PHENOLIC COMPOUND AND PROXIMATE ANALYSIS AND DETERMINATION OF ANTI-OXIDANT ACTIVITY OF "*MARSILEA QUADRIFOLIA*"

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DEDICATED TO MY RESPECTED AND BELOVED FAMILY AND TEACHERS

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Abbreviations

CHO	: Carbohydrate
DPPH	: 2,2 – diphenyl – 1 –picrylhydrazyl
UV	: Ultraviolet
℃	: Degree celcius
RPM	: Revolutions Per Minute
HPLC	: High Performance Liquid Chromatography
ROS	: Reactive oxygen species
LDL	: low-density lipo-protein
AOAC	: Association of Official Analytical Chemists
USDA	: United States Department of Agriculture

ABSTRACT

Marsilea quadrifolia is a unique plant with high medicinal value. The paper is a snapshot of the quantitative analysis of total carbohydrate, protein, fat, flavonoids, ash in the plant extract. Our analysis showed the highest value for total carbohydrate, followed by protein, amino acid, flavonoids and fat. Therefore, the goal of the current study is to evaluate the antioxidant properties of *Marsilea quadrifolia's* methanolic extract (MEMQ). By using a qualitative technique and a quantitative DPPH (1,1-diphenyl-2-picryl-hydrazyl) scavenging experiment at 517 nm, the antioxidant potential of MEMQ was evaluated. Additionally total phenolic content, total flavonoid content and proximate analysis were also assessed. The amount of antioxidant activity when employing DPPH increased with concentration, with an IC50 value that was 419.84 µg/ml higher than the normal value of (16.59±0.59µg/ml) and a total antioxidant capacity was equivalents of ascorbic acid 9 µg/ml. Both the total phenolic content (6.9603 ± 1.222 mg/100gm) and the total flavonoid content (400.20 ± 1.538 mg/100gm) have been identified. The percentages of carbohydrate, protein and fat were 26.8%, 20.3%, 8.1% respectively for the stem and leaves contain 30.9%, 19.8%, 14.4% respectively. Additionally, the extract demonstrated strong anti-oxidative efficiency. According to the current research, MEMQ may be an useful source of natural antioxidants with good proximate quantity.

Keywords : Antioxidant, DPPH, Flavanoid, Carbohydrate, Amino acid, Methanolic extract, Proximate quantity.

Chapter 1: Introduction

Many modern pharmaceuticals have been isolated from natural sources, which have been used as a source of treatments for thousands of years. In developing nations, plants are a key element of the traditional medical systems that have also played a significant role in their history and culture. Approximately 80% of the world's population still relies primarily on traditional medicines, which means that plant-based medicinal systems continue to play a significant role in basic health care **(AOAC, 1990)**. Since ancient times, higher plants that are sources of therapeutic chemicals have continued to play a significant role in influencing human life in Bangladesh.

Various bacterial, fungal, and viral infections are treated with medicinal plants. Because of their unrivalled availability of chemical variety, natural products—whether as pure chemicals or as standardized plant extracts—offer limitless prospects for the discovery of novel medications. About 87% of all category disorders are treated using plant-based products and related medications. Secondary metabolites are phytochemicals and chemical compounds created by a plant's normal metabolic activities. Alkaloids, flavonoids, glycosides, gums, polysaccharides, phenols, tannins, terpenes, and terpenoids are just a few kinds of secondary metabolites. There are 12,000 identified secondary metabolites, which is thought to be less than 10% of the total, that plants have the capacity to produce. These active components serve as molecules of the plant defence against attack by microorganisms as well as exhibit the medicinal properties for treating several diseases. In the present research programme, the aquatic fern *Marsilea quadrifolia* collected from Anowara, Chattogram was analysed to evaluate the levels of carbohydrate, protein, lipid, ash fiber, total flavonoids and ascorbic acid. The ethnic and urban societies of the world have conserved immense traditional knowledge of medicinal plants since primitive time. It is well known fact that most of the expensive drugs used in allopathic medicinal are derived from plant resources. In developing country like Bangladesh, nearly 60% of the population still rely on herbs, which are explored by the cultural societies, exploit them for the treatment of various diseases. *M. quadrifolia* Linn is an aquatic fern belongs to the family (Marsileaceae) commonly named has shushnia shak in Bangladesh, European water clover and four leaf clover in English, Sunsuniya in

Hindi. It is an aquatic fern bearing 4 parted leaf resembling ‘4-leaf clover’ (trifolium). In ferns, sporocarps are spheroid, to 3/16 long, dark brown, on stalks to 34 long, joined to the base of petioles. Leaflets are obtained to ¾long, glaucous, with petioles to '8' long. The rhizome's nodes and internodes are where the plant roots. Leaves floating in deep water or erect in shallow water or on land. It grows in artificial habitats such as permanent lakes, seasonal ponds, and thick clay to sandy substrates. It has a long stalked petiole with four clovers-like lobes that is either held above the water or buried (ditches and rice fields). It can be found across India, in marshy areas, along the banks of canals and rivers, and in both tropical and temperate regions of the world. Additionally, it is rooted in the clayey soil at the base of overwhelmed water.

All plant components contain a variety of phytochemical secondary metabolites that have outstanding effects on things like neurodegenerative diseases, anticonvulsants, and cholinesterase. For various pharmaceutical applications, a variety of extracts including aqueous, chloroform, ethanol, methanol, and petroleum ether have been utilized. The leaves juice is diuretic and febrifuge and it can also be administered to abscesses, snake bites, and other conditions. The herb has inflammatory-blocking, diuretic, purgative, febrifuge, and refrigerant properties. Vitiated pitta, cough, bronchitis, diabetes, psychological disorders, eye disorders, diarrhoea, and skin conditions are all soothed by plants. **(AmeyawY, Duker-Eshun G).**

DPPH Free Radical Scavenging Activity**:** Antioxidants are compounds that prevent oxidation and can get rid of the potentially harmful oxidizing agents in a living thing. Numerous phytochemicals found in plants can lessen or stop oxidative damage to human cells, which can even lead to cancer in people. It is highly vital to know about the antioxidant activities of each plant and the phyto-compounds responsible for that. The DPPH free radical scavenging capability of *Marsilea quadrifolia* extracts is examined in this work.

The medicinal plant *Marsilea quadrifolia* is significant. The *M. quadrifolia* plant is useful for reducing nitrogen levels in freshwater lakes and wetland restoration has advanced significantly. It is also referred to as four leaf clover, water shamrock, pepperwort, and clover. The *M. quadrifolia* plant has a variety of medical uses and has been consumed as food for more than 3000 years. It is utilized in the Ayurvedic medical system to treat a variety of illnesses. *M. quadrifolia* is used to treat cough,

Cough, bronchitis, diabetes, psychological disorders, eye disorders, diarrhea, and skin disorders are all treated using *M. quadrifolia*. In India, it typically grows in muck, on soggy soils, or frequently in small ponds and pools. *M. quadrifolia* arrived from Europe in the Northern United States more than a century ago. *M. quadrifolia* is widely acknowledged as a major weed in rice fields across the world, but especially in Asia. A green vegetable native to South India, *M. quadrifolia* is also found in Afghanistan, Central and Southern Europe, Western Siberia, China, Japan, and North America. There are only a few research on the phytochemical makeup of *M. quadrifolia*, and none have quantified the phytocompounds found in the plant's stem and leaves. Hence, the current study was aimed to determine the amount of phytocompounds such as phenolic compounds along with proximate value, antioxidant activity in leaf and stem of *M. quadrifolia.*

1.1 Aims and Objectives:

To determine the Phenolic Compound, Anti-oxidant Activity, total flavonoid as well as Proximate Analysis of *Marsilea Quadrifolia*.

Chapter 2: Literature Review

Marsilea Quadrifolia is a pteridophytic plant that tribal people in the region sell and utilize for its ability to soothe the nerves, its ability to treat numerous other nervous system conditions, and its nutritional worth. *M. quadrifolia* is classified in the International Union for Conservation of Nature's (IUCN) Red Data Book **(Tuba, 1995)**. European *M. Quadrifolia* is a rare plant that is listed among the floras on the Red List **(Wraber and Scoberne, 1989)**. *M. Quadrifolia* Linn. possess strong antibacterial, cytotoxic, and antioxidant properties and have potential medical applications. **(Singh et al, 2014)**

2.1 Taxonomy of the Plant

Domain	**:** Eukaryote
Kingdom	**:** Planate
Phylum	**:**Sterptophyta (Polypodiophyta)
Class	**:** Pteridopsida
Order	**:** Salviniales
Family	**:** Marsileaceae
Genus	**:** Marsilea L.
Species	**:** Marsilea quadrifolia
Division	**:** Pteridophyta – ferns

(*Mohanraj Subramanian* /J Compr Phar 2016;3(2):38-44)

M. quadrifolia is recognized in the International Red Data Book. *M. quadrifolia* Linn is a member of the family of aquatic ferns (Marsileaceae). Common names for Marsilea include "pepper wort" and "water fern" (although it is a fern but hardly resembles a true fern). It is represented by roughly 53 species, which have a wide geographic range but are particularly numerous in tropical regions like Africa and Australia. From Bangladesh, reports of four species or so have been made. The species either grow rooted in mud, marshes, and shallow pools or are wholly submerged, partially submerged, or entirely out of water in moist habitats, depending on whether they are hydrophytic or amphibious. *M. quadrifolia* was discovered in shallow water on a clay- and silt-rich bottom. It develops more or less monospecies

communities **(Hulina, 1998)**. It is also rooted in the bottom of clayey soil in submerged water.

Fig 2.1 *Marsilea Quadrifolia* (shushnia shak)

Local stores sell the plant's leaves and petioles for shushnia shak, a common name for the dish. The rains determine whether this Shak is accessible in the market. This Shak is accessible in the market by the latter week of July through the last week of November, or until the soils remain moist, if rain begins in the first week of June.

Table 2.1 Nutritive value of *Marsilea quadrifolia* per 100gm

Constituent	Amount µg, mg, g, or percent daily value
Energy	430.1kcal/g
Water	69.5g
Carbohydrates	18.84g
Protein	26.78g
Dietary fiber	2.6g
Vitamin c	5.4g
Minarel	2mg
β- carotene	1.4mg

Source: Wikipedia, Internet, USDA databases

2.2 Traditional Uses:

Petiole and leaves for salted & spiced while being cooked in oil: Regular use eases headache, insomnia, and hypertension (present investigation). Petiole and leaves along with warm mustard oil and garlic cooked in a bamboo basket while covered,

then seasoned with salt and 4-5 grams of desi masala (a blend of red chilies, turmeric, coriander, and zeera). Consume solely in the evening to relieve physical and mental tension and promote restful, tension-free sleep for at least 12 to 14 hours. All types of physical pains, sleeplessness, and hypertension are symptoms of neurological illnesses (present investigation). Entire fresh plant Juice with Garlic can treat coughing and convulsive disorders of the muscles and limbs (present investigation), whole plant Juice or paste: Applying it externally to the head provides relief from hypertension and sleep disorders (present investigation). Young leaves Migraine treatment: two drops of leaf juice in the nostrils twice daily (present investigation). whole plant Crushed plant with candy or honey with sugar to cure infantile diarrhoea. whole plant The entire *Centella asiatica* plant, including the roots, is prepared into a paste and administered twice daily for seven days around the nipple: Improving lactation after childbirth. With one breath, leaves the body's 10 tendons should be massaged twice daily for two to three days with the juice squeezed from many leaves. Tribe of Bangladesh rheumatism and mouth or tongue lesions. Young stems, boiled leaves, and petioles are famine foods that are only consumed during times of shortage. While making bread, for example, flour and ground spores are combined.

2.3 *Marsilea quadrifolia* Phenolic Compound:

Naturally occurring phenols in biochemistry refers to the phenol functional group that is present in natural compounds. Plants and bacteria both create phenolic chemicals. In response to environmental stresses like UV radiation, wounding, insect and disease attack, and other pressures, organisms will occasionally produce phenolic chemicals. Research is being done on their effects on human health and disease because they are found in foods used in human diets and in plants utilized in traditional medicine of various cultures. Some phenols have antimicrobial properties and are used to make disinfectants.

Plant phenolics may have health advantages in part because of their antioxidant capabilities, which include metal chelation, ROS scavenging and inhibition, electrophile scavenging, and ROS scavenging and inhibition. Consumption of foods high in phenols is associated with a lower incidence of coronary heart disease, atherosclerosis, some types of cancer, and stroke, according to epidemiological

research. Numerous reviews of phenolic compounds' chemistry and dietary benefits have been done.

Almost all plant-based meals contain phenolic compounds, which cover a wide range of molecules with an aromatic group and one or more hydroxyl groups on the aromatic ring. Phenolic chemicals are typically secondary metabolites in plants that are generated from phenyl alanine either tyrosine. The spectrum encompasses the complex family of flavonoids and tannins as well as simple phenolic acids, which are benzoic acid or cinnamic acid derivatives. More than 8000 distinct chemicals belonging to the phenolic family have been discovered. Cereals, tea, coffee, chocolate, vegetables, fruits, and nuts are among the foods that are frequently linked to high polyphenol content. Plants contain polyphenolic chemicals that have many functions, including color, antibacterial and antifungal activity, antioxidant defense against free radicals and phytoalexins among others. The color, flavor, and texture of liquids like beer, wine, and cocoa are all defined by polyphenols from a food viewpoint. In recent years, the amount of literature citations on polyphenolic substances has increased logarithmically across all journals. Numerous polyphenolic substances have been investigated as antioxidants, which have been linked to positive health effects. The bitter and astringent tannin compounds to the sweet chalcones and some glycosylated flavonoids are only a few of the flavor responses that the phenolic and polyphenolic chemicals can produce. There are a vast range of amounts and specific chemicals present in plants. Growing conditions, wetness, and disease attack all have an impact on the phenolic and polyphenolic contents in plants. Germination, degree of ripeness, processing, and storage conditions all have an impact on concentrations. The historical evolution of interest in these molecules from their colors in flowers, fruits, and vegetables, their enzymatic browning, and the impact on flavor, particularly in wine, to the present-day interest in the compounds' health advantages. A diet rich in fruits and vegetables has been linked to a lower chance of developing cancer, according to epidemiological data. Increased consumption of vegetables lowers the risk of many different cancers, including lung, stomach, oral, and colon cancers, as well as a likely drop on other forms of cancers, according to a review panel led by the American Institute for Cancer Research. Fruit eating also reduced the risk of the majority of the afore mentioned malignancies. A lower risk of cardiovascular disease has also been linked to eating fruits and vegetables. The cardio protection has been repeatedly linked to flavonoids and anti-inflammatory

polyphenols. Phenolic chemicals may act as a preventative measure against a number of illnesses, including diabetes, Alzheimer's disease, cancer, and cardiovascular disease. According to studies, the average food intake of all poly phenolic compounds is 780 mg/day for females and 1058 mg/day form ales with half of these composed of hydroxy cinnamates, 20% to 25% of the total flavonoids and about 1% of the anthocyanins. To draw any firm conclusions on the influence of interventions on health, there are presently insufficient intervention studies. There is strong proof that polyphenols from berry fruits are absorbed to varying degrees in the body and have advantageous effects on people. To ascertain which substances or mixtures of substances offer the benefits, more research must be done. More study and comprehension are needed to fully comprehend the methods by which they operate in vivo.

2.3.1 Phenolic Classes and Structures:

Plant polyphenols can be classified into a number of categories or classes. The non flavonoid classes are mainly composed of simpler compounds, beginning with stilbenes, cinnamic acids, and benzoic acids. In addition to these "simpler" structures, these simpler polyphenols also give rise to more complicated compounds like stilbene oligomers, Gallo tannins, lignans, procyanidins, and ellagitannins. These structures are exemplified by examples in Figure1. A variety of glycosides as well as hydroxyl and methoxy groups can be added to each of these backbones. The benzoic acid and cinnamic acid-based simple monocyclic phenolics are part of the plant defense systems and function as antioxidants, although they are not necessary for plant growth. Recent reviews of the chemistry and occurrence of monocyclic phenolic acids were made by **(Khadem and Marles, 1999)**. The conjugates of the majority of the monocyclic phenolic acids are present. Gallic acid, for instance, can be conjugated as a dimer and infrequently as a trimer (tergallicacid) or tetramers (gallagic acid). When these compounds are esterified on glucose, they are classified as hydrolysable tannins. Polymerized gallic acid is considered to be a hydrolysable tannin. The building blocks for structural polymers in plant cell walls are provided by cinnamic acids. Tomas-Barber and Clifford examined and compiled the main sources of hydroxy benzoic acids that are produced naturally and artificially. The main food sources include barley, caneberries, strawberries, cane wine, tea, and red wine. The innately existing

hydroxy benzoic acids are 4-hydroxy benzoic, gallic acid and protocatechuic. Stilbenes are commonly present in plants and are especially prevalent in red wine and peanuts. Stilbenes, especially trans-resveratrol, have been linked to a variety of health advantages. Anti-inflammatory, anti-tumor and antioxidant properties are among these advantages. There are two different types of resveratrol: free and glucoside. The main class of polyphenols, flavonoids, is broken down into subgroups as shown in Figure1. Plants contain thousands of naturally occurring chemicals called flavonoids, at least 2000 of which are present. The strongly colored anthocyanins, which are frequently found and concentrated in particular in berry crops, are included in the class of chemicals known as flavonoids. There are six other types of flavonoids in nature, including flavones, flavanones, flavanols, and flavanonols and flavones. The Plant source, food manufacturing and preparation methods, and food storage conditions all have an impact on the kind and concentration of flavanols in foods. Wine, cocoa, and tea are just a few examples of foods that contain flavanols, which also play a significant antioxidant role and have been demonstrated to improve the enzymatic oxidative stress system. The A- and B-type dimers and C-type pro anthocyanidins dimers are primarily found in wine. Despite the fact that beans contain them in little amounts. consumption of pro anthocyanidins can reach 0.5g per day, the majority of it being the B-1 and B-2types and pro anthocyanidin ins and DP>3. It has been demonstrated that berries only have trace amounts of flavanols and flavan-3-ols. Flavanols, procyanidins, and epicatechin, which are assumed to be responsible for the beneficial protective effects linked to chocolate consumption, are found in abundance in cocoa or meals that include cocoa. Foods containing chocolate have flavanol contents ranging from 43mg/100g in milk chocolate to 2500mg/100ga chocolate bake. Between 50 and 100 mg of flavanols and pro cyanidins are typically consumed daily in a typical Western diet, with 58 mg in the United States. With four primary catechins that vary in concentration depending on the method of tea preparation and are absorbed in the small intestine, tea is another good source of flavanols in the diet. Flavanones give fruits and vegetables their colors and flavors while also having the potential to have antioxidant properties. Flavanones, Frequently present in citrus fruits, they function as antioxidants and aid in the body's response to inflammation. Citrus fruits contain flavanones in both the juice and the tissue. Total flavone content is lower in citrus when the peel and albedo are more clearly distinguished from the fruit. The most prevalent and widely dispersed plant flavonoids in the diet are

flavonols. They are present in the majority of fruits and vegetables, especially leafy greens, grapes, onions, wine, and tea. **(AmeyawY, Duker-Eshun G, 1970)** The most prevalent flavonol is quercetin, followed by myricetin, morin, and kaempferol. The consumption of fruits and vegetables that are high in flavonols has been shown to reduce the risk of cardiovascular disease. Herbs, cereals, and green vegetables all contain modest levels of flavones. Increased consumption of flavones has been associated with a decreased risk of cardiovascular disease. Some common forms of flavones are pig eninandluteolin. Iso flavones, the main sources of flavones are soy and soy-derived products. It is commonly acknowledged that iso flavones can help with menopausal symptoms and lower LDL cholesterol. Anthocyanins are water-soluble pigments that give many different plants their distinctive red, blue, and purple hues. They are related to red fruits, including berries, and vegetables. Conjugated anthocyanidins, which are found in berries, are the anthocyanins that give them their vibrant colors. While some berries, like blueberries and blackcurrants, have varying amounts of these chemicals, some fruits, like elderberries and redcurrants, only contain one particular form of anthocyanin. Anthocyanin levels in muscadine grapes grown in the south-eastern United States, which stretches from Louisiana to North Carolina, are particularly high. In food plants, the six most prevalent anthocyanidin types are found as O-linked conjugates with various kinds of sugars. The most prevalent anthocyanins are malvidin, petunidin, cyanidin, peonidin, delphinidin, and pelargonidin. They are typically found as galactose, glucose glycosides. the acylated forms present in purple, black, and red carrots, red potatoes, red cabbage, and radishes. Sweet potatoes can be used as food colors in place of synthetic colours because they have a tendency to be more stable. Anthocyanins easily deteriorate, giving rise to color less or brown chemicals. In addition to their reactivity, their stability is also impacted by changes in pH, oxygen content, temperature, and light. Foods exhibit a wide range in the distribution and profiles of polyphenols. To determine the precise distributions and concentrations of polyphenols in foods, two databases are currently accessible. The USDA data base and Phenol Explorer both regularly update their quantitative values for polyphenols. It should be noted that numerous crops other than fruit contain polyphenols, including grains, legumes, nuts, tea, coffee, wine, and beer. **(Singh et al, 2014)**

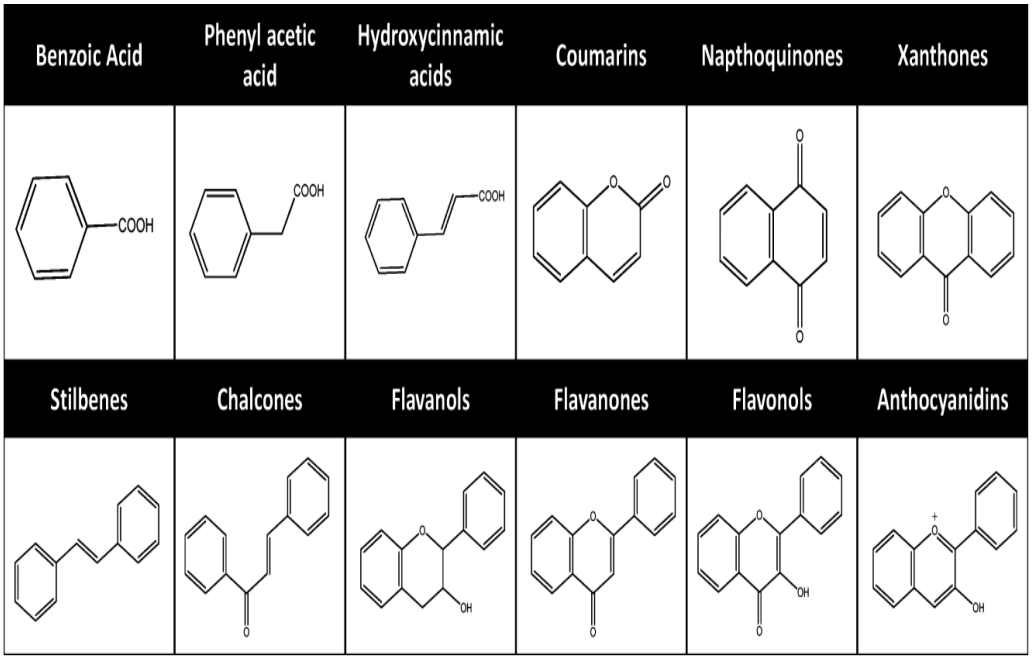

Fig 2.3.1: Polyphenolic Backbones Found in Plants (**Juarez-Garcia et al., 2006**)

2.3.2 Health Benefit of Phenolic Compound:

In the therapy of carbohydrate absorption, such as diabetes, phenolic compounds, which are chemical components taken from plants, can limit the absorption of amylase. Many fruits and vegetables, including grapes, berries, and tomatoes, contain phenolic chemicals. By lowering the risk of metabolic syndrome and the associated consequences of type 2 diabetes, phenolic substances, such as phenolic acids and flavonoids, may offer health advantages. There is still a need for more research because different phenolic chemical groups have diverse biological properties and little is known about the mechanisms through which they can help prevent disease. The highly reactive oxidized molecules reactive oxygen (ROS) and reactive nitrogen species (RNS) are continuously produced by normal cellular processes, such as the function of the mitochondrial respiratory chain and inflammation, which may cause harm to other biological molecules, such as proteins and DNA. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are three examples of antioxidant enzymes that all play a crucial role in eliminating these oxidants and preventing cellular damage. **(Khadem and Marles, 1999)**

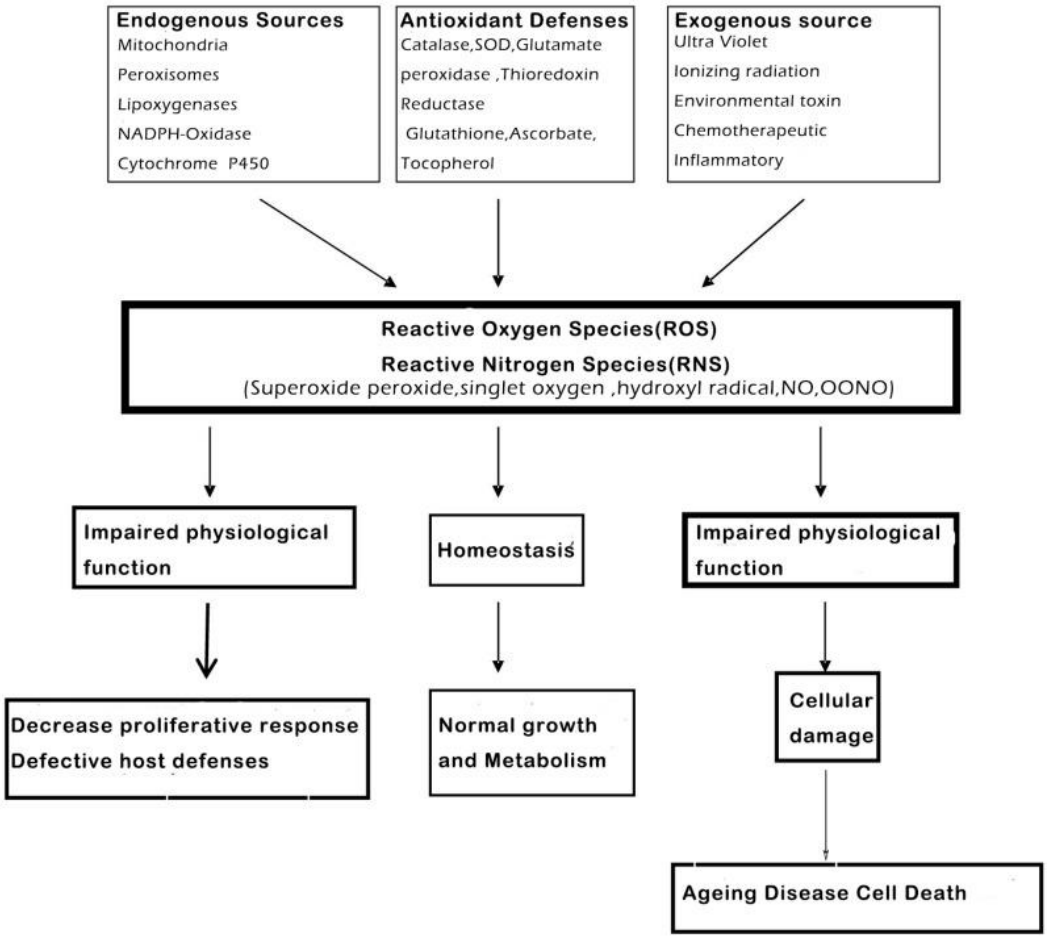

Fig 2.3.2: Flowchart showing of health benefit of phenolic compound

2.3.3: Quantitative Analysis of Phenolic Compound:

The Folin-Ciocalteu reaction is the basis for determining the total phenol content of dietary components and supplements. When combined with the phosphor molybdic, phenolic chemicals create blue complexes at high pH phosphor tungstic reagent. Because the analysis is straightforward, highly reproducible under strict controls and applicable to a wide range of applications. Strong connection with more difficult assays like DPPH, FRAP, TEA, and ORAC is reported in several articles. Unfortunately, a more thorough standardization of the procedure utilizing a recognized reference is required. Various organizations have employed various criteria, which are debatably appropriate to the matrix being evaluated, but makes comparing different approaches very challenging. The complete phenolic test quantifies the entire phenolic spectrum without taking into account polymerization or

other events that can change the effects on biological systems. Since the total phenolic test does not only measure phenolic chemicals, it is possible for additional reducing agents in the system to cause problems. The Folin method is a time-tested method for calculating total phenolic compounds in a range of matrices. Despite the method's lack of specificity, it is often used in biochemical, animal, and clinical trials as a measure of total phenolics. Because phenolics encompass abroad spectrum of classes of compounds, it is desirable to identify the classes of phenolics present such as flavonoids, chalcones, anthocyanins, and procyanidins. The phenolics that are present in food can be further distinguished using spectrophotometric methods, which wavelengths to watch for HPLC detection and quantification depends on the variations in ultra violet spectra. The best methods now available for identifying particular classes and structures of food phenolic are those that combine HPLC with UV detection and mass spectrometric (ESI, MS, MS/MS) measurement. **(Dung et al., 2008)**

2.4 *Marsilea quadrifolia* Anti-Oxidant Activity:

2.4.1 Antioxidants and their function

Antioxidants are compounds that prevent oxidation and can get rid of the potentially harmful oxidizing agents in a living thing. Numerous phytochemicals found in plants can lessen or stop oxidative damage to human cells, which can even lead to cancer in people. It is crucial to understand how each plant functions as an antioxidant and the phytocompounds that make it happen. They also play a significant role in the health landscape and have prospective applications in pharmaceuticals, neutraceuticals, functional foods, cosmetics, perfumes, and food preservation. **(Dung et al., 2008)**. Multiple diseases' onset and progression have been linked to oxidative stress as their primary cause. Exogenous antioxidant supplementation or strengthening the body's own endogenous antioxidant defenses are promising ways to counteract the negative effects of oxidative damage brought on by reactive oxygen species (ROS). The ability to biosynthesize a variety of non-enzymatic antioxidants that can reduce ROS-induced oxidative damage is intrinsic in plants. Numerous in vitro techniques have been employed plants' ability to act as antioxidants, and the majority of these assays showed strong antioxidant activity. However, plant antioxidants must first undergo a number of tests to determine their in vivo medicinal effectiveness on physio

pharmacological processes. Consequently, the findings of in vitro and in vivo antioxidant potential evaluation investigations are not usually the same. However, without conducting enough in vivo research, the outcomes of in vitro experiments have been irrelevantly extended to the therapeutic application of plant antioxidants. In order to better comprehend plant antioxidants as therapeutic agents, we have reviewed briefly the physiology and redox biology of both plants and people. **(Singh et al, 2014)** To determine the exact course to be taken for future study in the field of plant antioxidants, the applications and limitations of antioxidant activity measuring assays were also addressed. When present in lower concentrations than the oxidizable substrate, antioxidants dramatically slow down or stop oxidation. In vivo antioxidant synthesis is possible (e.g., reduced glutathione (GSH), For example, superoxide dismutase (SOD). or consumed as food antioxidants. Exogenous (dietary, i.e., plant-based) antioxidants have long been found in plants. Two-thirds of all plant species on earth are thought to have medicinal value, and almost all of them have excellent antioxidant potential. The discovery and subsequent separation of ascorbic acid from plants originally sparked interest in the exogenous plant antioxidants. Since then, increased oxidative stress has drawn a lot of attention because it has been found to be a major contributing factor in the onset and progression of many serious diseases, including cardiovascular and neurodegenerative disorders. Additionally, it has been discovered that strengthening the body's natural antioxidant defenses or taking supplements of exogenous antioxidants can help promising method of countering the undesirable effects of oxidative stress. **(Dung et al., 2008)**

Currently, there are about 19 in vitro and 10 in vivo techniques used to measure antioxidant activity that are frequently used to assess the antioxidant activity of plant samples. The majority of these in vitro tests revealed strong antioxidant activity in plant sample. This is most likely because of their natural capacity to produce secondary metabolites like phenolic compounds as well as non-enzymatic antioxidants like ascorbic acid and glutathione. Only a few number of these antioxidant properties have been verified or studied in vivo, despite the fact that many plants have been found to have antioxidant potential by in vitro experiments. The use of in vitro experiments to confirm the antioxidant activity of plant samples within specific reaction systems makes it unclear if the results of these assays apply to in vivo systems. Moreover, in vitro tests have revealed that a number of phytochemicals

exhibit antioxidant potential. However, due to their interference with certain processes, only a few number of them have been demonstrated to be therapeutically beneficial in vivo physio pharmacological processes as digestion, metabolism, absorption, distribution, and excretion. The outcomes of these research are then immediately extrapolated to the medicinal value of the phytochemicals. Nevertheless, phytochemicals are being evaluated for their in vitro antioxidant activity. The significance of plants as exogenous sources of antioxidants and their medicinal efficacies may be called into serious doubt by this misconduct. As a result, we covered briefly in the current paper the physiology and redox biology of both plants and people. Discussion is also had regarding the uses and restrictions of assays used to detect antioxidant activity. The knowledge presented here will allow accurate interpretation of the results of research assessing the antioxidant potential of plants using both in vitro and in vivo experiments. The DPPH free radical scavenging activity of the *Marsilea quadrifolia* extract was examined in this work.

2.4.2 Why do all plants have antioxidant potential?

Non-enzymatic antioxidant synthesis is a natural ability of plants. However, under biotic and abiotic stress circumstances, the plants produce more reactive oxygen species (ROS), which induces oxidative stress. In response to increased oxidative stress, plants increase the production and accumulation of a number of low-molecular-weight antioxidants (such as vitamin C, vitamin E, phenolic acids, etc.) and high-molecular-weight secondary metabolites that are antioxidants, such as tannins. These substances confer antioxidant protection to the majority of plants in in vitro studies by acting as free radical scavengers, reducing agents, and metal chelators.

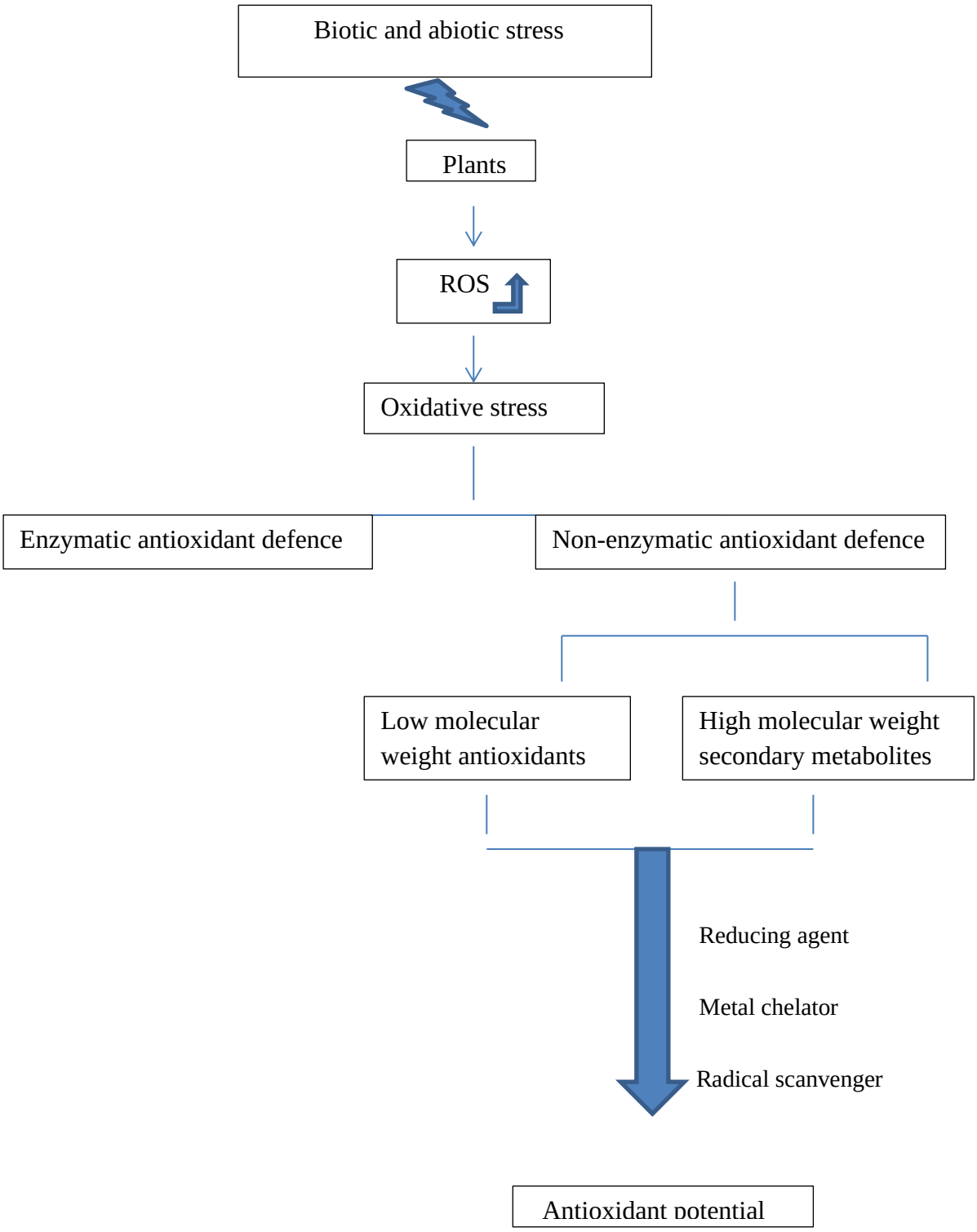

Fig 3: Source of antioxidant potential in all plants (**Juarez-Garcia et al., 2006**)

2.4.3 Assessment of antioxidant potential of plants

In vitro assays

In ethano pharmacological In vitro antioxidant activity evaluation techniques are frequently used to screen and confer antioxidant potential to plants or their phytochemicals, as well as nutraceutical investigations, and occasionally to identify the likely mechanism of action of plant antioxidant. These assays are used to provide plants the ability to scavenge free radicals in the case of medicinal plants, which is crucial for understanding how plants can reduce the oxidative stress connected pathophysiology of diseases. **(Dung et al., 2008).** Plants antioxidant activity is assessed using a variety of in vitro tests. Each of these, however has restrictions on how widely they can be used. Thus, the conferring of antioxidant potential has frequently involved the adaptation of different test techniques. Plants are typically evaluated in these assays for their ability to act as metal chelators, singlet oxygen quenchers, reducing agents, hydrogen donors, or donors of hydrogen, after which they are categorized as primary (chain-breaking) and secondary (preventive) antioxidants. While secondary antioxidants work by binding metal ions that can catalyze oxidative processes and scavenge oxygen, absorb UV radiation, inhibit enzymes, or break down hydroperoxides, primary antioxidants work by giving a hydrogen atom. Approaches for measuring antioxidant activity are divided into hydrogen atom transfer (HAT) and electron transfer (ET) reaction-based methods depending on the inactivation mechanism at play. The mechanism and effectiveness of antioxidants are primarily affected by bond dissociation energy and ionization potential. HAT-based techniques assess an antioxidant's capacity to scavenge free radicals by donating hydrogen to generate stable molecules. While SET-based methods test an antioxidant's capability to transfer one electron to decrease any molecule, including metals, carbonyls, and free radicals, they are more pertinent to the antioxidant capacity that breaks radical chains. HAT-based techniques include assays for carotene or crocin-bleaching, total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), lipid peroxidation inhibition capacity (LPIC), and these. SET processes are also involved in other frequently used techniques for measuring antioxidant activity, including the copper reduction assay and ferric reducing antioxidant power (FRAP). Others, such 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), however, combine the HAT and SET mechanisms. **(Beverly CD, 2011)**

In vivo assays

Plant antioxidants are typically tested in in vivo experiments for their effects on the activity of endogenous antioxidant enzymes or oxidative damage indicators both before and after experimental animals are exposed to oxidative stress. Some of these frequently employed techniques involve the measurement of oxidative damage indicators, while others directly assess the enzymatic activity of endogenous antioxidants including SOD, CAT, GPx, and GR. Quantitative oxidative damage biomarker approaches are used to determine the creation of certain end products as a result of ROS interaction with physiologically significant macromolecules such as DNA, protein, and lipids. By counting the amount of 8-hydroxydeoxyguanosine, DNA damage is identified. As indicators of protein and lipid oxidation, respectively, the levels of carbonyl and aldehydes (for example, malondialdehyde) are examined. **(Beverly CD, 2011)**

2.5 *Marsilea quadrifolia* Proximate Analysis:

Proximate are employed in the breakdown of a product for human consumption into its essential components while analyzing biological materials. They serve as an affordable and simple means of verifying nutritional panels and provide a good approximation of the contents of packaged edible commodities. This means that while testing can be used to validate batches, it cannot validate a food processor or food processing facility; rather, a nutritional assay on the product is required to certify those producers. In order to assure the accurate and exact content of nutrients, nutritional panels in the United States are subject to FDA regulation and must pass stringent testing. This should stop food producers from misleading the general population. The norm proximate in the industry are

- Moisture content
- Volatile matter
- Ash content
- Fixed carbon
- Carbohydrates (calculated)

Four of the five ingredients are obtained analytically by chemical processes and tests. Carbohydrates, the fifth component, are derived using the results of the analysis of the first four. Proximate should almost always comprise 100% of a food product; any divergence from 100% demonstrates the chemical test's resolution since minute differences in how each test is carried out aggregate or overlap the compositional make-up.

The category of one of the five constituents may also include other ingredients. Examples of carbohydrates include, but are not limited to:

• Food fibers

• Sugars

• Sugar and booze

Whereas ash may consist of but is not limited to:

- Dietary minerals (sodium, potassium, iron, calcium)

Although proximates do not provide a complete nutritional analysis, they offer a cheap tool to monitor changes in food quality. Around 80% of the world's marginal communities use medicinal herbs, which play a vital role in delivering basic health care services to rural residents. In addition to possessing phytochemicals that are crucial for pharmacology, each species of medicinal plant has a unique composition of nutrients. These nutrients are necessary for the body's physiological processes. Such nutrients and biochemicals as proteins, lipids, and carbohydrates are crucial for supplying the human body with the energy it needs to function **(Adnan et al., 2010)**. The proximate analysis of many medicinal herbs was published by several researchers **(*Amaranthus viridis* Falade et al., 2004; *Sonchus eruca, Withania coagulans* and *Fagonia indica,* Hussain et al., 2010; *Zingiber officinale, Allium sativum* and *Parkia biglobosa* Odebunmi et al., 2010)**.

Tribulus terrestris is an annual or biennial herb, while *Fagonia cretica* is a perennial plant with corymbose branches and *Peganum harmala* is an annual **(Shah and Khan 2006; Hussain et al., 2011)**. Uses for fagonia species include anticancer, antioxidant,

analgesic, febrifuge, and preventative treatment for kidney illnesses, fever, asthma, urine discharges, and smallpox agents **(Alam, 2011)**. The herb *Chrozophora tinctoria* is a yearly plant. In Iran, it is used to heal warts, as a cathartic, an emetic, and to treat fever **(Delazar et al., 2006)**. A soft-wood little tree known as *Ricinus communis* was established throughout the tropics and warm climate zones. Its leaf, root, and seed oil exhibits therapeutic potential for treating liver problems, inflammation, hypoglycemia, and laxative effects **(Zarai et al., 2012)**. In this nation, several diseases are treated with the help of these herbs. Evaluating the nutritional relevance of medical plant species can assist to comprehend the value of these plant species, as many medicinal plant species are also consumed as food in addition to their medicinal effects **(Hussain et at., 2011)**. Only a few studies have been done to indicate the proximate composition of these plants in terms of seasonal fluctuation, and none have been done for *Fagonia cretica* L., *Tribulus terrestris* L., and *Ricinus communis* L.

Conclusion

From the proximate analysis, shushnia shak have high protein amount. In particular, their phenolic compounds also directly enhanching the antioxidant activities.

Chapter -3: Materials and Method

3.1 Study period and study area

The research work was conducted for a period of five months from January 2022 to May 2022. The research work was conducted in the laboratory of the Department of Applied Food Science and Nutrition, Department of Food Processing and Engineering at Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Experimental procedures were also carried out at BCSIR LABORATORIES, Chattogram.

3.2 Experimental design

At first shushnia shak were collected from local area of Chattogram. After collection of samples, they were used for processing of plant materials. Then the samples were ready for plant extraction. After processing of plant extraction, samples were used to determine the total phenolic compound and total flavonoid content (TFC). Besides that, proximate composition (moisture, ash, crude fat, protein, crude fiber and carbohydrate) contents along with antioxidant activity were also measured.

3.3 Samples collection

The collections were carried out during 2nd January to 3rd February 2022.

3.3.1 Location Map

Sample of *Marsilea quadrifolia* has been collected from the following source

Location: Anowara, Chattagram

Coordinate: N 22.232988, E91.866508

Fig 3.3.1 (a): Co-ordinate with Location Map

Fig 3.3.1 (b): Site Location for Sample Collection

3.4 Processing of plant materials:

To get rid of dirt and any other unwanted objects, the plant material was thoroughly washed under running water. Next, the samples were put on a tray, exposed to the sun for seven days straight, and dried. The stale plant matter was ground to a fine powder using an electric blender and then sieved them. After that, the ground powder was kept in a zip-lock plastic bag and refrigerated at 4°C for further testing.

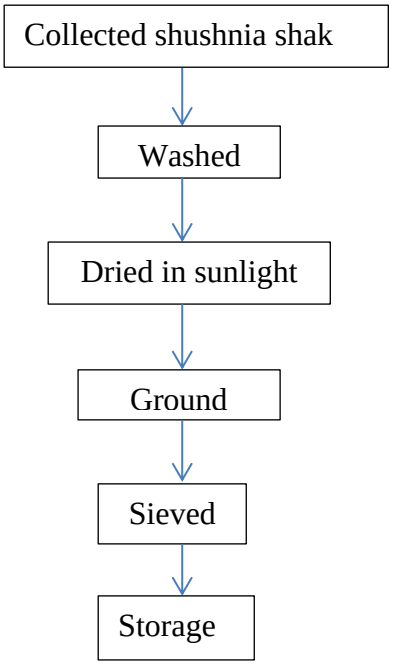

Figure 3.4: Flow sheet for *Marsilea quadrifolia* powder production

3.5 Preparation of Plant Extract:

Hexane, ethyl acetate, ethanol, and methanol were the four distinct solvents employed for the sequential extraction, which began with low polarity and progressed to high polarity. Three different plant powder to solvent (wt/vol) ratios were used for the sequential extraction. [50g of the plant sample were combined with 200ml of solvent (1:4), 300ml of solvent (1:6), and 50g of the plant sample were combined with in order to determine the greatest experimental yield, use 400ml of solvent (1:8). Only the solvent volume rose in each ratio while the weight of the plant sample remained unchanged. The extractions were carried out using an orbital shaker at 120 rpm for 72 hours at room temperature. With each solvent, the extraction process was performed three times. The extracts were concentrated at low pressure in a rotary vacuum evaporator after being filtered with Whatmann No. 1 filter paper. The glass vials

containing the dried crude extracts were maintained in the refrigerator at 4 °C until use. Calculating the extracting values: The extracting values provide information on the type of chemical phytoconstituents that are present in the crude medicine. Information on the quality of a certain drug sample can be obtained by using a particular solvent.

The following formula was used to determine the extractive values:

W1 + W2 x 100 = Yield (%)

Where:

W1 is the weight of the extract following hexane, ethyl acetate, ethanol, and methanol evaporation.

W2 = The plant sample's dry weight

3.6 Methods for Total Phenolic Compound Analysis

The extract will be mixed with 10 ml of ethanol, and 3 ml of the resultant solution will be placed to a test tube and incubated for 15 minutes in a water bath. Then 3 freshly made drops of ferric cyanide are to be added. The development of a blue-green hue will signal the presence of polyphenols. The Folin-Ciocalteu technique was used to quantify the amount of total phenolic compounds in methanolic extracts of *M. quadifolia*'s leaf and stem. 1 ml aliquots of 1 ppm, 2 ppm, 3 ppm, 4 ppm, 5 ppm, 6 ppm, 7 ppm, and 8 ppm ethanolic gallic acid solutions were used to create the calibration curve will be mixed with 5 ml of Folin-Ciocalteu reagent and 4 ml (75g/l) of sodium carbonate. After 30 minutes, the absorbance will be measured at 765 nm in 20°C, and a calibration curve will be constructed. The same reagent as stated before will be combined with 1 ml of mathanolic extract (10g/l), and after 1 hour, the absorption will be evaluated to determine the amount of plant phenolics present. There will be three copies of every determination made. The total amount of phenolic components in methanolic extracts will be calculated as gallic acid equivalents (GAE). **(Tuba, 1995)**

The Folin-Ciocalteu method was used to quantify the amount of total phenolic compounds present in methanolic extracts of *M. quadifolia*'s leaf and stem.

Total phenolic content (mg/g of plant extract in GAE) =

gallic acid concentration determined by the calibration curve (mg/ml) x [extract volume (ml) / weight (g) of pure plant methanolic extracts]

Using quercetin as a reference chemical, the Pharmacopoeia method (1989) was used to calculate the amount of flavonoids in the sample.

Toyal flavonoid content (mg/g of plant extract in QE) =

(Absorption of plant extract solution/Absorption of standard quercetin solution) X [Weight of quercetin in the solution (g) / Weight of plant extract (g)] X 10

3.7 Methods for Total Flavonoid Content Analysis

Using querectin as a reference, the total flavonoid content was calculated using the aluminium chloride method. A volumetric flask was filled with 4 ml of water and 1 ml of plant extract (10 ml volume). Add 3 milliliters of 5% sodium nitrite After 5 minutes, 3 cc of 10% aluminum chloride was added. The reaction mixture received 1 ml of sodium hydroxide after 6 min of room temperature incubation. With pure water, the final volume was quickly increased to 10 ml. Spectrophotometer measurement of the sample's absorbance at 510 nm in comparison to a blank. For accuracy, the entire experiment was run three times.

3.8 Methods for Antioxidant Activity Determination

In both a qualitative and a quantitative experiment, the antioxidant activity of the aerial portion of the methanol extract will be assessed based on its capacity to scavenge the stable DPPH free radical.

- **Qualitative Assay:** To resolve the polar and non-polar components of the extracts, a suitably diluted stock solution will be spotted on pre-coated silica gel TLC plates. The plants will then be grown in solvent systems of different polarity (polar, medium polar, non-polar). The plates will be 0.02% DPPH in ethanol sprayed on them after being dried at room temperature. For ten

minutes, the resolved band's DPPH will be monitored, and color changes (a backdrop of yellow and purple) will be noted **(Sadhu et al., 2003)**.

- **Quantative Assay:** The Blois method will be used to assess the antioxidant activity of the aerial component extract of *M. quadrifolia* utilizing the 1,1 diphenyle-2-picrylhydrazyl (DPPH) free radical scavenging test (1958). For determining the concentration of oxidizable groups in either natural or synthetic anti-oxidants, DPPH provides a practical and precise approach (Cao et al., 1997). 95% methanol will be used to create the DPPH solution. The stock solution (5 mg/50ml) will be made by combining the *M. quadrifolia* crude extracts with 95% methanol. The sample solutions will have a concentration of 100 g/ml. A stock solution will be used to create the test sample, which will then be diluted with methanol to achieve concentrations of 20, 40, 60, 80, and 100 µg/ml, respectively. In each of these test tubes containing *M. quadrifolia* extract, freshly made DPPH solution will be added. After 20 minutes, the absorbance will be measured at 517 nm. The control will be ascorbic acid. The blank will be made of 95% methanol. Using the following equation, the percentage of the DPPH free redical that has been scavenged will be determined.

  %DPPH radical scavenging (%)= [1-(As/Ac)]*100

  Here, Ac= absorbance of control, As=absorbance of sample solution.

  Then, % inhibitions will be plotted against the relevant concentrations used and the IC50 will be determined from the graph.

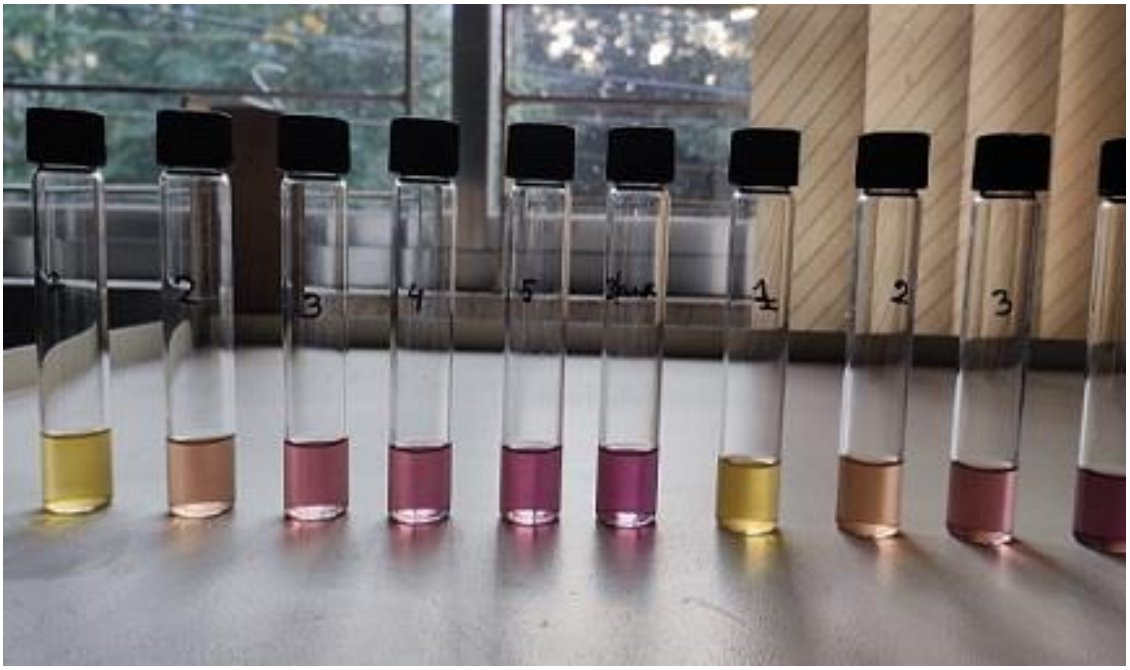

Fig 3.8: Anti-oxidant Activity Determination of *Marsilea Quadrifolia* by Methanol Extract.

3.9 Methods for proximate analysis

3.9.1 Determination of Protein

0.5 g of the dry ground material was collected and put in a Kjeldahl flask. 18 ml of H2SO4, 1 g of CuSO4, and 20–25 ml of concentrated H2SO4 were added to it. For six hours, it was digested in the Kjeldahl digestion unit. The substance was chilled until it reached room temperature. A receiving flask containing 50ml of 4% boric acid solution was filled with it, along with 3–5 drops of mixed indicator, and Kjeldahl's condenser was placed on top of it. Distillation unit introduced 50 ml of water and 60 ml of a 32% NaOH solution to the Kjeldahl flask, making sure the condenser tube extended beneath the surface of the acid in the flask. 200°C of the distilled product is collected in the receiving flask removed for titration. Titrate the contents of the flask against 0.1 N HCl in a burette.

Make a note of the reading and calculate the protein content using the formula below.

% Protein = % Nitrogen x 6.25.

3.9.2 Determination of Fiber

Weigh and transfer a precise amount of the sample into a clean filter crucible (approximately 1.0 g = W0). The crucible was set in the crucible stand, a few drops of

octanol were added to stop foaming, and it was heated for 30 minutes to boiling. With hot water from a spray device (30 ml of water), it was filtered and cleaned three times while being kept as dry as possible. 150 ml of KOH solution that had been heated in the second reagent system was added to each sample.

Add a few drops of octanol and continue to boil for 30 more minutes as before.

% Crude fiber = W1 – W2 x 100

Wt of the sample

3.9.3 Determination of Fat

In this process, the extraction thimble was first weighed using Soxhelt's instrument, which weighs 2-4 g. The thimble was filled with cotton wool that was absorbent. The extraction chamber received the thimble. Weighing a 250 ml round bottom flask that had been cleaned and dried, 1/3 of the flask was filled with solvent, and the extraction tube was attached. Place the sample in filter paper and secure. Then position the entire contraption and activate the burner and water faucet. The extraction was carried out over the course of 5–6 hours, with siphoning taking place after 5–6 minutes at a condensation rate of 3–4 droplets per second. Remove the thimble from the extractor once the procedure is complete, then boil the flask so that all of the solvent may be collected for analysis. Finally, it was cooled and weighed again.

% Crude fat = Weight of flask with fat - weight of empty flask x 100

Weight of original sample

3.9.4 Determination of Ash

A clean, flat-bottomed silica dish was used, held in a hot burner flame for one minute, then moved to a desiccator, cooled, and weighed (W). A reasonable amount of plant components (a sample) were weighed out onto a dish (W1), roasted gently on the Bunsen burner, and then the charred mixture was ready to be transferred to a muffle furnace at 550°C (AOAC, 2000). Until all of the carbon had been burned away, the heating was maintained. I moved the dish with the ash to a desiccator, let it cool, and then weighed it (W2)Weight of the empty dish = W

Weight of the empty dish + sample = W1

Weight of the empty dish + ash = W2

% of Ash = W1 – W2 X 100

Wt of the sample

3.9.5 Determination of the Moisture

A clean Petri dish (W1) that has been correctly weighed and filled with 1-2 g of sample was placed in the oven at 105°C for 4-6 hours to achieve a steady weight. After that, take out the petri dish with the lid on and cool it for 30 minutes in desiccators. Weigh the dish after cooling (W2).

Following are the calculations for percent moisture:

% Moisture = W1 – W2 X 100

Wt of the sample

3.9.6 Carbohydrates Content

The weights of crude protein, crude fats, crude fiber, ash, and moisture content can be subtracted from 100 to get the carbohydrate content (100 - moisture + ash + fibre + fat + protein).

Gross Energy

The formula used for gross energy is as follows:

GE (Kcal/g) = 5.72 x (protein) + 9.5 (fat) + 4.79 (fibre) + 4.03 (carbohydrate) = Gross Energy Value. **(Garrett and Johnson 1983)**.

Chapter 4: Result

4.1 Total phenolic content and total flavonoid content (TPC, TFC) of *Marsilea quadrifolia*

The result of the analysis of Phytocompounds such as total phenolic compound and total flavonoid content of methanolic extract of *M. quadrifolia* were showed in table 4.1

Table 4.1: Total phenolic content and total flavonoid content of *M. quadrifolia*

Sample	TPC (mg GAE / 100 g)	TFC (mg QE / 100 g)
MEMQ	6.9603 ± 1.222	400.20 ±1.538

Legends: Total phenolic content is measured using gallic acid equivalents (GAE, mg/100g of extract), while total flavonoid content is measured using quercetin equivalents (QE, mg/100g of extract).

ME±SD, which stand for Mean and Standard Deviation, were displayed for all values.

4.2 Antioxidant activity of *Marsilea quadrifolia*

The percentage (%) scavenging of DPPH radical was found to be concentration dependent. The results of DPPH scavenging activity with IC50 value of the experimental extract and the standard ascorbic acid are given in Table 4.2. The percentage (%) scavenging of DPPH radical was found to be concentration dependent. The results of DPPH scavenging activity with IC50 value of the experimental extract and the standard ascorbic acid are given in Table 4.2

Table 4.2: DPPH scavenging power of free radical of MEMQ and ascorbic acid

Sample	DPPH method IC50 (µg/ml)
MEMQ	419.84
Ascorbic acid	9

4.3 Proximate composition of *Marsilea quadrifolia*

Proximate composition of roots, stems and leaves of the plant were shown in the Table 4.3. Roots had the highest moisture content (9.7 ± 0.17) % whereas leaves had the lowest (8.6 ± 0.45) % . The highest value of ash content was found in leaves (15.3 ± 1.00) % and the lowest value (8.1 ± 0.85) % was for stems. Protein content was higher in leaves (19.8 ± 0.14) % comparatively than stems and roots. Fat content (14.4 ± 1.04) % was also higher in leaves than parts. Fiber content was higher in stems (26.6 ± 0.03) % and lower in leaves (11.0 ± 0.07) %. Carbohydrate content was higher in roots (35.0 ± 0.65) % and lower in stems (26.8 ± 1.05) %. So the result of this analysis showed that the leaves of *M. quadrifolia* could be important green leafy vegetable as a source of nutrients to supplement other major sources.

Table 4.3: The table shows the results of proximate analysis of *Marsilea quadrifolia*

Plant	**Parts**	**Moisture Content (%)**	**Ash Content (%)**	**Protein Content (%)**	**Fat Content (%)**	**Fiber Content (%)**	**Carbohydrate (%)**	**Gross Energy (Kcal/g)**
Marsilea Quadrifolia	Roots	9.7 ± 0.17^{a}	11.2 ± 0.92^{b}	11.2 ± 0.07^{c}	11.8 ± 2.05^{b}	21.1 ± 0.01^{b}	35.0 ± 0.65^{a}	418.1
	Stems	9.3 ± 0.39^{b}	8.1 ± 0.85^{c}	20.3 ± 0.09^{a}	8.9 ± 1.36^{c}	26.6 ± 0.03^{a}	26.8 ± 1.05^{c}	436.0
	Leaves	8.6 ± 0.45^{c}	15.3 ± 1.00^{a}	19.8 ± 0.14^{b}	14.4 ± 1.04^{a}	11.0 ± 0.07^{c}	30.9 ± 0.51^{b}	427.2

Legends: All numbers represent the ME±SD of the data. Mean is ME. SD stands for standard deviation. Different superscripts in a row signify a significant difference, but the identical superscripts do not differ substantially at $P<0.05$.

Chapter 5: Discussions

5.1 proximate composition of *Marsilea Quadrifolia*

From the test results, the raw *Marsilea quadrifolia* contained (8.6 ± 9.7) % moisture content. This percentage may vary as it depends on different parts of it. Total moisture content of shushnia shak considerably less than what was cited by (**Juarez-Garcia et al., 2006)** for the climate change. These readings were within the allowed range (less than 20%) for a long shelf life **(Mahloko et al., 2019)**. The leaves have high moisture content which is similar to the observation made by (**Larksome et al., 1980)**. Increased moisture promotes microbial activity. Low moisture content plants are simpler to keep.

The leaves contained higher amount of carbohydrates content. Total carbohydrate content was found to be (30.9 ± 35.0) %. **(Facciola et al., 1990)** reported 34% carbohydrate in *M. quadrofolia*. That is similar to the following assessment. The carbohydrate contents of leaves was higher than reported by (**Rodriguez-Ambriz et al., 2008)**. In addition to, the leafy parts of carbohydrates content was lower than the root (35%) and higher than the stem (26.8%). Carbohydrates are a significant class of naturally occurring organic molecules that support and sustain life in both plants and animals as well as serving as a source of raw materials for numerous businesses. Given that it satisfies the Recommended Dietary Allowance (RDA) requirements, the leaf is a good source of carbohydrates when ingested.

The amount of crude protein in *Marsilea quadrifolia* was quantified to be (11.2 ± 19.8) % which shows an excellent source of protein. The outcome is consistent with the 5.8% observed in a related investigation by **(Hedrick et al. in 1972)**. Apparently, **(Pearson et al., 1976)**, The leaves were provided more protein than 12% of the caloric content of protein, leaves are regarded as a good source of protein. Shusnia shak was high in crude protein, about 35% reflects a significant and different amount of amino acids throughout all harvesting seasons **(Jafari et al., 2012)**. But this plant was not particularly promising in terms of nutrient composition when compared to other widely utilized green leafy vegetables and showed significant seasonal variations.

The value of the crude fat (11.8 ± 14.4) % that keeps Dietary fat enhances the flavor of food by absorbing and holding onto the flavor. **(Tanaka et al. 1976)** claimed that crude lipid was present in the leaves of *M. quadrifolia* as 15.0%. The findings showed that the leaves are a poor source of plant lipid, supporting the overall finding that leafy vegetables are low in lipids **(Lintas et al., 1992)**. A diet that contains 1.20 % of its calories as fat is considered inadequate for humans because excessive fat consumption has been linked to a number of cardiovascular problems.

Numerous complex carbs must contain fiber. It was only ever found in plants, especially in vegetables, fruits, and nuts and legume. The crude fiber values for the leaves of (21.1 ± 11.0) % that contains a good source of fibre. the fibre content was found higher than that of the results of other studies. Other research showed (18.0±0.01) % fiber content **(Oliver et al., 2002)**. The result of crude fiber was lower than that of the results of (**Rodriguez-Ambriz et al., 2008).** The recommended amount of fiber per day is 25 to 30 g, with insoluble fibers accounting for 70 to 75 percent of that amount **(Figuerola et al., 2005)**. When ingested, plants are good sources of crude fiber because dietary fiber can reduce the risk of diabetes, breast cancer, heart disease, hypertension, constipation, and other conditions by lowering serum cholesterol levels. It is advised that an average adult take 18–32 grams of fiber per day. **(Ram et al., 1994)**.

Ash still contains the majority of the minerals or inorganic components. The total ash content measured (11.2 15.3) % was rather close to those of other researchers. The values were in good agreement with those found in various Nigerian green crops. **(Ifon and Bassir, 1980).** It showed that ash content of shushnia shak were higher than the Nigerian leafy vegetables. The plants included a good amount of minerals because the ash content is a reflection of the quantity of mineral elements found in the samples.

It was clearly notified that the gross energy was found to be (418.1 ± 427.2%) so it might be said that shushnia shak could meet the daily energy need of an adult.

5.2 Total phenolic content (TPC) and total flavonoid content (TFC) of *Marsilea quadrifolia*

One of the biggest and most common families of plant metabolites is the phenolic chemicals. Phenolic substances have medicinal qualities like anti-apoptosis, anti-aging, anti-cancer, anti-inflammation, anti-atherosclerosis, cardiovascular protection, improvement of endothelial function, and inhibition of angiogenesis and cell proliferation activities. Plant polyphenols are extensively found throughout the plant kingdom and can occasionally be found in very large concentration. Polyphenolic substances are linked to antioxidant activity and play a significant role in stabilizing lipid oxidation. The phenolic chemicals may directly support the antioxidative effect. When up to 1.0 g of polyphenolic compounds are consumed daily from a diet high in fruits and vegetables, it is hypothesized that these compounds have inhibitory effects on human mutagenesis and carcinogenesis **(Rodríguez-Ambriz et al., 2008)**. Phenolic compounds were referred to be primary antioxidants or free radical terminators and have the capacity to donate hydrogen atoms to free radicals. Tannins, anthocyanins, catecholamines, phenolic acids, epicatechin, gallic acid and flavonoids were some of the phenolic compounds found in shushnia shak **(Singh et al., 2016)**. The most essential antioxidants were polyphenols, which were found in fruits and vegetables. Both edible and non-edible plants contain phenolic chemicals, which have been shown to have biological effects, such as antioxidant activity. The redox characteristics of phenolic compounds, which could be extremely important in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or dissolving peroxides, are what give them their antioxidant qualities. The results of total phenolic content (TPC) were ranged from (6.9603 ± 1.222 mg GAE/100gm) for *M. quadrifolia* were higher than reported by **(Savlak et al., 2016)** for climate change.

Flavonoids-rich plants have been said to have antioxidant qualities. Flavonoids indicate the role of the floral species in improving the health of the human beings. Widespread secondary metabolites having antioxidant, anti-inflammatory, antibacterial, antitumor and antiradical properties. Shushnia shak is high in TF and the tocopherol, alpha-tocopherol, and kaempherol are the main types of flavonoids found in *M. quadrifolia*. Flavonoids are substances that have anti-infective (bacterial and viral illnesses), anti-cancer, and anti-heart disease properties.Total flavonoid content was found (400.20 ±1.538 mg QE/100gm) that indicates a high content for

antioxidant properties compared to other plants. Total flavonoid content of *M. quadrofolia* was lower than the other research reported by (**Khoza et al., 2021)**.

5.3 Antioxidant capacity of *Marsilea quadrifolia*

The amount of ascorbic acid equivalents represents the crude extract's overall antioxidant capacity. In order to convert into a stable diamagnetic molecule, the comparatively stable nitrogen-centered free radical DPPH can readily take an electron or hydrogen radical. By increasing the concentration of the sample extract, this action was enhanced. DPPH was a widely used substrate to assess antioxidant activity, particularly for examining the free radical scavenging capacities of both chemical and biological compounds. Antioxidant activity using DPPH was discovered to rise in a concentration-dependent manner, with a greater IC50 value (419.84 µg/ml) than the conventional one (16.59 µg/ml) and the antioxidant capacity of ascorbic acid 9 µg/ml, From the result of antioxidant capacity it can be said that shushnia shak is an excellent source of antioxidants. *M. quadrifolia* contains tocopherols and tocotrienols, two potent antioxidants that can neutralize highly reactive radicals by releasing H+ ions from their ring **(Bharti et al., 2013)**.

Chapter 6: Conclusion

Marsilea quadrifolia is a prospective candidate for use as a leafy vegetable since it has a high protein content but low mineral and carotene values, low ash values, and was a strong source of carotene. Through all harvesting seasons, this plant had a high crude protein content. It is advisable to promote the broad eating of this plant in areas where it is discovered to flourish. Edible aquatic plants should be employed more extensively as expanding populations force the use of marginal agricultural regions, especially as a cheap supply of nutrient-dense greens that would improve the diets of the local population. The extract demonstrated substantial antioxidant potency in all respects. Their phenolic components and antioxidant properties in particular may be helpful for type 2 diabetic meal planning. They might help maintain plasma antioxidant levels because antioxidants in plants and herbs stop the vascular disorders associated with type 2 diabetes from developing. According to the current research, MEMQ may be a possible source of natural antioxidants due to its high levels of protein, ash, fat, CHO, and total phenolic and flavonoid content. According to the current research, medicinal plants with high antioxidant capacity are the ideal supplements for illnesses related to oxidative stress.

Chapter 7: Recommendations and Future Perspectives

In recent years, shushnia shak have received considerable attention due to its nutritional value. Half of people are suffering from malnutrition in our country and in this case raw *Marsilea Quadrifolia* could be a great source of nutrients and energy. In our country shushnia shak are widely available and we can cook this shak with randon ingridients. This may be helped as a good choice for undernourished by taking into account the nutritional aspects, both children and adults. The following recommendations and research opportunities are provided based on the current investigation:

It is important to examine vitamin C and fat-soluble vitamins including A, D, E, and K.

• Anti-diabetic qualities need to be considered.

• The shelf life of shushnia shak and the physical features test should be examined.

• Since the discoveries have medicinal significance, they will be useful from a therapeutic standpoint.

• For improved taste, the cooking procedure may be adjusted using different flavors.

• To extend the shelf life, availability and storage conditions would be improved.

The health advantages of shushnia shak and its potential for use in the food business, in addition to its wide range of applications as a folk remedy, should be made more widely known.

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Appendices

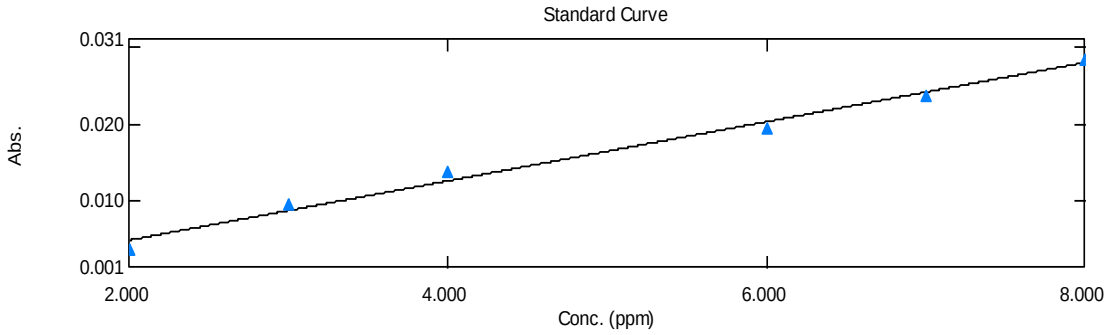
Appendix A: Determination of phenolic compound and Analysis of total flavonoid content (TFC)

Table 1: Analysis of TFC (Total Flavonoid Content)

Standard Table of Qwercetin:

	Sample ID	Type	Conc(ppm)	WL415.0	Wgt.Factor	Comments
1	Std_1	Standard	2.000	0.004	1.000	Dilution Factor 1
2	Std_2	Standard	3.000	0.010	1.000	Dilution Factor 1
3	Std_3	Standard	4.000	0.014	1.000	Dilution Factor 1
4	Std_4	Standard	6.000	0.020	1.000	Dilution Factor 1
5	Std_5	Standard	7.000	0.024	1.000	Dilution Factor 1
6	Std_6	Standard	8.000	0.029	1.000	Dilution Factor 1

Standard Curve:



y = 0.00385110 x - 0.00271158
r2 = 0.98868

Sample Table:

	Sample ID	Type	Conc(mg/100g)	WL415.0	Comments
1	Sample1.1	Unknown	400.143	1.538	
2	Sample1.2	Unknown	400.738	1.541	
3	Sample1.3	Unknown	399.726	1.537	

Sample Graph:

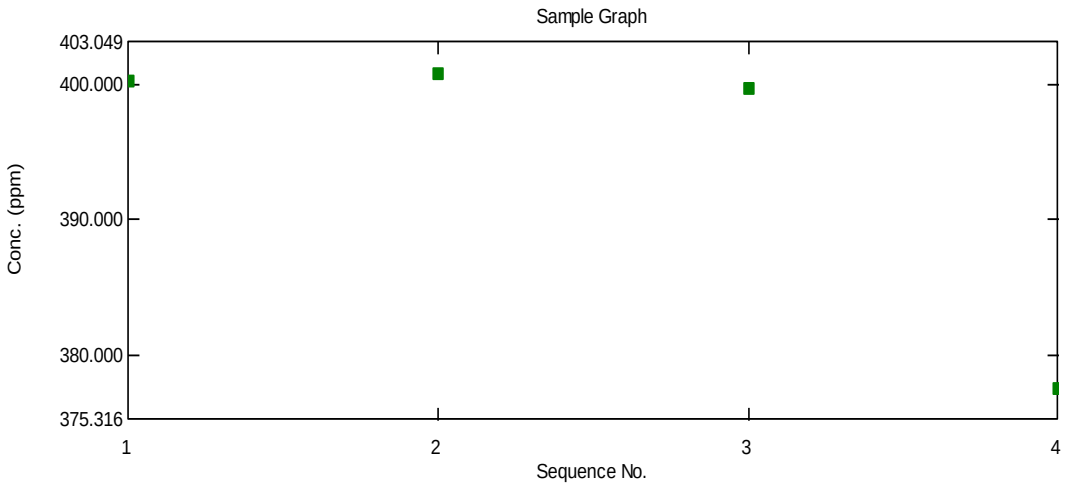
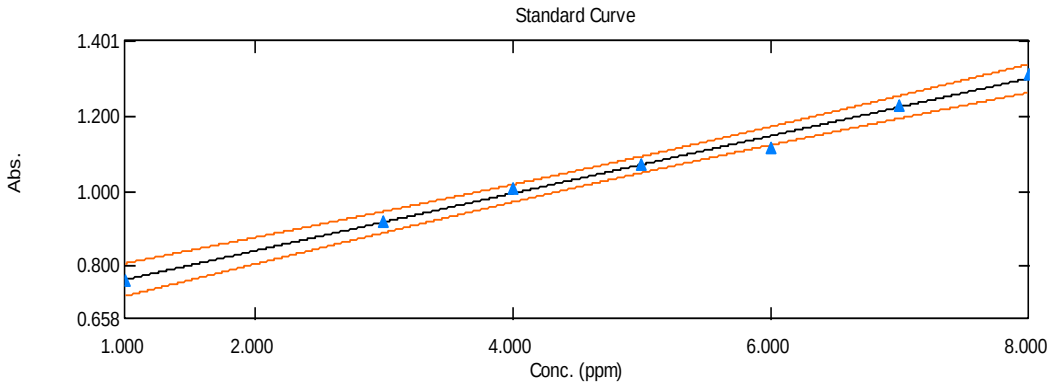


Table 2: Determination of TPC (Total Phenolic Content)

Standard table of Gallic Acid:

	Sample ID	Type	Conc(ppm)	WL760.0	Wgt.Factor
1	STD1	Standard	1.000	0.763	1.000
2	STD2	Standard	2.000	0.780	1.000
3	STD3	Standard	3.000	0.920	1.000
4	STD4	Standard	4.000	1.007	1.000
5	STD5	Standard	5.000	1.074	1.000
6	STD6	Standard	6.000	1.115	1.000
7	STD7	Standard	7.000	1.230	1.000
8	STD8	Standard	8.000	1.314	1.000

Standard Curve:

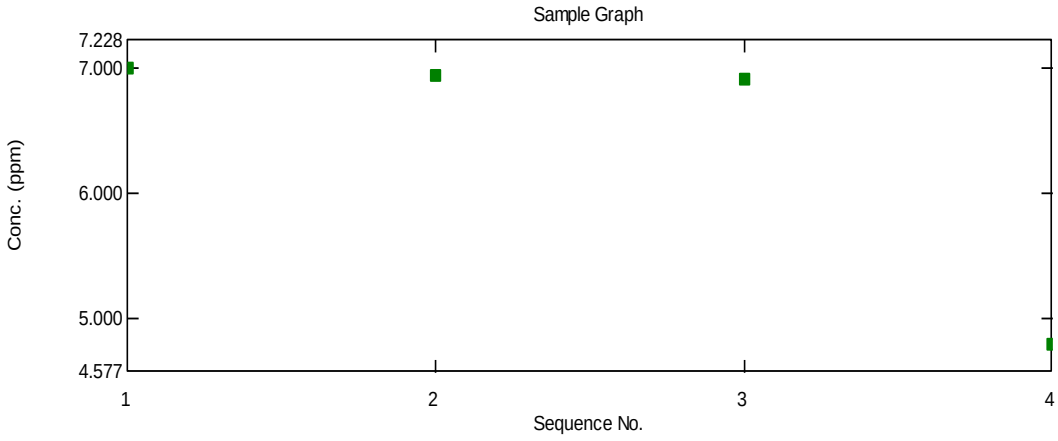


y = 0.0768527 x + 0.687090
r2 = 0.99301

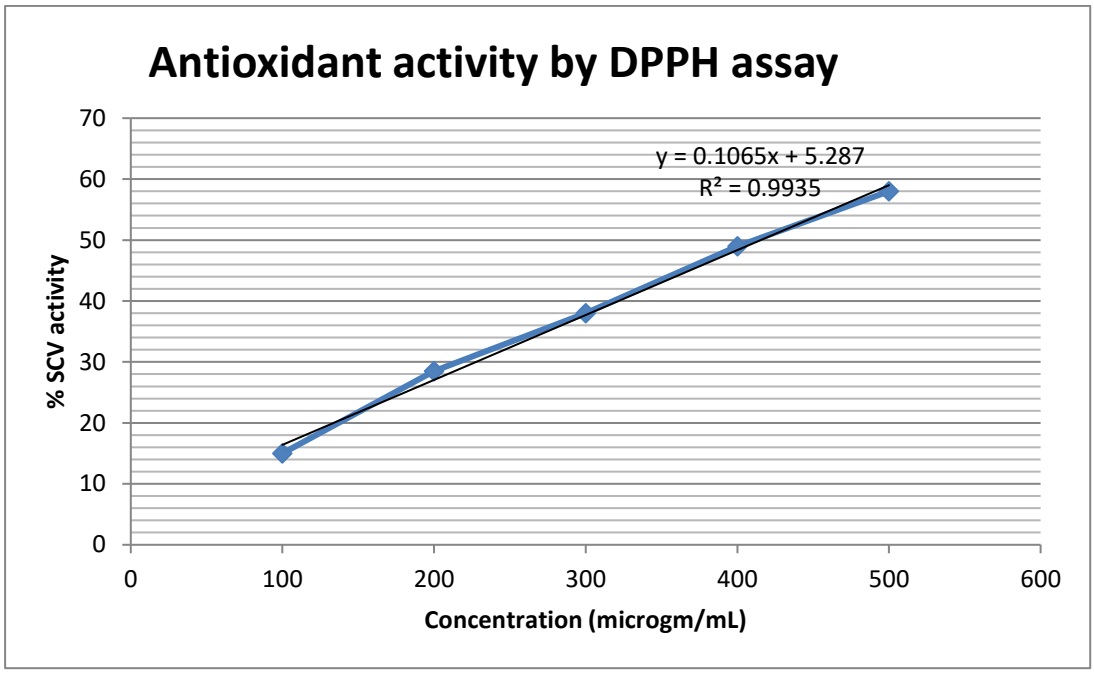
Sample table

	Sample ID	Type	Conc(mg/100g)	WL760.0	Comments
1	Sample1.1	Unknown	7.007	1.226	
2	Sample1.2	Unknown	6.950	1.221	
3	Sample1.3	Unknown	6.924	1.219	

Sample Graph:



Appendix B: Determination of Anti-oxidant Activity and Ascorbic Acid:



For IC50 value,

Y = 0.1065X + 5.287

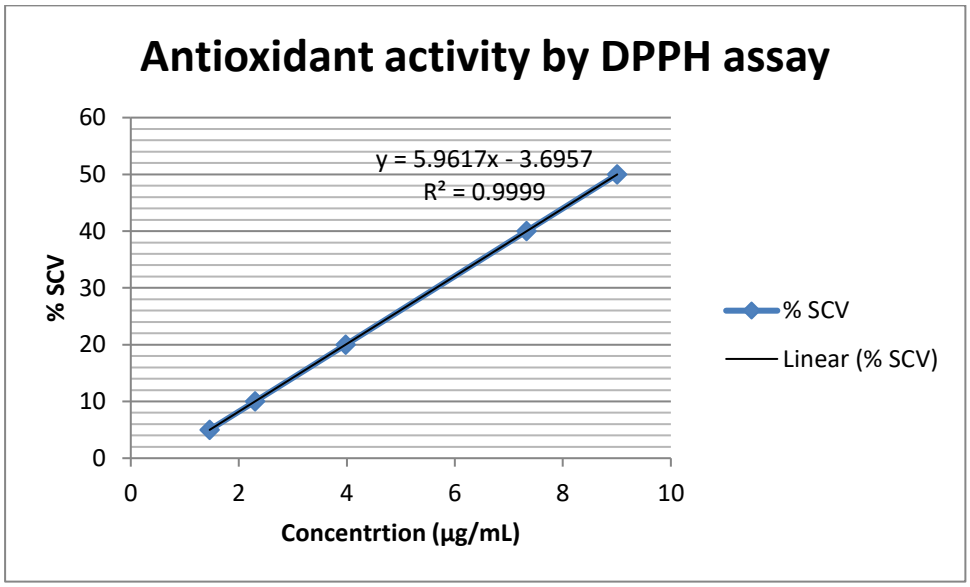
Or, 50 = 0.1065X + 5.287

Or, X = 50 – 5.287/0.1065

Or, X = 419.840

So, the IC50 value of *Marsilea quadrifolia* is 419.84 µg/mL.

Antioxidant Activity of Ascorbic Acid (Standard)



For IC50 value,

Y = 5.961X – 3.6957
Or, 5.961X = 50+3.6957
Or, X = 9.007

So, the IC50 value of *Marsilea quadrifolia* is 9 µg/mL

Appendix C: Photo Gallery

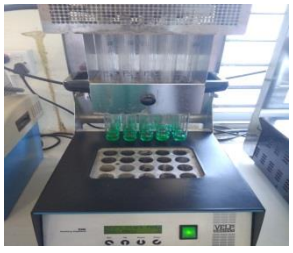
Sample collection

Sun drying

Grinding

Ground Powder

Fat determination



Protein determination

 **Ash determination**	 **Titration** **Protein determination**
 **Distillation** **Protein determination**	 **Fibre determination**
 **Preparing samples for spectrophotometric**	 **Working in UV - spectrophotometer**
 **Soxhlet extractor**	

Brief Biography

Mousumi Das passed the Secondary School Certificate Examination in 2010 and then Higher Secondary Certificate Examination in 2012. She obtained her B.Sc (Hon's) in Food Science and Technology from the Faculty of food Science and Technology of Chattogram Veterinary and Animal Sciences University, Chattogram, Bangladesh. Now, she is a candidate for the degree of Master of Science in Applied human Nutrition and Dietetics under the Department of Applied Food Science and Nutrition, Faculty of Food Science and technology, Chattogram Veterinary and Animal Sciences University (CVASU). She has immense interest to work in improving health status of poor people through proper guidance and suggestions and create awareness among people about food science and nutrition.