Investigation of *In-vitro* Antioxidant Potential of Methanolic Extract of *Marsilea minuta*

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 (B. Pharm.)

School of Pharmacy

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Declaration

It is hereby declared that

- The thesis submitted is my/our own original work while completing degree at BRAC University.
- 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.
- The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
- I have acknowledged all main sources of help.

Student's Full Name & Signature:

_____

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Approval

The thesis titled **"Investigation of *In-vitro* Antioxidant Potential of Methanolic Extract of *Marsilea minuta*"** submitted by Samiha Sabah Chaity (21146008), of Summer, 2024 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

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Ethics Statement

The study does not involve any animal or human trial.

Abstract

The present study focuses on the in vitro evaluation of the antioxidant potential of *Marsilea minuta*, a widely distributed aquatic fern known for its traditional medicinal applications. This study focuses on DPPH free radical scavenging activity and total phenolic content (TPC). Oxidative stress, resulting from the accumulation of reactive oxygen species (ROS), is a major contributor to various degenerative diseases. Natural antioxidants, particularly phenolic compounds, play a crucial role in mitigating oxidative damage. In this work, different extracts of *Marsilea minuta* were prepared, and their antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The total phenolic content was quantified using the Folin–Ciocalteu method. Among the tested extracts, the methanolic extract exhibited the highest DPPH radical scavenging activity and the greatest TPC, indicating a strong correlation between phenolic content and antioxidant capacity. These findings suggest that *M. minuta* contains significant levels of phenolic compounds contributing to its antioxidant activity, highlighting its potential as a natural source of antioxidants for therapeutic and functional food applications. Further studies are warranted to isolate active compounds and evaluate their in vivo efficacy.

Key words: *Marsilea minut*a, Antioxidant potential, DPPH assay, Total phenolic content (TPC), Natural antioxidants, Oxidative stress, Reactive oxygen species (ROS).

Dedication

I dedicate this thesis to my beloved parents for their unwavering support and sacrifices laid the foundation of my journey and to my dear husband for being my greatest cheerleader. This achievement is as much yours as it is mine.

Acknowledgement

I am grateful to almighty Allah for providing me the opportunity to work with such wonderful peoples from the school of pharmacy who have always been idealistic and encouraging throughout my journey.

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Chapter 1: Introduction

A chemically identifiable molecule that is either synthesized or sourced from plants or animals is referred to as a drug. For ages, plants have been chosen and utilized empirically as medicines, first as traditional concoctions and then as pure active ingredients. This knowledge and accumulated practice have been passed down from one generation to the next. The study of novel traditional medicines and phytotherapy is once again gaining scientific attention with the goal of creating affordable, non-toxic, and effective new medications—the latter of which is especially crucial for developing nations. Due to the exorbitant expense of Western medications and medical care, or because traditional remedies are more acceptable from a cultural and spiritual standpoint, a sizable section of the populace in developing nations consumes them (Taylor et al., 2001).

Traditional medicine has an extensive history in Bangladesh. Although the percentage of practitioners and their visiting patients have somewhat faded into the background with the rise of modern medicine and allopathic physicians, traditional medical practices have not disappeared yet. In actuality, traditional medicine is used in the treatment of a wide range of illnesses by the vast majority of Bangladesh's rural residents, who occupies the majority of the country's population. As of right now, Bangladesh has at least four types of traditional medical practices. These include the folk medical system, Ayurveda, Unani, and homeopathy. Prehistoric India is the origins of Ayurvedic medicine, which bases its diagnosis and treatment on alleged complications in the body's three "humors," or "Tridoshas" (Vata, Pitta, and Kapha). Bangladesh residing in Indian sub-continent, has a long tradition of using Ayurvedic medicine, with practitioners known locally as Kavirajes or Vaidyas. During the spread of Islam, Arab medical professionals adopted and developed unani medicine from Greeks, which had Hellenistic origins. In Unani medicine, the four humors, Phlegm (Balgham), Blood (Dam), Yellow Bile (afrā'), and Black Bile (Saudā') are the foundation for both disease diagnosis and treatment. One thing that unites Bangladeshi folk medicine with the Ayurvedic and Unani medical systems is that all three uses medicinal plants to cure patients. Folk medical practitioners, however, rely on straightforward plant formulations for treatment, in contrast to the Ayurvedic or Unani systems, which have their own established concepts of disease their causes and treatments. This can be an extremely complex formulations and processings of plants, animal parts, and minerals. Virtually all folk medicinal practitioners, often referred to as Kavirajes or Vaidyas, depends on their personal experiences to identify

illnesses and recommend medicinal plants for their cures because they lack authorized medical treatises. A Kaviraj's expertise is highly secured and passed on to a family member who has shown interest and worked as a pupil to the Kaviraj for a certain amount of time. Folk medicine is typically limited to the immediate family. As a result, even though two regions may have comparable plant species, the process of formulating medicinal plants for the treatment of different diseases is highly varied and might differ significantly between them (Akber et al., 2011).

1.1 Plant as drug source:

Natural items with medicinal qualities are being used for as long as human civilization, and for a long time, the primary sources of pharmaceuticals were mineral, plant, and animal goods. Synthetic products are preferred for pharmacological therapy as a result of the Industrial Revolution and the advancement of organic chemistry. This was due to the ease with which pure substances could be obtained, the ease with which structural changes could be made to create potentially safer and more active medications, and the growing economic might of pharmaceutical corporations. However, the significance of natural products is evidently significant, impact of the discovery of penicillin can be taken into account for the development of anti-infection therapy. Approximately 25% of medications prescribed globally are derived from plants among which 121 active compounds now in use. World Health Organization (WHO) has designated 11% of the 252 medications as basic and essential that are solely extracted from plants, while a generous portion are synthetic medications made from natural precursors. Digoxin from Digitalis spp., quinine and quinidine from Cinchona spp., vincristrine and vinblastine from Catharanthus roseus, atropine from Atropa belladona, and morphine and codeine from Papaver somniferum are a few examples of significant medications derived from plants. 60% of anti-tumor and anti-infectious medications currently marketed or undergoing clinical trials are thought to be natural (Yue-Zhong Shu, 1998). Most of these are still derived from wild or cultivated plants because they are not yet cheaply synthesized. Natural substances can serve as lead compounds, enabling the formation of biomimetic synthesis, the design and logical planning of novel medications, and the identification of novel therapeutic qualities not yet associated with established compounds (Hamburger and Hostettmann, 1991). Furthermore, substances derived from plants, including muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine, and phorbol esters, are crucial

instruments for pharmacological, physiological, and biochemical research (Williamson et al., 1996).

Alternative therapies and the therapeutic use of natural products, particularly those derived from plants, have gained popularity in recent years (Goldfrank et al., 1982, Vulto and Smet, 1988, Mentz and Schenkel, 1989). This interest in plant-based medications stems from a number of factors, including the fact that conventional medicine can be ineffective (e.g., side effects and ineffective therapy), that improper and/or abusive use of synthetic drugs can result in side effects and other issues, that a significant portion of the global population lacks access to conventional pharmaceutical treatment, and that folk medicine and ecological consciousness imply that "natural" products are safe. Legal agencies that oversee safety and efficacy protocols, however, do not always approve the use of these compounds, and numerous published studies highlight the poor quality of phytomedicinal product manufacturing, distribution, and prescriptions. The global market for over-the-counter phytomedicinal medicines was predicted to be worth $10 billion in 1997, growing at a rate of 6.5% annually (Soldati, 1997). In its health initiatives, the WHO takes phytotherapy into account and recommends fundamental protocols for the verification of medications derived from plants in underdeveloped nations (Vulto and Smet, 1998; OMS, 1991). There are now more publications in this area, and both private and public institutions are funding research projects all over the world. These factors include the contemporary social and economic perspective on health services, the demands of the pharmaceutical industry, and the realization that studying medicinal plants used in traditional medicine is a viable strategy for creating new medications (Elisabetsky, 1987a, Calixto, 1996). Over 50,000 plant samples have been investigated for anti-HIV activity and 33,000 samples for anti-tumor activity by the National Cancer Institute (NCI, USA). In order to promote natural products in Latin America and Africa, the International Program of Co-operation for Biodiversity (IPCB) was established in 1993. It links governments, businesses, and universities in a multidisciplinary program for the long-term development and conservation of the environment (Rouhi, 1997). Nowadays, major pharmaceutical firms like Syntex, Boehringer, Glaxo, CIBA, and Merck have specialized divisions devoted to researching novel medications derived from natural sources (Reid et al., 1993). Nevertheless, little research has been done on the possibility of using higher plants as a source of novel medications. Only a small portion of the estimated 250,000–500,000 plant species have undergone phytochemical investigation, and even fewer have had their pharmacological

characteristics thoroughly examined; in the majority of cases, only pharmacological screening or preliminary studies have been conducted. An estimated 5,000 species have been investigated for potential medical applications (Payne et al., 1991). Around 20,000 plant species from Asia and Latin America were examined for anti-tumor activity by the NCI between 1957 and 1981, but none of them were checked for additional pharmacological properties (Hamburger and Hostettman, 1991).

1.2 *Marsilea minuta* as Medicinal Plant:

The aquatic or sub-aquatic fern *Marsilea minuta*, sometimes referred to as pepperwort, little water clover, water clover, or Gelid water layer, is an extremely soft and irregular pteridophyte that is a member of the Marsileacae family.(Iqbal et al., 2021)**.** The common water fern, or genus Marsilea, is found all around the world. One of the common Indian plants that is frequently found in flooded and wet lowlands is water clover, or *M. minuta Linn* (Marsileaceae). The entire plant has hypnotic, cooling, digestive, diuretic, astringent, sweet, and expectorant properties. Rich in antioxidant activity, the phytochemical chemicals include terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites endow plants.(De Britto et al., 2013)

Due to its aphrodisiac properties, a whole-plant extract is utilized as an antifertility treatment. The herb was also used as an anticonvulsant and to cure drowsiness. In addition to being used to avoid epilepsy, migraine pain, and sleep disorders, the plant is suggested for the treatment of insomnia. Folk medicine practitioners recommend the extraction of leaves as a nose bleeding inhibitor. The leaves of *M. minuta* and *Shorea robusta* were boiled to reduce swelling. The extract from leaves lowers the levels of triglycerides and cholesterol in the liver and blood when given to gerbils for consumption. The fresh plant was cooked and applied to avoid spasmodic contractions of the bladder, leg, and urethra muscles. *M. minuta* root extract was utilized to treat atopic dermatitis. (Iqbal et al., 2021)According to studies, a large number of these substances contain antiviral, antibacterial, anticancer, antimutagenic, anticarcinogenic, anti-inflammatory, and antiatherosclerotic properties. Because of this, the *M. minuta* plant was thoroughly investigated for possible phytochemical components that could support its therapeutic benefits.(De Britto et al., 2013). Marsilea quadrifolia is believed to be closely related to *M. minuta*. Both belong to the broad Old-World subgroup known as "Marsilea" along with *M. angustifolia, M. drummondii, M. crenata,*

and *M. fadeniana,* according to molecular phylogenetic analysis of the genus Marsilea. It also shows that *M. crenata* is a synonym of *M. minuta* (Table 1).(Iqbal et al., 2021)

Table 1. Taxonomy Classification of M. minuta (Iqbal et al., 2021)

Sl no	**Kingdom**	**Plantae**
1.	Subkingdom	Tracheobionta
2.	Division	Pteridophyta
3.	Class	Polypodiopsida/Pteridopsia
4.	Order	Hydropteridales
5.	Family	Marsileaceae
6.	Genus	Marsilea L
7.	Species	M. minuta

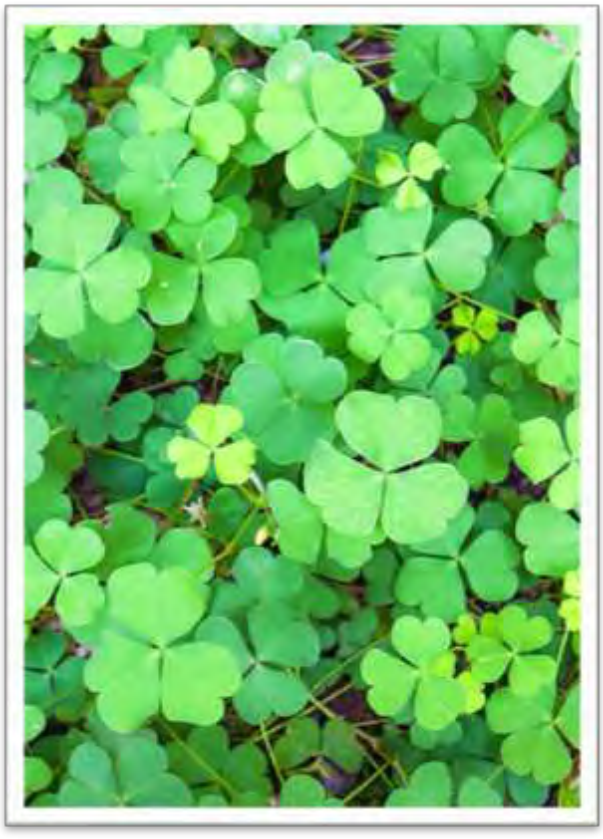

Figure 1. Structure of Marsilea

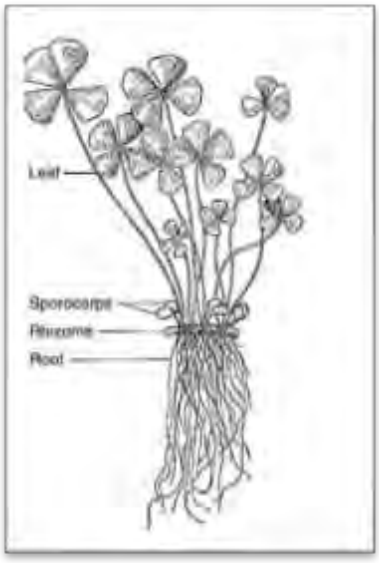

Figure 2. Leaves of M. minuta

1.2.1 Geographical Distribution of *M. minuta*:

M. minuta is predominantly found in nearly all Indian states, especially in tropical Africa and Asia. It typically grows on sandy or clay substrates in freshwater or brackish water (Trinidad). It can be found in a swamp as well.(Sajini et al., 2019)

1.2.2 Other Species of *M. minuta*:

Different species of *M. minuta* are *M. aegyptica, M. ancyclopoda, M. apposita, M. burchellii, M. coromandelina, M. crenata, M. crotophora, M. deflexa, M. distorta, M. drummondii, M. ephippiocarpa, M. farinosa, M. fenestrata, M. hirsuta, M. aminuta L., M. mollis, M. mutica, M. nubicam, M. oligospora, M. quadrifolia L., M. schelpeana, M. unicornis, M. vera, M. vestita M. villosa* and *M. villifolia*.(Sajini et al., 2019)

1.2.3 Phyto-Constituents of *M. minuta*:

M. minuta contains anthroquinones, terpenoids, phenol, flavonoids, saponins, quinones, tannins, coumarins, and total sugar. It is also said to contain Marsiline. A. Marsileagenin. Chalcone-O-glucoside and quercetin-3-O-glucoside Flavonoids such as quercetin-3-O-galactoside and kaempferol-3-O-glucoside were reported. Quercetin-3-rutinoside (rutin) and naringenin-7-O-glucoside Hentriacontane and β-sitosterol are also reported to be present. The methanolic extract

of *M. minuta* leaves contained about 36 phytocompounds, including phytol, n-hexadecanoic acid, 9-Pentadecadien-1-ol, 2-Cyclohexane-1-one, 4-hydroxy-3, 5, 6-trimethyl-4 (3-oxo-1-butenyl), 9, 12, 15-Octadecatrienoic acid, 3, 7, 11, and 15-Tetramethyl-2-hexadecane-1-ol. Glycerin, 1,19-Eicosadiene, n-Hexadecanoic acid, methyl ester, and 10-Octadecenoic acid methyl ester, as well as benzofuran, 2, 3-dihydro-3, 7, 11, and 15- Tetramethyl-2 hexadecene-1-ol, were among the 27 bioactive chemicals found in the methanolic extract of *M. minuta* stem.(Sajini et al., 2019)

1.2.4 Pharmacological Activity of *M. minuta*:

Table 2. Pharmacological activity of M. minuta

Sl no.	Pharmacological Activity	Parts Used	Extracts used
1.	Antidiabetic activity	Leaves	Ethanolic extract
2.	Antimicrobial activity	Whole plant	Petroleum ether, chloroform-ethyl acetate, alcohol, and water.
3.	Antipyretic and analgesic activity	Leaves	Ethanolic extract
4.	Anti-amnestic activity	Whole plant	Ethanolic extract
5.	Anti-aggressive activity	Whole plant	-
6.	Hepatoprotective activity	Whole plant	Methanolic extract
7.	Antifertility activity	Whole plant	Methanolic extract
8.	Antitumor activity	Whole plant	Ethanolic extract
9.	Antidepressant activity	-	Ethanolic extract
10.	Antioxidant activity	Leaves	Methanolic extract

1.3 Rationale of the project:

The importance of finding and assessing bioactive chemicals from medicinal plants has been brought to light by the growing interest in plant-based therapies around the world. The aquatic fern *M. minuta* is found throughout Bangladesh and other tropical and subtropical areas. This herb, which has long been utilized in folk medicine for its alleged health benefits—such as reducing inflammation, anxiety, and insomnia—has gained notice for its possible pharmacological qualities.

But there is still little scientific proof of it, especially when it comes to its cytotoxic and antioxidant properties.

Numerous chronic diseases, such as cancer, heart problems, and neurological disorders, are linked to oxidative stress, which is brought on by an imbalance between the body's antioxidants and free radicals. Therefore, in the continuous search for therapeutic medicines, it is essential to screen natural sources for antioxidant molecules. Similar to this, cytotoxicity screening is essential for finding new anticancer drugs since it finds compounds that can specifically stop cancer cells from proliferating.

Using well-established in vitro tests, this project seeks to examine the cytotoxic and antioxidant capabilities of extracts from *M. minuta*. It is anticipated that the results will offer scientific proof for its conventional applications and could result in the identification of novel lead compounds for drug development. Furthermore, the findings may stimulate more research on the medicinal uses of *M. minuta* and add to the expanding corpus of knowledge in ethnopharmacology and natural product studies.

1.4 Aim of the study:

In order to observe *M. minuta*'s potential as a source of bioactive chemicals for medicinal applications, the purpose of this study is to examine the antioxidant and cytotoxic qualities of plant extracts by using in vitro tests.

1.5 Objectives of the Study:

- To arrange and use suitable extraction technique to create plant extracts from *M. minuta*.
- To identify the presence of useful elements in extracts using both qualitative and quantitative phytochemical methods.
- To use in vitro tests like the DPPH free radical scavenging assay, to identify the antioxidant property of *M. minuta*.
- To use the appropriate techniques to evaluate the plant extracts' cytotoxic activities.

- To evaluate and contrast the outcomes in order to observe *M. minuta*'s possible therapeutic efficacy as a natural source of cytotoxic agents and antioxidants.

Chapter 2: Methodology

2.1 Collection and authentication of the plant:

M. minuta was preferred for the study. Despite some prior research on its cytotoxic and antioxidant properties, it was selected for study by switching between the previously investigated solvent and plant sections. It was selected for availability and comparison with the earlier study.

2.1.1 Chemical Investigation of *M. minuta*:

Table 3. Plant Introduction

Name of the plant	Family	Plant part
Marsilea minuta	Marsileaceae	Whole plant

The plant *M. minuta* was collected from Rajshahi, Bangladesh in January 2025. The plant was collected and was submitted to the National Herbarium of Bangladesh (NHB) for the verification of the plant. The plant was verified (ACCESSION NO.: DACB-121316) there and its voucher specimen was collected and the taxonomist of National Herbarium of Bangladesh, Mirpur, Dhaka authenticated it.

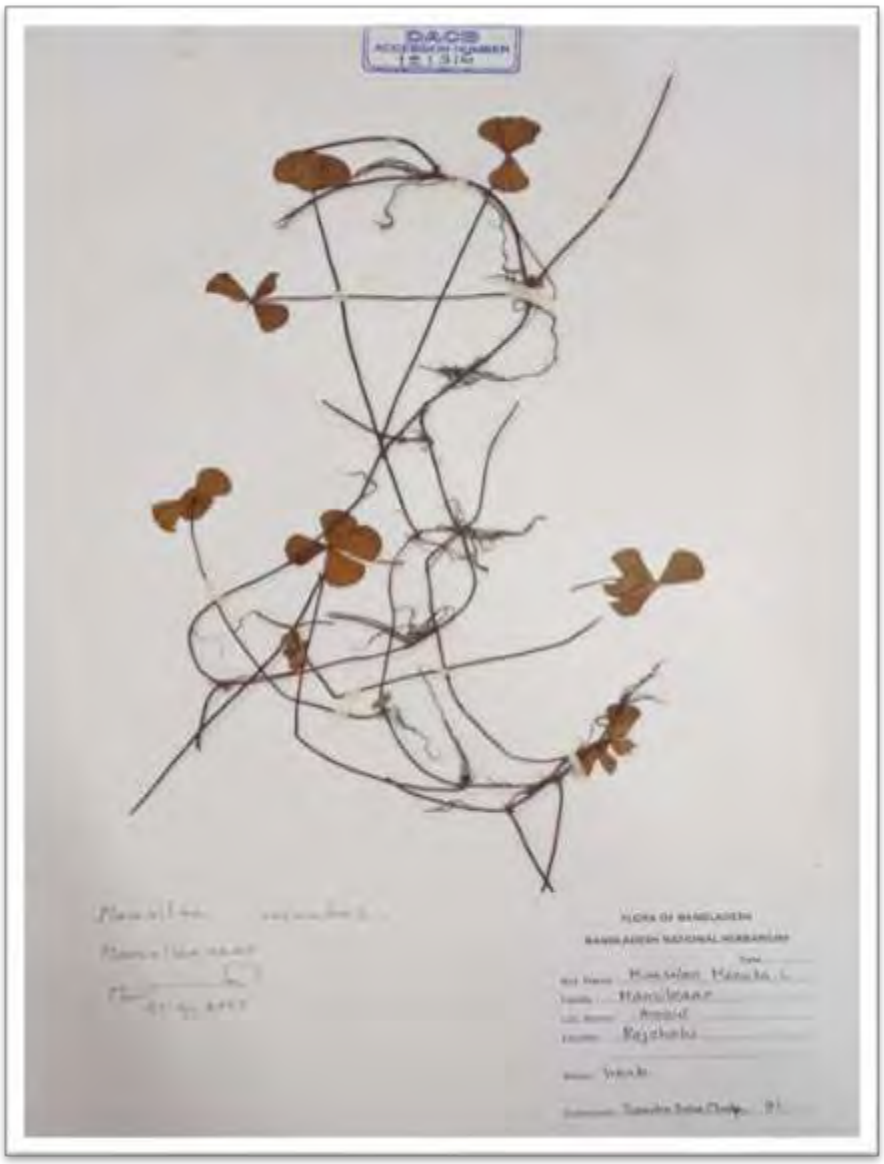

Figure 3. M. minuta plant obtained from National Herbarium

2.2 Extraction Process:

Numerous steps are involved in the process of extraction of medicinal plant:

The total extraction process can be alienated in two parts virtually which is shown by flow chart.

a) Preparation of plant material and its drying
b) Extraction technique

2.2.1 Preparation of plant material and drying

The whole plant was washed thoroughly with clean water so that any dirt or dust can be removed. Thereafter, the washed plant was shed dried for some days until they were properly dried and ready for size reduction.

2.2.2 Extraction Technique

A. Preparation of the plant material

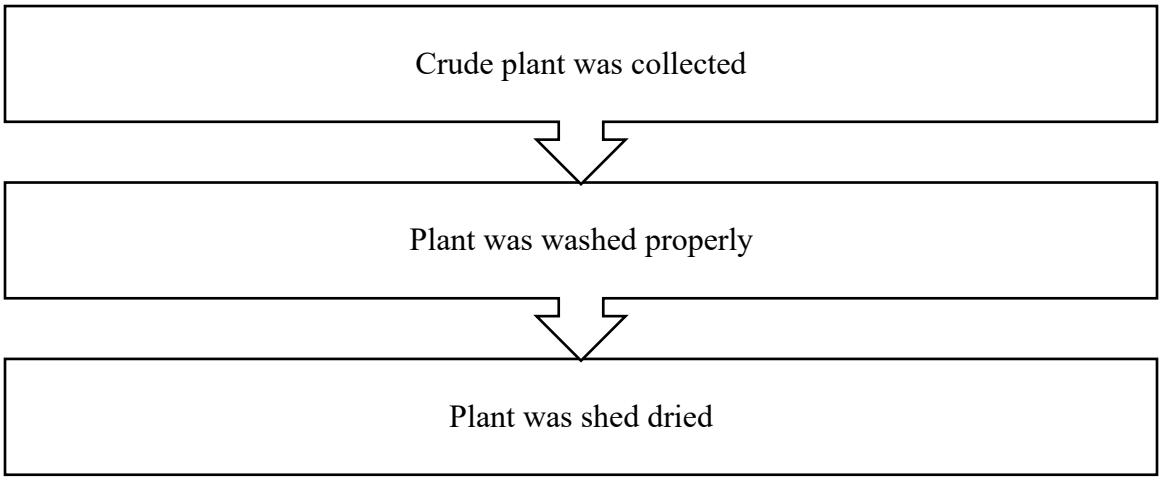

Figure 4. Collected M. minuta

B. Extraction procedure

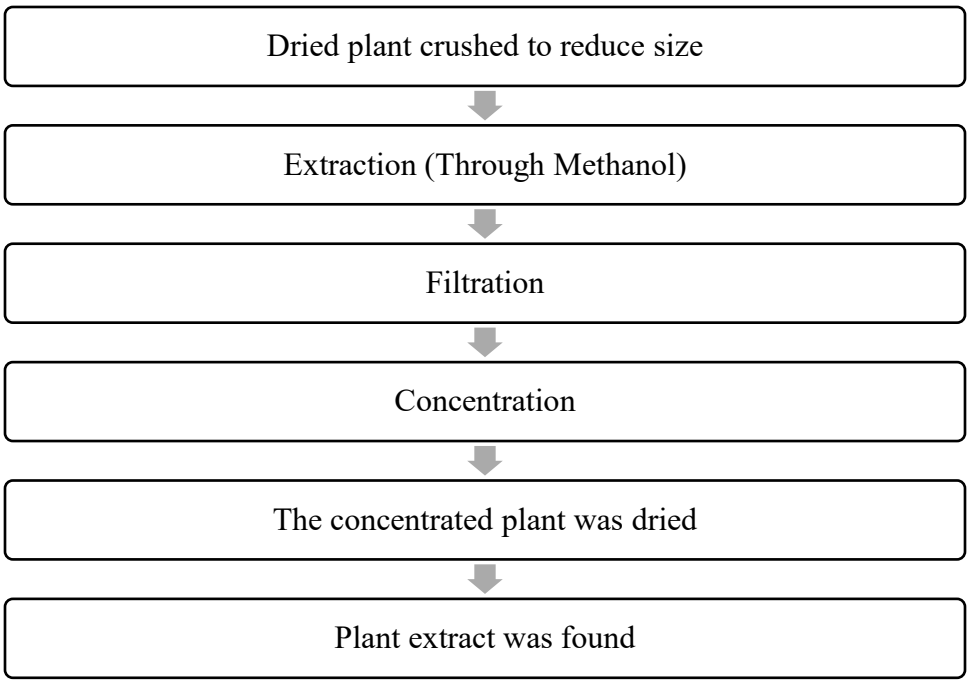

Figure 5. Dried and Crushed M. minuta

2.3 Reduction of plant size and weighing

A high-capacity grinding machine was utilized to process the crisp, clean plants into a coarse powder. The coarse powder was securely later on and was stored in an airtight plastic container to prevent contamination. After labeling two plastic containers with the relevant information about the leaves and stem, they were placed in a dark, cool location to await the need for further investigation. The entire amount of plant powder was then calculated by weighing the plants and noted.

2.4 Extraction

Methanol was used as an organic solvent to macerate the extraction process. The powdered plants were steeped in methanol for three days at room temperature (22–25 °C), in a different beaker with periodic stirring.

Table 4. Amount of methanol used in the maceration process and the weight of the powdered plants:

Weight of plant powder(gm)	200
Volume of Methanol(ml)	1500

The maceration procedure was a two-layer phase. Filtration separates the superior phase with the methanolic solution, resulting in a cloudy precipitate of plant parts in the lowermost phase.

Figure 6. Solvents

Figure 7. Plant powder emerged in solvent (Methanol)

2.5 Filtration

After three days of maceration, the contents of the beaker were decanted after first being filtered through cotton cloth and filter paper.

2.6 Concentration

Following filtration, the filtrate was collected and concentrated using a Heidolph rotary evaporator set at 100 rpm and 30-degree Celsius. The mixture was then moved onto the petri dishes in order to be dried under LAF. The same process was used for the stem, which was gathered in a different petri dish.

2.7 Drying of extracts

To eliminate the possibility of microbiological amplification during the extract's drying process, Laminar Air Flow (LAF) was utilized to evaporate the solvent. Petri dishes were placed beneath laminar air flow immediately after the concentration procedure in order to distribute the extract's solvent. Following the effective drying of the extract, the petri plates were securely covered with aluminum foil and refrigerated for further analysis.

Figure 8. Filtrate was collected and concentrated using a Heidolph rotary evaporator

Figure 9. Laminar Air Flow (LAF) was utilized to evaporate the solvent

Figure 10. Extract in Petri dish

2.8 In-Vitro antioxidant activities:

Numerous in vitro techniques have been created to assess the effectiveness of natural antioxidants, whether they are plant extracts or pure molecules. There are two main categories of in vitro techniques: 1) Hydrogen atom transfer reactions, such as those involving oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant potential (TRAP), and carotene bleaching; 2) Electron transfer reactions, such as those involving trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), diphenyl-picryl-hydrazyl radical scavenging assay (DPPH), superoxide anion radical scavenging assay, hydroxyl radical scavenging assay, nitric oxide radical scavenging assay, and total phenol assay. These techniques are widely used because of their great sensitivity and rapidity. However, due to the complexity of phytochemicals, it is imperative to employ many methods to assess the antioxidant capability of plant materials (Salazar et al., 2008). The following describes the most popular and unusual antioxidant assays as well as a number of standards that can be used as positive controls (Chanda & Dave, 2009). There are several in-vitro techniques for determining a plant extract's antioxidant activity. Two techniques were chosen from a variety of approaches to assess the antioxidant activity of stem extract.

The techniques are:

1. DPPH or diphenyl-picryl-hydrazyl radical scavenging assay
2. TPC, or total phenolic content.

2.8.1 DPPH scavenging activity:

According to Braca et al., 2002, the DPPH free radical scavenging assay of the extract of *M. minuta* was determined by using this method.

Figure 11. DPPH

Reagent and chemicals

Name of Reagent/Chemical Suppliers

1. DPPH Sigma Aldrich, U.S.A.
2. Methanol, Active Fine Chemicals Ltd., Bangladesh
3. L-ascorbic acid, Merck, German

Reagent preparation

2 mg DPPH was dissolved in 50 mL methanol and 0.004% (w/v) DPPH solution was prepared. The solution was then kept at -4°C in the refrigerator till before use.

Sample and standard preparation

The sample stock solution was prepared by dissolving 120 mg of stem extract in 10 mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 6 serially diluted concentrations of the sample concentrations were prepared as 1200, 800, 400, 200, 100 and 50 µg/mL

Using L-ascorbic acid, the standard was prepared in the same manner. The extract was made by 6 serially diluted concentrations which ranges from 1200-50 µg/mL.

Preparation of blank solution

3 mL methanol was used to prepare the blank solution.

Experimental procedure

a) In test tubes 1 mL of each of the fractions of sample and standard (L-ascorbic acid) were taken.

b) 2mL of 0.004% (w/v) DPPH solution was added to each of the test tube.

c) After that, at room temperature for 30 minutes the test tubes were incubated. Then, the absorbance of the resulting solutions and control (DPPH and methanol was measured at 517nm against blank (methanol) using a spectrophotometer (U-2910 UV-Vis Spectrophotometer).

d) Thereafter, the percentage of free radical scavenging activity (%FRS) was calculated from equation given below:

% Inhibition of free radical scavengers = (A0 – A1) x 100/A0

Where, A0 = The absorbance of the control

A1 = The absorbance of the sample/standard

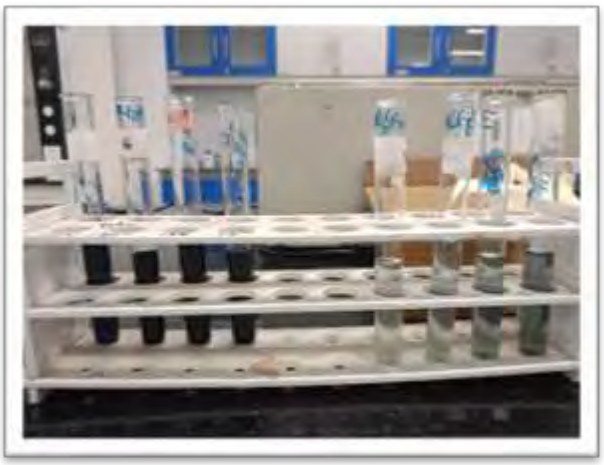

Figure 12. Diluted sample and standard

2.8.2 Total Phenolic Content:

According to Wolfe, Wu and Liu (2003), the TPC of the stem extract of *M. minuta* was determined by using this method.

Reagents and chemicals

Name of Reagent and the chemical Suppliers

1. Folin-Ciocalteu Reagent (FCR), LOBAChemie Pvt. Ltd., India
2. Gallic acid monohydrate (Standard), Sigma Aldrich, USA
3. Sodium carbonate,MerckSpecialities Pvt. Ltd., Mumbai
4. Methanol, Active Fine Chemicals Ltd., Bangladesh

Reagent preparation

25 mL of FCR was taken in a 250 mL volumetric flask and then it was diluted with distilled water to 250 mL mark. In this way 250 mL of 10 % FCR solution was prepared.

7.5 g of Sodium Carbonate was taken in a 100 mL volumetric flask and diluted with distilled water to 100 mL mark. This makes 100 mL of 7.5 % (w/v) Sodium Carbonate.

Sample and standard preparation

The sample stock solution was made by dissolving 120 mg of stem extract in 10 mL of methanol to produce 12 mg/mL of concentration.

By serial dilution of the sample stock solution so that 4 serially diluted concentrations of the sample concentrations were prepared as 120, 80, 40 and 20 µg/mL.
Using Gallic acid, the standard was prepared in the same manner. The extract was made by 4 serially diluted concentrations which ranges from 120 to 20 µg/mL.

Preparation of the blank

The solution of blank was prepared by 5 mL FCR solution and 4 mL Sodium Carbonate and 1 mL methanol to make the volume up to 10 mL.

Experimental procedure

a) 1 mL of each of the fractions of sample and standard (gallic acid) were taken in test tubes.

b) 5mL of FCR solution was added in each test tube separately.

c) Following that 4ml sodium carbonate was added.

d) Each of the mixture was vortexed in a vortex machine for 15s and followed by stand for 30 min at 40 °C in a water bath.

e) After that, the standard and sample absorbance was measured against blank measured at 765 nm using spectrophotometer (U-2910 UV-vis spectrophotometer).

f) The total phenolic content, C, for each of the fractions were expressed as Gallic

g) Acid Equivalents using the following equation:

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

Where, C = Total content of phenolic compounds, milligram of gallic acid per gram of dried plant extract, expressed as gallic acid equivalent (GAE)
c = concentration of gallic acid obtained from calibration curve (mg/mL)
V = Volume of sample solution (ml)
m = weight of the sample (g)

Chapter 3: Results and Calculation

The formula mentioned in the methodology was used for the calculation and determination of the results.

3.1 DPPH (1,1-diphenyl-2-picryl hydrazyl) free radical scavenging assay

Table 5. DPPH free radical scavenging assay (Absorbance vs. concentration) (Standard L- ascorbic acid)

Standard (L-ascorbic acid) concentration(µg/ml)	Absorbance	% of inhibition
50	0.359	59.16%
100	0.230	73.83%
200	0.156	82.25%
400	0.073	91.70%
800	0.029	96.70%
1200	0.011	98.75%

3.1.1 Graphical presentation of DPPH % of inhibition of Standard vs. concentration

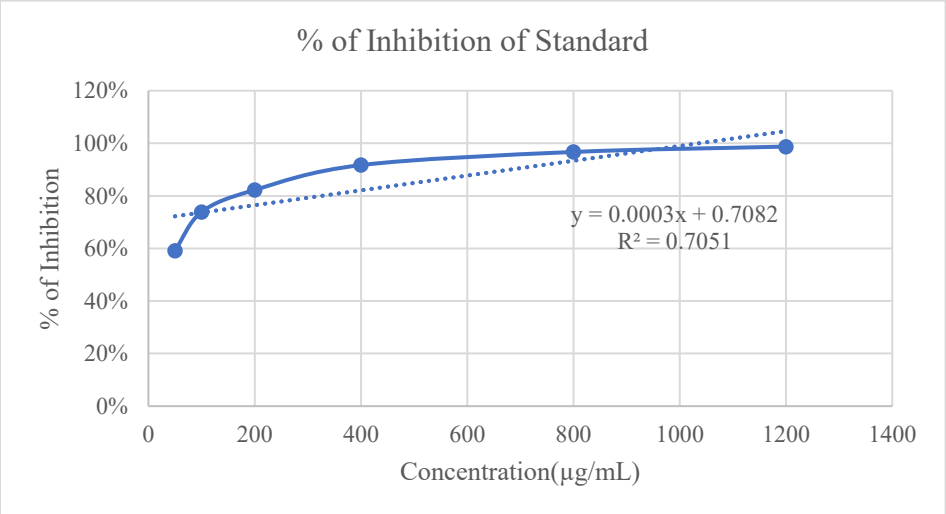

Figure 13. % Of Inhibition of Standard

Table 6. DPPH free radical scavenging assay of M. minuta (Absorbance vs. concentration) (Sample)

Concentration(µg/mL)	Absorbance	% Of Inhibition
50	0.793	9.70%
100	0.687	21.84%
200	0.516	41.30%
400	0.485	44.82%
800	0.397	54.84%
1200	0.306	65.19%

3.1.2 Graphical presentation of DPPH % of inhibition of sample vs. concentration

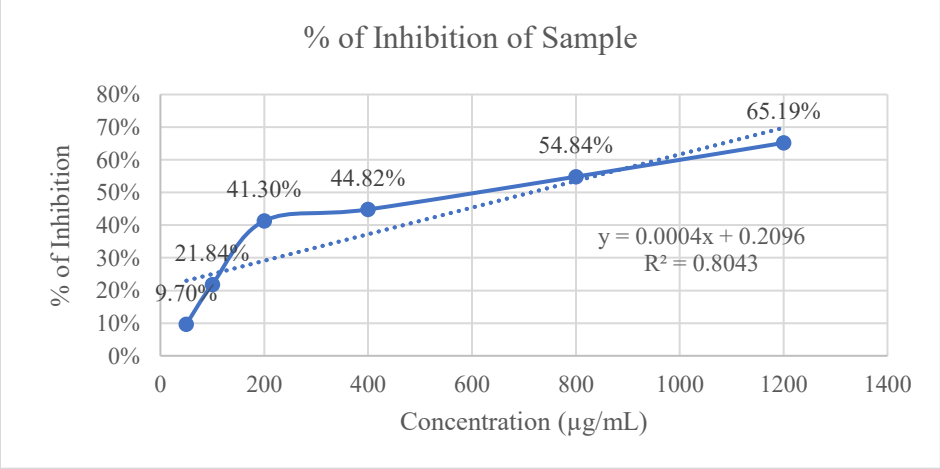

Figure 14. % of Inhibition of Sample

Interpretation: From the table and figure, it was observed that with an increase in concentration of *M. minuta* from 50 to 1200 µg/mL, the absorbance decreased slowly from (0.793 to 0.306) in comparison to that of ascorbic acid (0.359 to 0.011). Therefore, with a decrease of absorbance the

% free radical of inhibition increases. The highest % of inhibition was shown at the highest concentration of 1200 µg/mL. At the lowest concentration of 50 µg/mL, the extract showed notable % of inhibition which was 9.70 %.

3.2 Determination of total phenolic content (TPC)

3.2.1 Calibration curve of gallic acid

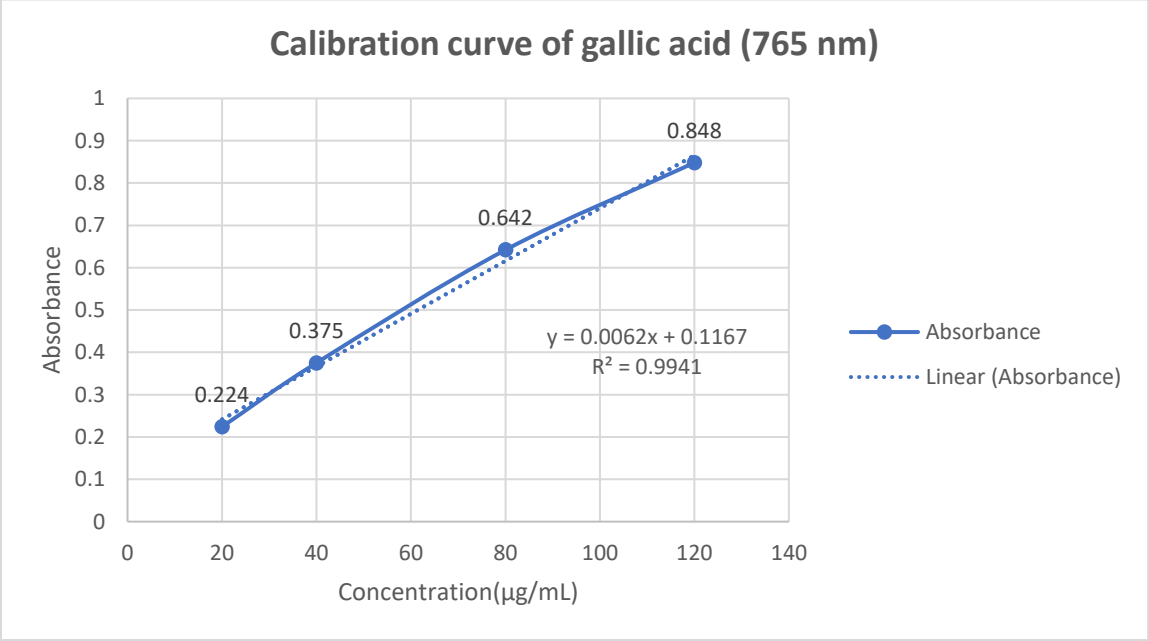

Figure 15. Calibration curve of gallic acid (GA) at 765 nm for determining TPC in M. minuta

Table 7. Total phenolic content (TPC) in M. minuta

Concentration of *M. minuta* (µg/mL)	**TPC (GAE) mg of gallic acid per gram of dried extract**
20	0.008
40	0.084
80	0.346
120	0.550

Interpretation: It is observed that the above concentration of *M. minuta* was raised from 20 to 120 µg/mL, the total phenolic substance also raised from 0.008 to 0.550 mg of gallic acid per gram

of dried extract. Therefore, it indicates that with increases in total phenolic content, its antioxidant activity also increases. At the lowest concentration extract showed 0.008 mg of gallic acid per gram of dried extract. With the highest concentration extract showed 0.550 mg of gallic acid per gram of dried extract.

Chapter 4: Discussion

The project began with the selection of the plant by literature review and online search. Based on that *M.minuta* was chosen for this study. For the determination of antioxidant potential methanolic plant extract was chosen as previous study was done on plant extract. The Total Phenolic Content (TPC) assay and the DPPH free radical scavenging method were used to assess *M. minuta*'s antioxidant activity. The information supports the idea that *M. minuta* has a significant capacity to scavenge free radicals by showing a concentration-dependent increase in antioxidant potential.

From the obtained result of DPPH test, it was observed that with an increase in concentration of *M. minuta* from 50 to 1200 µg/mL, the absorbance decreased slowly from (0.793 to 0.306) in comparison to that of ascorbic acid (0.359 to 0.011). Therefore, with a decrease of absorbance the % free radical of inhibition increases. The highest % of inhibition was shown at the highest concentration of 1200 µg/mL. At the lowest concentration of 50 µg/mL, the extract showed notable % of inhibition which was 9.70 % and this signified that it has slight antioxidant potential. Other concentrations also showed notable % of inhibition and by comparing them with the standard it was observed that the sample showed notable inhibition which was close to standard. This pattern points to a dose-dependent antioxidant action, which is frequently seen in phenolic compounds originating from plants (Debnath & Das, 2016). A greater availability of hydrogen-donating antioxidants that neutralize DPPH free radicals is shown in the increasing inhibition percentage as extract concentration rises (Nath et al., 2023).

In support of this, the TPC results (Table 7) demonstrate that phenolic content rises with concentration as well. It is also observed that the concentration of *M. minuta* was raised from 20 to 120 µg/mL, the total phenolic substance also raised from 0.008 to 0.550 mg of gallic acid per gram of dried extract. Therefore, it indicates that with increases in total phenolic content, its antioxidant activity also increases. At the lowest concentration extract showed 0.008 mg of gallic acid per gram of dried extract. With the highest concentration extract showed 0.550 mg of gallic acid per gram of dried extract. Because they can stabilize free radicals by giving them electrons or hydrogen atoms, phenolic substances are well-known for their antioxidant qualities (Rahman et al., 2018).

Therefore, we have noticed that small amount of gallic acid per gram of dried extract can provide significant % of inhibition. The result of DPPH and TPC of *M. minuta* can be attributed to the fact that it possesses antioxidant potential (Keen et al., 2005). This study's substantial association between TPC and DPPH activity supports earlier research showing that phenolic content plays a significant role in medicinal plants' antioxidant activity (Bhowmik et al., 2020).

The findings in this experiment is the scientific proof of the presence of medicinal value in *M. minuta.* The results confirm that *M. minuta* includes bioactive substances that greatly enhance its antioxidant capacity, particularly phenolics. As a result, this plant may provide a natural supply of antioxidants for use in the food, pharmaceutical, or nutraceutical industries.

Chapter 5: Conclusion

The entire plant *M. minuta* has hypnotic, cooling, digestive, diuretic, astringent, sweet, and expectorant properties. Rich in antioxidant activity, the phytochemical chemicals include terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites endow plants (De Britto et al., 2013) . Two different investigations were done rarely to decide the *in vitro* antioxidant activity on plant extract. To evaluate the free radical scavenging capacity DPPH test was performed which showed that at the lowest concentration 50 µg/mL the extract showed highest inhibition 9.7%% which signified that it has antioxidant potential. Total phenolic content showed that at the highest concentration extract showed 0.55 mg of gallic acid per gram of dried extract. So, it is established that *M. minuta* has antioxidant potential after performing four individual tests.

Direction for the future

Since *M. minuta* has antioxidant capacity, an in-vivo pharmacological assessment of its antioxidant potential should be conducted. In the future, it will be possible to isolate additional compounds and examine other bioactivities of this plant that have not been done before. Working with *M. minuta,* a fast-growing aquatic fern, could have a lot of uses in the future in medical, farming, nutrition, and managing the environment. Working with the rapidly growing aquatic fern *M. minuta* has enormous potential for use in environmental management, agriculture, medicine, and nutrition in the future. Because it contains bioactive substances like flavonoids and phenolics, it has a considerable antioxidant capacity, which is one of its most important advantages. These antioxidants can aid in the fight against oxidative stress, which has been connected to cardiovascular disorders, cancer, and aging (Debnath & Das, 2016; Nath et al., 2023). Furthermore, *M. minuta* shows potential as a natural substitute for synthetic antibiotics in the pharmaceutical or food preservation industries by demonstrating broad-spectrum antibacterial activity against a variety of bacterial and fungal diseases (Bhowmik et al., 2020). *M. minuta* is well-known for its efficacy in phytoremediation from an agricultural and environmental standpoint, especially when it comes to removing heavy metals like arsenic from contaminated soils and water. This makes it beneficial for enhancing the security of water bodies and agricultural

ecosystems in areas where heavy metal pollution is a problem (Rahman et al., 2018). Its possible application in bioremediation and environmentally friendly farming systems may potentially support international sustainability objectives. Additionally, it is a green vegetable that is palatable in many cultures, has nutritional value, and may be marketed as a functional food that provides inexpensive vitamins and antioxidants to local diets (Nath et al., 2023).

Depending on the research aim, several phytochemicals, pharmacological, antimicrobial, and environmental approaches can be used to conduct experimental studies on the therapeutic aquatic fern *M. minuta*. To find important bioactive substances such as flavonoids, alkaloids, tannins, saponins, and phenolic acids, are the most popular first step is phytochemical screening. Quantitative tests such as Total Phenolic Content (TPC) and Total Flavonoid Content (TFC), which are helpful markers of antioxidant capacity, usually come next (Debnath & Das, 2016; Nath et al., 2023). In vitro tests like the Ferric Reducing Antioxidant Power (FRAP) assay and the DPPH radical scavenging assay are frequently used to assess antioxidant capacity. These tests aid in assessing the plant's capacity to lessen oxidative stress and eliminate free radicals (Rahman et al., 2018). Chromatographic methods such as Gas Chromatography-Mass Spectrometry (GC-MS), High-Performance Liquid Chromatography (HPLC), and Thin Layer Chromatography (TLC) are also used to separate and describe certain chemical components (Nath et al., 2023). Agar well diffusion, disc diffusion, or Minimum Inhibitory Concentration (MIC) experiments are used in antimicrobial research to evaluate methanolic, ethanolic, and aqueous extracts of *M. minuta* against bacterial and fungal strains. These investigations have confirmed the plant's potential as a natural antibacterial agent by showing noteworthy effectiveness against pathogens such as E. coli, Staphylococcus aureus, and Fusarium spp (Bhowmik et al., 2020). *M. minuta* is being employed more and more in environmental trials, particularly in phytoremediation, in addition to pharmaceutical applications. It has demonstrated the capacity to absorb harmful heavy metals from contaminated soil or water, including arsenic. It is appropriate for ecological restoration efforts because pot studies have been done to assess how well it lowers arsenic levels when co-cultivated with rice plants (Rahman et al., 2018). In conclusion, a multidisciplinary strategy that incorporates biochemical analysis, pharmacological screening, antimicrobial testing, and environmental assessment can be used to conduct tests on *M. minuta*, thereby advancing its application in sustainability research, medicine, and agriculture. Furthermore, funding *M. minuta* research and

development may lead to affordable, long-term solutions for environmental preservation, food security, and healthcare.

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