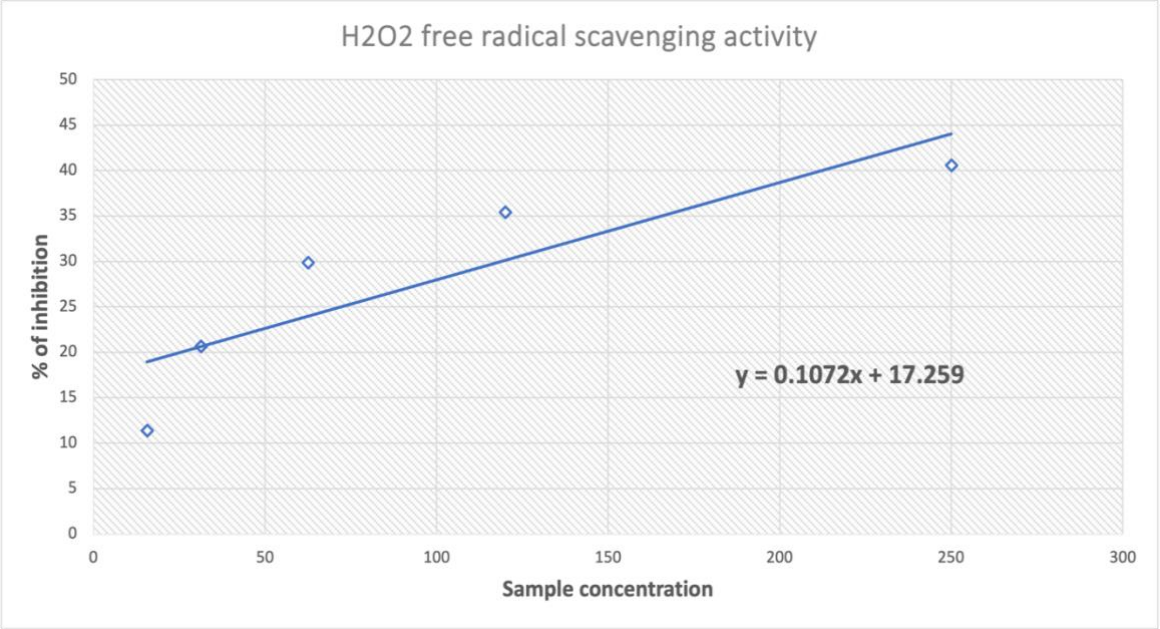

Figure 9: % Inhibition vs. Concentration Curve of Marsilea minuta (Sample)

The absorbances of the sample were recorded across concentrations ranging from 250 to 15.63 µg/mL. A clear trend was observed where the absorbance decreased gradually with the reduction in sample concentration.

As the concentration decreased, it was seen that the H_2O_2 scavenging activity was decreasing. Additionally, it was found that the lowest level of inhibition was shown at a concentration of 15.63µg/mL, while the highest level of inhibition was seen at a dosage of 250µg/mL. The graph's y = 0.1072x + 17.259 was used to determine the IC_{50} value.

Now, to determine the value of IC_{50}, we find the concentration (x) where % of inhibition (y) is 50.

Now, from the equation: y = 0.1072x + 17.259;

Therefore, 50 = 0.1072x + 17.259; and x= 305.41963 µg/mL

The sample had an IC_{50} value of 305.41963 µg/mL

3.1.2 Evaluation of H_2O_2 scavenging activity of L-ascorbic acid

Now, for the standard solution, again we use the % of inhibition determination formula to find out the values of % of inhibition or the H_2O_2 scavenging activity.

H_2O_2 scavenge (%) = [(A230 Control−A230 Sample)⁄A230 Control] ×100

Table 13: Ascorbic acid % of inhibiion Value Calculation (µg/mL)

Standard concentration (µg/mL)	Absorbance of control (standard without H_2O_2)	Absorbance of standard	% of inhibition
250	0.481	0.243	49.480
120	0.316	0.171	45.886
62.5	0.261	0.148	43.295
31.25	0.149	0.095	36.241
15.63	0.103	0.068	33.980

The standard concentration was then placed on the x-axis of a graph, while the percentage of inhibition was put on the y-axis. A linear equation with the formula "y = mx + c" was obtained from this. The IC_{50} value was determined by calculating this equation for the value of "x" when "y" equals 50. The extract concentration required to neutralize 50% of the hydrogen peroxide present is indicated by this figure.

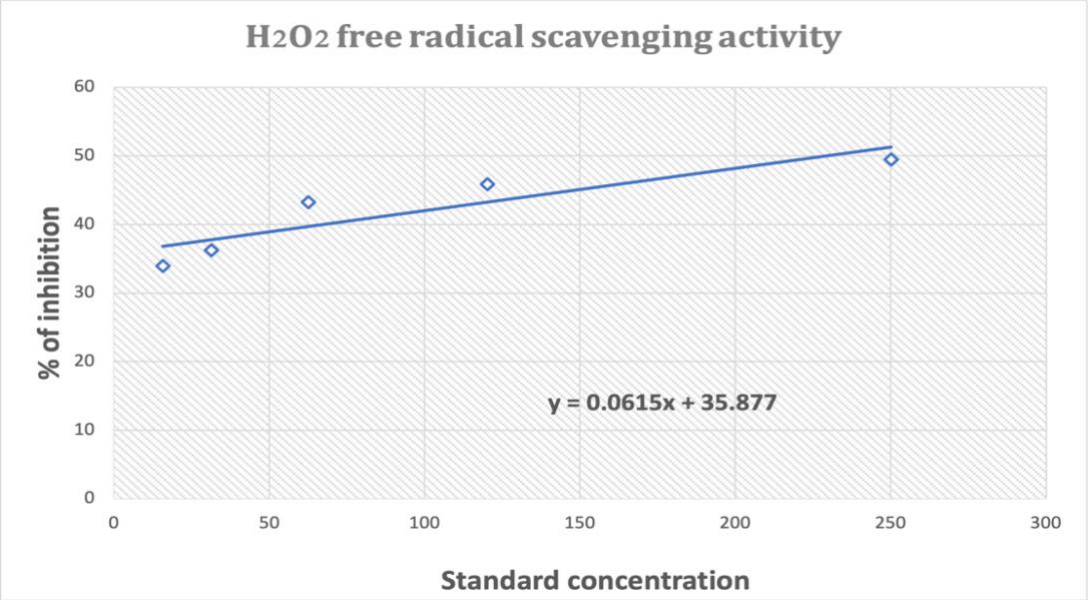

Figure 10: % Inhibition vs. Concentration Curve of Ascorbic acid (STD)

The absorbances of the sample were recorded across concentrations ranging from 250 to 15.63 µg/mL. A clear trend was observed where the absorbance decreased gradually with the reduction in sample concentration.

Additionally, it can also be observed that the H_2O_2 scavenging activity decreased as the concentration decreased. Furthermore, it was observed that the lowest level of inhibition was shown at a concentration of 15.63µg/mL, while the highest level of inhibition was seen at a dosage of 250µg/mL. The graph's y = 0.0615x + 35.877 was used to determine the IC_{50} value.

Now, to calculate IC_{50}, we find the concentration (x) where % of inhibition (y) is 50.

Now, from the equation: y = 0.0615x + 35.877;

Therefore, 50 = 0.0615x + 35.877; and x= 229.64227 µg/mL

The sample had an IC_{50} value of 229.64227 µg/mL.

3.1.3 Comparison of the sample and standard

According to the data, the percentage of H_2O_2 scavenging activity inhibited by the ethanol crude extract of *Marsilea minuta* plants was less than the concentration-corresponding L ascorbic acid. *Marsilea minuta*, the sample, demonstrated efficacious H_2O_2 free radical scavenging activity from the solutions. IC_{50} was 305.419 63 µg/mL for the sample. 229.64227 µg/mL was the IC_{50} value for the standard ascorbic acid. This suggested that in order for *Marsilea minuta* to have the same antioxidant effect as ascorbic acid, a greater quantity was needed.

3.2 Study of *Marsilea minuta's* total antioxidant capacity

3.2.1 Ascorbic acid calibration curve

From the experiment, the absorbance values of different concentrations of the sample and standard solution had been determined through the UV-Vis spectrophotometer.

Table 14: Ascorbic acid and Marsilea minuta absorbances of different concentrations

Concentration (µg/mL)	Standard absorbance	Sample absorbance
75	0.004	0.029
150	0.019	0.072
300	0.028	0.116
600	0.092	0.164
1200	0.126	0.285

By plotting the standard concentrations in x-axis and standard absorbances in y-axis, we get the calibration curve of ascorbic acid having the "y=mx+c" line value of y= 0.0001x + 0.0022.

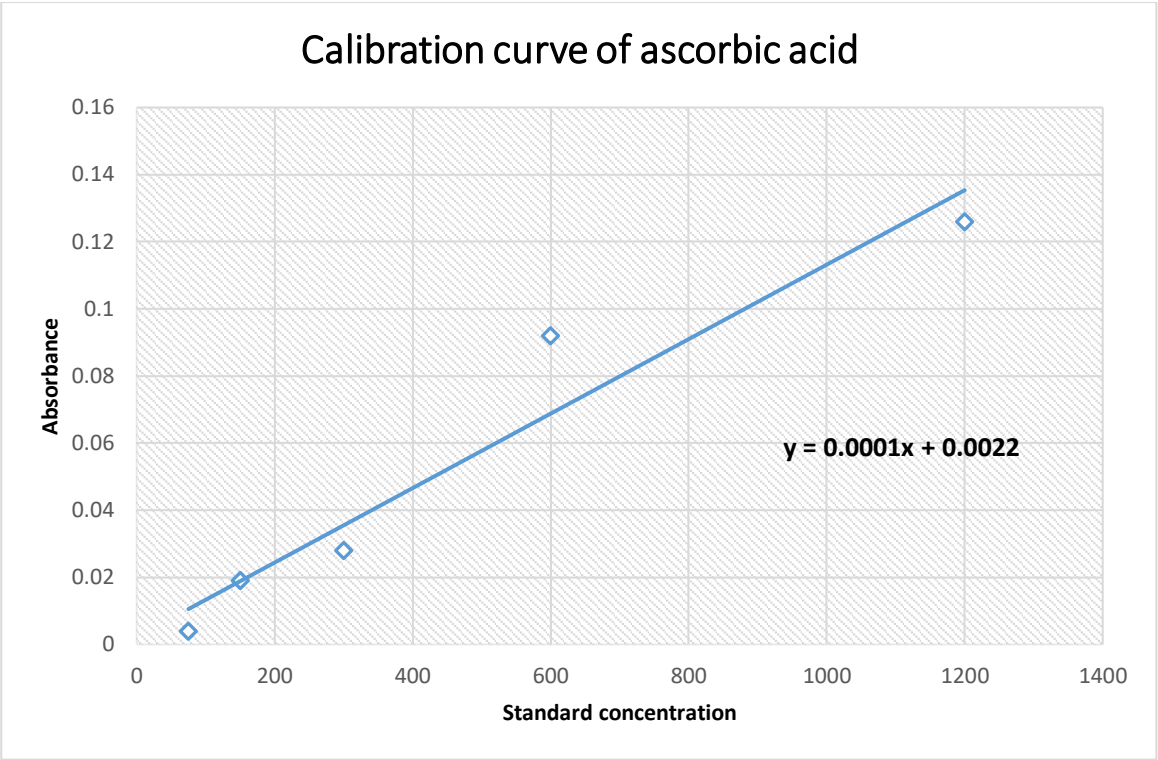

Figure 11: Absorbance vs concentration curve of ascorbic acid

The equation for ascorbic acid was determined to be y = 0.0001x + 0.0022 based on the calibration curve above. The formula was used to calculate *Marsilea minuta'*s total antioxidant capacity (TAC).

We know that TAC is expressed as mg of ascorbic acid equivalent (AAE) per gram of dry extract. And the formula to determine this is: TAC (mg AAE/g) = (c × V) / m

Where:

- *c* = concentration of ascorbic acid from the calibration curve (mg/mL) corresponding to the sample's absorbance,
- *V* = volume of extract used in the assay (mL),
- *m* = weight of the dry extract used (g)

Now, in the equation "y = 0.0001x + 0.0022", putting the values of standard absorbances (y) correspond to their standard concentrations, we will get the value of "x", that is the value of "c" – in the formula to determine the TAC (mg/g).

3.2.2 Table showing the total antioxidant capacity in *Marsilea minuta*

Table 15: Values of total antioxidant capacity of Marsilea minuta plant extract

Standard concentration (µg/mL)	Value of "c" (mg/mL)	TAC present (mg) of ascorbic acid per gram of dry extract in the sample
75	0.018	1.5
150	0.168	14
300	0.258	21.5
600	0.898	74.8334
1200	1.268	105.6667

The overall antioxidant capacity improved from 1.5 mg to 105.6667 mg of ascorbic acid equivalent (AAE) per gramme of the dried extract when the concentration of Marsilea minuta plant extract raised from 75 µg/mL to 1200 µg/mL. Furthermore, it was evident from the data that antioxidant activity increased gradually as concentration rose. Remarkably, the extract at 1200 µg/mL exhibited the highest antioxidant potential, corresponding to 105.6667 mg AAE/g of dry sample.

3.2.3 Graph of TAC of L-Ascorbic acid and *Marsilea minuta*

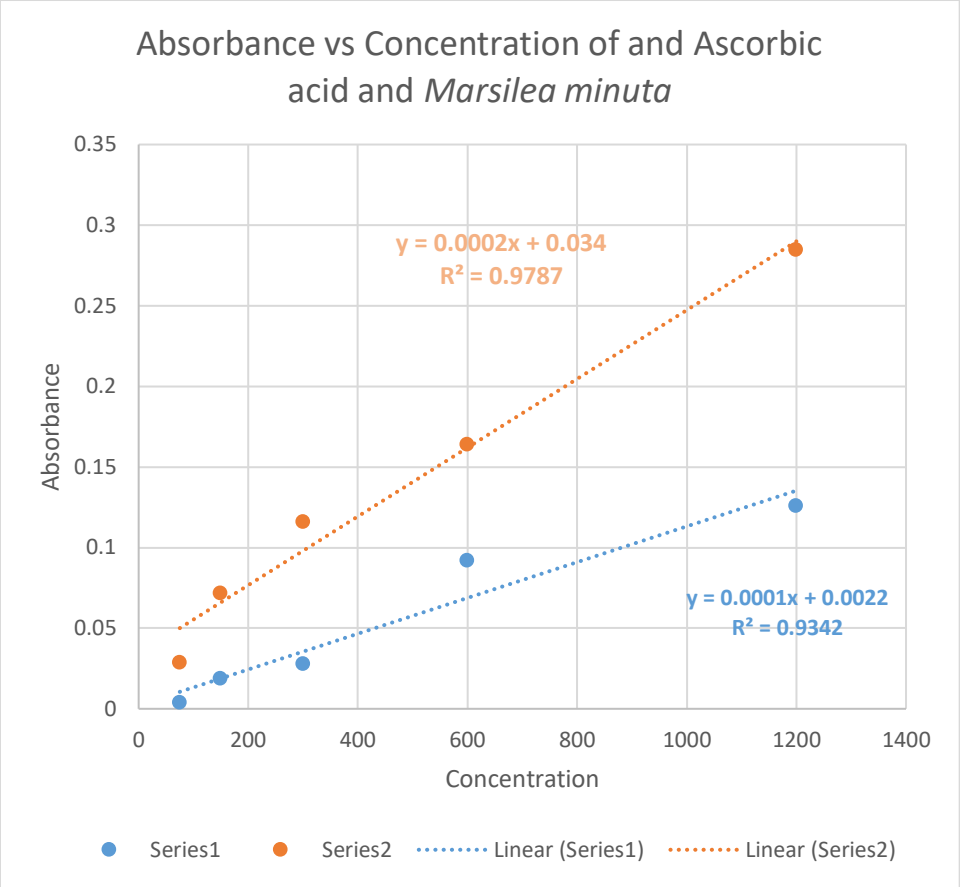

Figure 12: Absorbance vs. Concentration (µg/mL) graph of Ascorbic acid and Marsilea minuta

It was clear from the prior figure that both the sample's and the standard's absorbance increased noticeably with concentration. The calibration graphs were observed to be close to linear. Notably, the rise of the calibration curve of the sample (y=0.0002x + 0.034) is higher than that of standard (y=0.0001x + 0.0022). To conclude, we can tell that the sample had good total antioxidant capacity.

Chapter 4

Discussion

Marsilea minuta L., a pocket-sized fern species belonging to the family *Marsileaceae*, is commonly found in moist environments such as marshes, the edges of ponds, and rice fields. Various species within the *Marsilea* genus, including *Marsilea minuta*, are known to exhibit numerous pharmacological effects such as antioxidant, anti-inflammatory, analgesic, antimicrobial, antipyretic, hepatoprotective, and neuropharmacological activities. For example, *Marsilea quadrifolia* is reported to have antioxidant, anti-inflammatory, antidiabetic, hepatoprotective, and neuroprotective functions. Likewise, *Marsilea crenata* shows potential as an antioxidant and possesses anxiolytic and anticonvulsant properties, while *Marsilea aegyptiaca* has demonstrated notable antibacterial, antifungal, and wound-healing capabilities.

Given that *Marsilea minuta* shares similar phytochemical constituents with these related species—including flavonoids, alkaloids, tannins, phenolics, saponins, triterpenoids, and glycosides—it is expected to offer comparable therapeutic benefits. Therefore, this research aimed to explore the bioactive potential of *Marsilea minuta*, focusing primarily on its antioxidant efficacy. To achieve this, both qualitative and quantitative assessments were carried out using hydrogen peroxide (H_2O_2) scavenging activity and the total antioxidant capacity (TAC) assay, respectively.

The H_2O_2 scavenging assay revealed that *Marsilea minuta* possesses effective antioxidant properties, likely due to its ability to neutralize reactive oxygen species such as peroxides and hydroperoxides—compounds known to contribute to degenerative diseases like diabetes, cardiovascular disorders, and neurodegeneration. The IC_{50} value of *Marsilea minuta* was calculated to be 305.42 µg/mL, closely aligning with the standard ascorbic acid IC_{50} value of 229.64 µg/mL, suggesting that the plant extract is a strong free radical scavenger.

In the TAC assay, the maximum antioxidant capacity of *Marsilea minuta* extract was observed to be 104.67 mg of ascorbic acid equivalent (AAE) per gram of dry extract, while the lowest concentration measured 1.5 mg AAE/g. These findings confirm the plant's strong potential as a natural antioxidant, adding valuable knowledge to the fields of phytotherapy and oxidative stress-related disease management.

Chapter 5

Conclusion

Medicinal plants are widely recognized as valuable sources for developing treatments for numerous diseases. This study was therefore undertaken to evaluate the biological activities of the plant extract of *Marsilea minuta*. The findings demonstrated that the plant extract possesses considerable hydrogen peroxide (H_2O_2) scavenging activity, indicating strong antioxidant potential. Furthermore, the total antioxidant capacity (TAC) of *Marsilea minuta* was found to be significantly high. When these antioxidant results were compared with the reference standard L-ascorbic acid, the values were observed to be closely aligned. Additionally, the antioxidant activity showed a clear concentration-dependent relationship, as reflected by a linear trend in the graph. Based on these promising outcomes, it is highly advisable to conduct further in-depth studies to explore the pharmacological properties of *Marsilea minuta* assess its therapeutic effectiveness in vivo, and establish a reliable safety profile.

Chapter 6

Future Studies

Research into the bioactive properties of *Marsilea minuta* has demonstrated promising antioxidant activity. Similar antioxidant potential has also been observed in other species of the same genus, such as *Marsilea crenata* and *Marsilea quadrifolia*. The strong antioxidant effect suggests that this plant could be beneficial in managing diseases caused by oxidative stress. Future research could expand this evaluation by incorporating enzyme-based assays such as peroxidase activity tests and anthocyanin content assays.

Further chemical analysis, particularly through Gas Chromatography-Mass Spectrometry (GC-MS), could help identify the specific compounds responsible for the plant's antioxidant and cytotoxic activities. These findings could pave the way for advancements in pharmacognosy and pharmacology, especially when tested across various solvents like methanol.

Additionally, advanced analytical techniques such as High-Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), Infrared Spectroscopy (IR), and Nuclear Magnetic Resonance (NMR) could be used to isolate and characterize individual bioactive molecules.

Building on these findings, in vivo experiments and clinical evaluations would provide deeper insight into the therapeutic potential of *Marsilea minuta*. Furthermore, its potential antivenom, antidiabetic and antiviral, properties remain unexplored. By investigating into them, new medication candidates from natural sources may be developed and isolated.

Gaining insights into the antioxidant capacity can support future studies by clarifying the structural characteristics of active compounds. Additionally, using methanol extract of *Marsilea minuta* may effectively demonstrate synergistic interactions.

References

Ang-Lee, M. K., Moss, J., & Yuan, C.-S. (2001). Herbal medicines and perioperative care. *JAMA, 286*(2), 208–216. https://doi.org/10.1001/jama.286.2.208

Bardhan, S., Ashrafi, S., & Saha, T. (2018). Commonly used medicinal plants in Bangladesh to treat different infections. *ResearchGate*. https://www.researchgate.net/publication/324295037

Chavan, M. J., Wakte, P. S., & Shinde, D. B. (2019). Total antioxidant capacity of *Marsilea quadrifolia*Linn. using phosphomolybdenum assay. *Journal of Pharmacognosy and Phytochemistry, 8*(1), 123–126. https://www.phytojournal.com

Cooke, M. S., Evans, M. D., Dizdaroglu, M., & Lunec, J. (2003). Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB Journal, 17*(10), 1195–1214. https://doi.org/10.1096/fj.02-0752rev

Davies, K. J. A. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie, 83*(3–4), 301–310. https://doi.org/10.1016/S0300-9084(01)01250-0

Dean, R. T., Fu, S., Stocker, R., & Davies, M. J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochemical Journal, 324*(1), 1–18. https://doi.org/10.1042/bj3240001

Firenzuoli, F., & Gori, L. (2007). Herbal medicine today: Clinical and research issues. *Evidence-Based Complementary and Alternative Medicine, 4*(Suppl. 1), 37–40. https://doi.org/10.1093/ecam/nel080

Forman, H. J., & Zhang, H. (2021). Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nature Reviews Drug Discovery, 20*(9), 689–709. https://doi.org/10.1038/s41573-021-00233-1

Ghani, A. (2003). *Medicinal plants of Bangladesh: Chemical constituents and uses* (2nd ed.). Asiatic Society of Bangladesh.

Ghosh, D., & Banerjee, A. (2021). Taxonomic study and habitat analysis of selected *Marsilea*species in Eastern India. *International Journal of Botany Studies, 6*(2), 12–16.

Haida, Z., & Hakiman, M. (2019). A comprehensive review on the determination of enzymatic assay and nonenzymatic antioxidant activities. *Food Science & Nutrition, 7*(5), 1555–1563. https://doi.org/10.1002/fsn3.1012

Halberstein, R. A. (2005). Medicinal plants: Historical and cross-cultural usage patterns. *Annals of Epidemiology, 15*(9), 686–699. https://doi.org/10.1016/j.annepidem.2005.02.004

Halliwell, B., & Gutteridge, J. M. C. (2015). *Free radicals in biology and medicine* (5th ed.). Oxford University Press. https://global.oup.com/academic/product/free-radicals-in-biology-and-medicine-9780198717478

Haque, M. I., Chowdhury, A. B. M. A., Shahjahan, M., & Harun, M. G. D. (2018). Traditional healing practices in rural Bangladesh: A qualitative investigation. *BMC Complementary and Alternative Medicine, 18*(1), 62. https://doi.org/10.1186/s12906-018-2129-5

Kehrer, J. P., & Klotz, L.-O. (2015). Free radicals and related reactive species as mediators of tissue injury and disease: Implications for health. *Critical Reviews in Toxicology, 45*(9), 765–798. https://doi.org/10.3109/10408444.2015.1074159

Kumar, D., Arya, V., Bhat, Z. A., Khan, N. A., & Prasad, D. N. (2010). The genus *Marsilea*: A review of the traditional uses, phytochemistry and pharmacology. *Asian Journal of Pharmaceutical and Clinical Research, 3*(4), 27–32.

Lushchak, V. I. (2021). Free radicals, reactive oxygen species, oxidative stress and its classification. *Chemico-Biological Interactions, 224*, 164–175. https://doi.org/10.1016/j.cbi.2021.108989

Maity, T., Banerjee, S., Bishal, A., & Maji, M. (2022). An overview of antioxidant: Classifications, therapeutic effect & its applications. *ResearchGate*. https://www.researchgate.net/publication/361717419

Mondal, M., Hossain, M. S., Das, N., & Khalipha, A. B. R. (2019). Phytochemical screening and pharmacological potential of traditional medicinal plant. *Journal of Pharmacognosy and Phytochemistry, 8*(3), 42–49.

Nabavi, S. M., Ebrahimzadeh, M. A., Nabavi, S. F., Hamidinia, A., & Bekhradnia, A. R. (2008). Determination of antioxidant activity, phenol and flavonoid content of *Parrotia persica* Mey. *Pharmacology Online, 2*, 560–567.

Nathiya, S., Manikandan, P., & Viswanathan, P. (2014). Neuropharmacological potential of *Marsilea minuta* L. in experimental animal models. *Indian Journal of Natural Products and Resources, 5*(3), 237–243.

Pham-Huy, L. A., He, H., & Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *International Journal of Biomedical Science, 4*(2), 89–96. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3614697/

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D., & Bitto, A. (2017). Oxidative stress: Harms and benefits for human health. *Oxidative Medicine and Cellular Longevity, 2017*, 8416763. https://doi.org/10.1155/2017/8416763

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry, 269*(2), 337–341. https://doi.org/10.1006/abio.1999.4019

Qiu, J. (2007). Traditional medicine: A culture in the balance. *Nature, 448*(7150), 126–128. https://doi.org/10.1038/448126a

Rahmatullah, M., Azam, K., Rahman, M., Seraj, S., Mahal, M. J., Mou, S. M., Nasrin, D., Khatun, Z., Islam, F., & Chowdhury, M. H. (2011). A survey of medicinal plants used by Garo and non-Garo traditional medicinal practitioners in two villages of Tangail district, Bangladesh. *American-Eurasian Journal of Sustainable Agriculture, 5*(3), 350–357.

Rajendran, P., Nandakumar, N., Rengarajan, T., Palaniswami, R., Gnanadhas, E. N., Lakshminarasaiah, U., Gopas, J., & Nishigaki, I. (2014). Antioxidants and human diseases. *Clinica Chimica Acta, 436*, 332–347. https://doi.org/10.1016/j.cca.2014.06.004

Sachdev, S., Ansari, S. A., & Ansari, M. I. (2023). *Reactive oxygen species in plants*. Springer Nature.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects. *Journal of Functional Foods, 18*, 820–897. https://doi.org/10.1016/j.jff.2015.06.018

Sies, H. (2017). Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology, 11*, 613–619. https://doi.org/10.1016/j.redox.2016.12.035

Sinha, P., Tiwari, A., & Singh, J. (2015). Phytochemical and antioxidant analysis of *Marsilea quadrifolia* L. *International Journal of Pharmacognosy and Phytochemical Research, 7*(5), 981–985.

Spickett, C. M., & Pitt, A. R. (2010). Protein oxidation: Role in signalling and detection by mass spectrometry. *Amino Acids, 42*(1), 5–21. https://doi.org/10.1007/s00726-010-0585-4

Taniyama, Y., & Griendling, K. K. (2003). Reactive oxygen species in the vasculature. *Hypertension, 42*(6), 1075–1081. https://doi.org/10.1161/01.HYP.0000100443.09293.4f

World Flora Online. (2024). *Marsilea minuta* L. https://www.worldfloraonline.org