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Pharmacognostic evaluation, phytochemical investigation and antioxidant activity on leaves of *Marsilea minuta* Linn. (Marsileaceae)

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Abstract

The present study deals with a detailed pharmacognostical study on the leaf of *M. minuta*. The preliminary phytochemical studies revealed the presence of carbohydrates, saponins, steroids, tannins and phenolic compounds. The morphological, microscopical and physicochemical standards developed in this study will provide referential information for identification of the crude drugs and standardization. QC standardization of the various medicinal plants used in traditional medicine is becoming more vital today in view of commercialization of formulations based on these plants.

In the present study, various standardization parameters of leaves like macroscopical, microscopical characters, physicochemical parameters like foreign matter, LOD, ash values, extractive values, swelling index and foaming index were performed. The in-vitro antioxidant study was performed by DPPH and H₂O₂ Method.

Keywords: *Marsilea minuta* Linn., Pharmacognostical study, Physicochemical analysis, Antioxidant activity.

1. Introduction

Though medicinal plants originate from almost every part of the globe so the use of plants for alleviation of human suffering is, perhaps, as old as humans themselves. People in India and China are known to have used plants in organised health care regimes for over 5,000 years. As traditional medicine, being non toxic, having no-side effects, easily available at affordable prices so much of the world's population rely on it to meet daily health requirements, especially within developing countries. The World Health Organisation estimates that as many as 80 per cent of the world's population depends on plants for their primary healthcare (Farnsworth *et al.*, 1995) [1, 2].

Marsilea minuta Linn. (Marsileaceae) is a common species of water fern and are widely found in wet and humid places. It is used as sweet, astringent, cooling, digestive and diuretic [3].

Standardization of plant material is the set of procedures which are used to specify the limits of various components present in the plant. It can be defined as the status of a drug that is determined by identity, purity, content and other chemical, physical or biological properties (WHO Guidelines). In case of herbal drugs and products, the word Standardization should encompass entire field of study from cultivation of medicinal plant to its clinical application [4]. Standardization of herbal drug is not an easy task as numerous factors influence the bio efficacy and reproducible therapeutic effect. In order to obtain quality oriented herbal products, care should be taken right from the proper identification of plants, season and area of collection, extraction and purification process [5].

Oxygen is a double-edged sword. We cannot live without oxygen but at the same time we are continuously exposed to oxygen toxicity. However molecular oxygen is neither very reactive nor very toxic. The apparent toxicity of oxygen is actually due to free oxygen radicals formed by partial reduction of molecular oxygen [6]. Antioxidants are the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance. Plants are the potential source of natural antioxidants. Natural antioxidants or phytochemical antioxidants are the secondary metabolites of plants. Carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols, tocotrienols etc., are some of the antioxidants produced by the plant for their sustenance. Beta-carotene, ascorbic acid and alpha tocopherol are the widely used antioxidants. The antioxidants generally used in foods are BHT, BHA and gallates propyl (PG), Octyl (OG) or dodecyl (DC).

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2. Materials and Methods

2.1. Plant Material

The plants were collected from Hooghly district of West Bengal (India). The plant materials were authenticated by Mr K Karthigeyan, Chief Scientist at Central National Herbarium, Shibpur, West Bengal, with Reference No. (CNH/67/2012/Tech. II/868), Dated 24/08/2012. The collected plant materials were washed under running tap water to remove foreign particles such as sand, clay, etc. and then sun and shade dried.

2.2. Macroscopical Characterization

Macroscopical studies of the plant were carried out using the naked eye, and the shape, colour, taste and odour of leaves were determined and reported [7].

2.3. Microscopical Characterization

Selected samples of dried leaves were stored in a solution containing Formalin, Glacial acetic acid and 70% Ethyl alcohol (FAA) in the ratio of (5: 5: 90) [8]. The Photomicrography of transverse section of the leaves was done after proper staining with phloroglucinol-hydrochloric acid (1:1) and mounting in glycerine [9]. Quantitative leaf microscopy to determine palisade ratio, stomatal number and stomatal index were carried out. Powder (Sieve mesh 60) of the dried leaves was used for the observation of powder microscopical characters after treating with phloroglucinol-hydrochloric acid (1:1) solution [10]. Photographs of different magnifications were taken with Nikon Camera [11].

2.4. Physicochemical study

Shade-dried powder of leaves was used for physicochemical analysis as per standard methods. The ash value represents the presence of inorganic matter in the drug. The foreign matter, moisture content [12], swelling index [13] and foaming index [14] were also determined. The dried powdered material (40 g) was defatted with petroleum ether at room temperature for 48 hrs. The percentage extractive value was found out. Then the defatted powdered material thus obtained was further extracted with chloroform and methanol for 48 hrs each respectively in a percolator. The solvent was filtered off and resulting residue and percentage extractive value was taken out. Extracts obtained by exhausting crude are indicative of approximate measures of certain chemical compounds, thus representing quality and purity of the drug.

2.5. Phytochemical analysis

The preliminary phytochemical analysis was performed for all 3 extracts to identify the phytoconstituents present in the extracts using standard procedures [15-17].

2.6. Antioxidant activity

2.6.1. DPPH Radical Scavenging Method

10 mg of extract and ascorbic acid were taken and dissolved in the methanol and final volume make upto 10 ml which was used as a stock solution with the concentration 1000 µg/ml. Then different concentrations like 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml and 200 µg/ml were prepared by diluting with the methanol from the stock solution.

In this assay, 1ml of varying concentrations (25, 50, 100, 150, 200 µg/ml) of methanol extract of *M. minuta* Linn. was mixed with 2 ml of 0.1 mM DPPH solution in methanol. The mixture was allowed to react at room temperature in the dark for 30 minutes. Blank solutions were prepared with each test sample

solution only when control was DPPH solution. L-ascorbic acid (Vitamin C) has been used as standard reference. The absorbance was measured against methanol as blank at 517 nm. In DPPH method percentage inhibition was measured by formula:

$$\% \text{ inhibition} = \frac{A_C - A_T}{A_C} \times 100$$

Where, A_C = absorbance of control sample; A_T = absorbance of test sample [18, 19].

2.6.2. Hydrogen Peroxide Scavenging Activity

A solution of hydrogen peroxide (H_2O_2) (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically. Samples (20, 40, 60, 80, 100 µg/ml) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after ten minute against a blank solution. The percentage of scavenging of hydrogen peroxide of various samples of *M. minuta* Linn. and standard compound (Ascorbic acid) was calculated using the following formula:

$$\% \text{ Scavenged} = \frac{A_0 - A_1}{A_0} \times 100$$

Where, A_0 was the absorbance of the control; A_1 was the absorbance in the presence of the sample of *M. minuta* Linn. and standard [20].

3. Results and discussions

3.1. Pharmacognostic Studies

Various Pharmacognostic parameters were reported after following the WHO guidelines.

3.1.1. Macroscopic evaluation

Table 3.1.1: Macro-morphological Description

S. No.	Characters	Observation
	Organoleptic Characters	
1.	Colour	Yellowish green, dull green
2.	Odour	Characteristic
3.	Taste	Astringent

Macroscopic Examination of Leaves

Macroscopy of leaves of *M. minuta* Linn. was done in which size, surface characteristics, texture, taste, colour and odour was examined. It was found that *M. minuta* Linn has four leaflets with smooth surface. The size of the leaf was 1.5-2.0 cm having light green colour with astringent taste and characteristic odour.

Table 3.1.2: Qualitative Macro morphology

S. No.	Characters	Observation
1.	Size	Length: 2.0 cm; Width: 1.5 cm
2.	Shape	Obovate
3.	Texture	Short
4.	Fracture	Easy
5.	Surface	Smooth and Glabrous
6.	Arrangement	Simple
7.	Apex	Retuse / Obtuse
8.	Base	Cordate
9.	Petiole	Long
10.	Margin	Entire / Dentate
11.	Venation	Radiating, Dichotomously branched

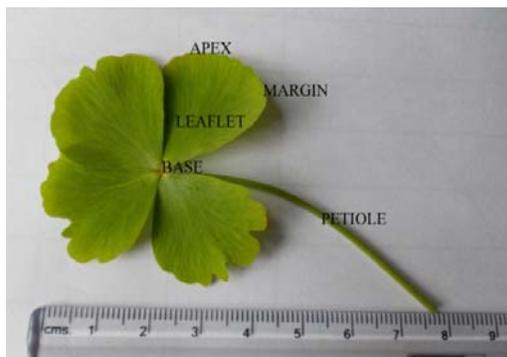
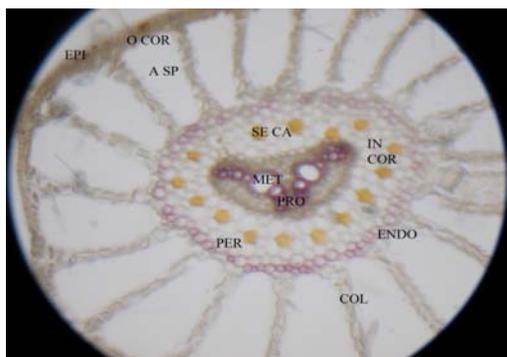


Fig 3.1.1: Macroscopy of *M. minuta* Linn.

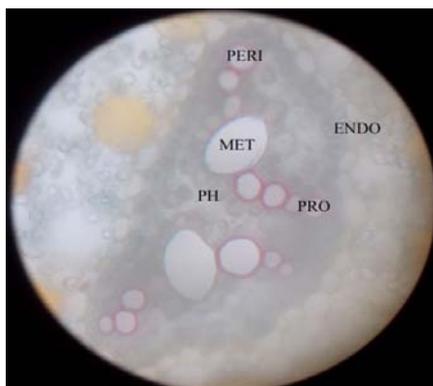
3.1.2. Microscopic Evaluation
Microscopic examination



O COR= Outer cortex; EPI= Epidermis; A SP= Air space; SE CA= Secretory cavity; IN COR= Inner cortex; ENDO= Endodermis; PER= Pericycle; PRO= Proto xylem; MET= Meta xylem; COL= Collenchyma

Fig 3.1.2: T.S of petiole of *M. minuta* Linn.

There is a single layered epidermis made up of rectangular cells. Beneath it are a few layers of thin walled cells that constitute the outer cortex and followed by middle cortex which consist of a ring of air chambers. The inner cortex is a solid, compact tissue. The outer cell layers are collenchymatous. The inner cortex is followed by a single layered endodermis. Within the endodermis is the stele which is somewhat boat shaped in outline. It lies in the centre and has a single vascular bundle. The xylem part of the bundle is shaped like the “V” with opening towards the axis. The protoxylem and metaxylem are observed and surrounding the xylem is the phloem followed by pericycle, bounded by the endodermis.



PERI= Pericycle; MET= Meta xylem; PRO= Proto xylem; PH= Phloem; ENDO= Endodermis

Fig 3.1.3: Petiole showing Vascular Bundle at 45X



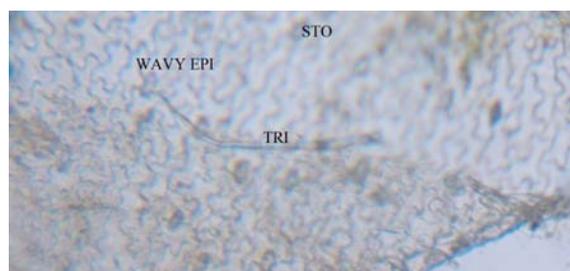
SE CA= Secretory cavity; PAR= Parenchyma; MET= Meta xylem; PRO= Protoxylem

Fig 3.1.4: Petiole showing metaxylem and protoxylem at 45X

Microscopy of fresh leaf of *M. minuta* Linn. was done under microscope. It was seen that it has wavy epidermal cells with anisocytic stomata. In the fresh leaf simple covering trichomes was also found out.

Table 3.1.3: Microscopic characters and their measurements

Part identified	<i>M. minuta</i> Linn.
Stomata	Anisocytic Stomata, Stomatal pore = 11.662 μ, With guard cells = 6.664μ.
Epidermal cells	Wavy walled, L = 46.64 μ; W = 16.66μ.
Trichomes	Unicellular, L = 49.98μ; W = 3.332μ.



STO= Stomata; WAVY EPI= Wavy epidermis; TRI= Trichome

Fig 3.1.5: Upper epidermis of leaf of *M. minuta* Linn at 10X



WAVY EPI= wavy epidermis; ANI STO= Anisocytic stomata

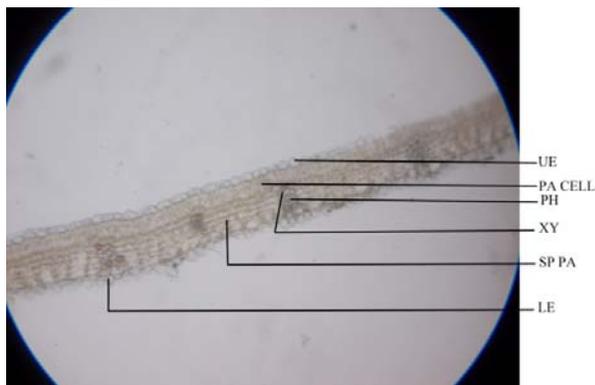
Fig 3.1.6: Lower Epidermis of leaf of *M. minuta* Linn. at 45 X

Table 3.1.4: Stomatal number and Stomatal index

Plant	Surface	Stomatal number (per mm ²)	Number of epidermal cells	Stomatal index (I=S/E+S x 100)
<i>M. minuta</i>	Lower	12	28	30
<i>M. minuta</i>	Upper	7	19	26.92

I= Stomatal index, S= No. of Stomata per unit area, E= No. of epidermal cells in the same unit area.

The Stomatal number and Stomatal index of *M. minuta* Linn was calculated. The Stomatal number comes out to be 30 and the number of epidermal cells was also found to calculate the stomatal index which comes out to be 26.92. Palisade ratio =5.9



UP=upper epidermis; PA=parenchyma cell; PH=phloem; XY=xylem; SP PA=spongy parenchyma; LO EPI= lower epidermis;

Fig 3.1.7: T.S of Leaf of *M. minuta* Linn.

3.1.3. Powder Characters Evaluation

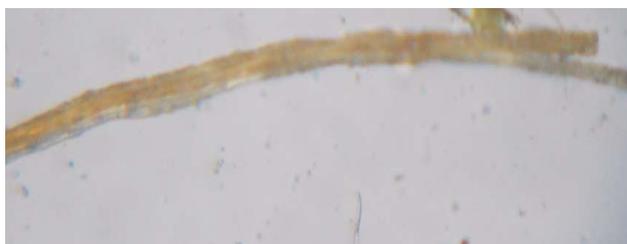


Fig 3.1.8: Unicellular trichomes at 10X



Fig 3.1.9: Non lignified fibres at 10X

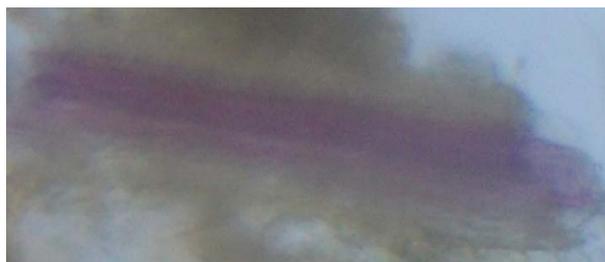
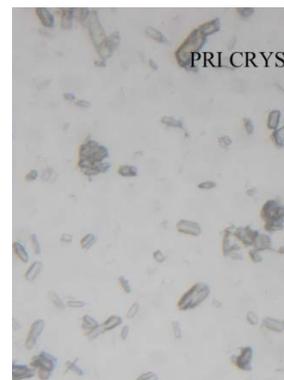


Fig 3.1.10: Phloem fibres at 45X



PRI CRY= Prismatic crystal

Fig 3.1.11: Prismatic crystals at 10 X

Powder Characters and Quantitative Microscopy

Table 3.1.5: Powder characters and their measurement

Part identified	<i>M. minuta</i> Linn.
Trichomes	Unicellular, W =1.66-3.33 μ , L=8.33-24.99 μ .
Phloem fibres	Lignified; W=1.66 μ ; L= 9.99 μ .
Non lignified fibre	L= 54.97 μ ; W=1.66 μ .
Epidermal cells	Wavy Walled, L= 36.65 μ ; W= 1.66 μ .
Crystals	Prismatic, L= 4.99 μ ; W= 3.33 μ .

Powder microscopy of *M. minuta* Linn. revealed the presence of epidermal cells. Fibres were observed in bundles as well as in individual state. Trichomes, unicellular trichomes were observed. On treatment with Phloroglucinol, non lignified fibres were observed. Phloem vessels were also present.

3.2. Proximal Studies

3.2.1. Foreign Matter

The Foreign Matter was calculated to be 0.2% w/w. Presence of part or product of an organism is very less amount.

3.2.2. Loss on drying

Loss on drying was found to be 9.8% w/w. Insufficient drying favours spoilage by moulds and bacteria and makes possible the enzymatic destruction of active principles. The rate at which the moisture is removed and the condition under which it is removed is of utmost important as determination of moisture content provide the method of preparation of drug.

3.2.3. Ash Values

The total ash is a measure of the presence of inorganic matter in the drug. A large value indicates that the plant material contains more of inorganic matter. The total ash is a measure of the presence of inorganic matter in the drug. A large value indicates that the plant material contains more of inorganic matter. The total ash was found to be 12.5% w/w. When concentrated acid was added to ash, it reacts with calcium oxalate crystals. If the plant material contains a large number of calcium oxalate crystals, the amount of substances remaining after acid treatment will be quite less. Thus a lower value of acid insoluble ash content indicates the presence of large number of calcium oxalate crystals in the plant material and vice versa. The acid insoluble ash also shows the amount of silica present in the plant material. The acid insoluble ash was found to be 1.5% w/w. Water soluble ash is part of the total ash content, which is soluble in the drug and good indicator of the water soluble salts. The water soluble ash was found out to be 5.5% w/w. The Sulphated ash was found to be 14.0% w/w.

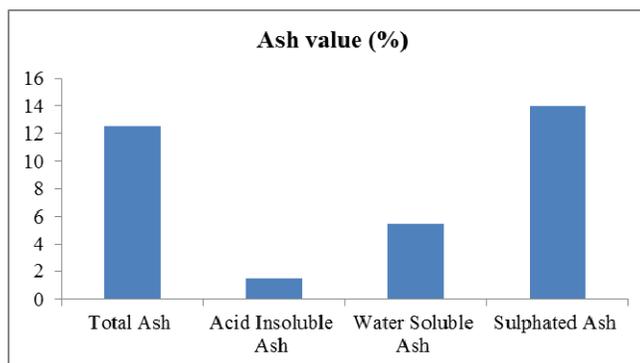


Fig 3.2.1: The result of ash values for *M. minuta* Linn.

3.2.4. Extractive Values

The alcohol soluble extractive value was found to be 3.2% w/w. Alcohol soluble extractive signifies that the large amount of constituents of leaf was soluble in alcohol. Water soluble extractive was found to be 16.8% w/w and it indicated the presence of sugar, acids and inorganic compounds.

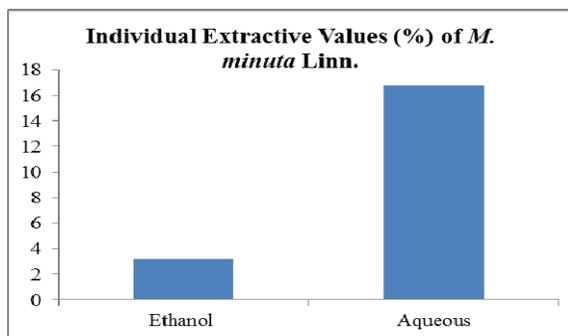


Fig 3.2.2: Individual Extractive Value of Solvents

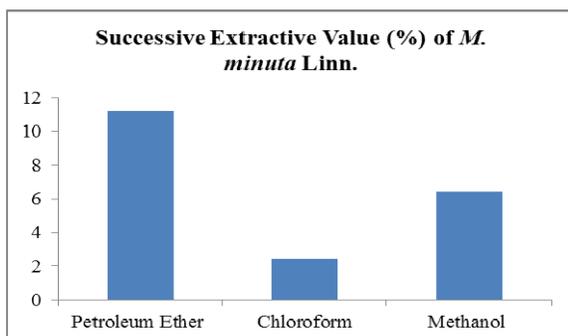


Fig 3.2.3: Successive Extractive Value of different Solvents

Table 3.2: Proximal Analysis

	Parameters	Results
1.	Foreign Matter	0.2% w/w
2.	Loss on Drying	9.8% w/w
Ash Values		
3.	Total Ash	12.5% w/w
4.	Acid Insoluble Ash	1.5% w/w
5.	Water Soluble Ash	5.5% w/w
6.	Sulphated Ash	14.0% w/w
Extractive Values		
7.	Alcohol (Ethanol) Soluble Extractive	3.2% w/w
8.	Methanol Soluble Extractive	6.4% w/w
9.	Water Soluble Extractive	16.8% w/w
10.	Chloroform Soluble Extractive	2.48% w/w
11.	Pet. ether Soluble Extractive	11.2% w/w

3.2.5. Swelling Index

The swelling index was calculated to know that how much plant material can swell after putting in water and also to know that the plant material contains some mucilaginous content. After adding water in the plant material two reading was taken, initial reading and final reading after 3 hours. The swelling index of *M. minuta* Linn. was found to be 1.67.

3.2.6. Foaming Index

The water and decoction of plant material was put in ten tubes in ratio and after shaking the test tubes, foam was measured with the scale. Height of forth measured was less than 1 cm in every test tube. Therefore the foaming index was found to be less than 100.

3.3. Qualitative and Chemical Examination of the Extracts (Phytochemical Screening)

The results of preliminary phytochemical screening were recorded in table 3.3.

Table 3.3: Qualitative Phytochemical analysis of *Marsilea minuta* Linn. Leaves

S. No.	Name of test	Petroleum ether Extract	Chloroform Extract	Methanol Extract	Ethanol Extract	Aqueous Extract
1.	Test for Alkaloids	-	-	-	-	-
2.	Test for Carbohydrates	+	+	+	-	-
3.	Test for Tannins	+	+	+	-	-
4.	Test for Phenolic compounds	+	+	+	+	+
5.	Test for Proteins	-	-	-	-	-
6.	Test for Amino acids	-	-	+	-	-
7.	Test for Glycosides	+	+	+	-	-
8.	Test for Steroids	+	+	+	+	+
9.	Test for Flavonoids	+	+	-	-	-
10.	Test for Oils & Fats	-	-	-	-	-
11.	Test for Saponins	+	+	-	+	+
12.	Test for Mucilage	-	-	-	-	-

Key: + = present; - = absent.

The chloroform and petroleum ether extracts of *M. minuta* demonstrated maximum occurrence of phytoconstituents. The Phenolic compounds and steroids are present in all the tested extracts of *M. minuta*. The tannins, glycosides and carbohydrates showed its presence in all the extracts of *M. minuta* except ethanol and aqueous. The amino acid is present in methanol extract. The flavonoids are present in petroleum ether and chloroform extract. The Saponins is present in all the extract except methanol extract.

3.4. Antioxidant Activity

3.4.1. DPPH Free Radical Scavenging Method

Table 3.4.1: Percentage free radical scavenging of various extracts of *M. minuta* Linn. by DPPH free radical scavenging method

Conc. (µg/ml)	% Inhibition MeOH Extract	% Inhibition Ascorbic acid
25	47.80	58.70
50	58.66	64.35
100	70.67	78.79
150	86.38	88.44
200	96.51	98.81

Values are the average of triplicate experiments and represented as Mean.

Free radical scavenging activity of MeOH Extract was evaluated by DPPH method. The sample was compared with the standard i.e ascorbic acid. DPPH is a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so the molecules do not dimerise, thus gives rise to the deep violet colour, characterized by absorption at 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this give rise to the reduced form with the loss of violet colour. The result from the present study revealed that the samples showed concentration effect with highest free radical scavenging was at concentration 200 µg/ml.

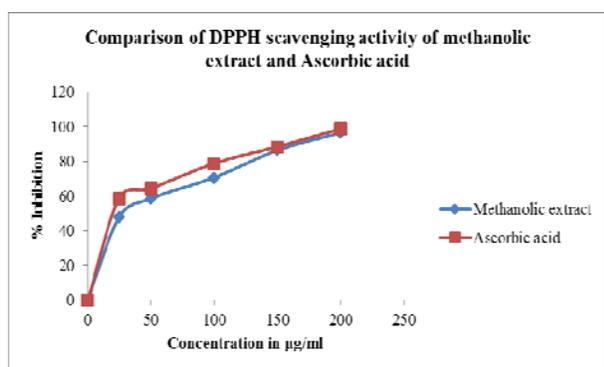


Fig 3.4.1: Graphical representation of DPPH Scavenging activity of methanolic extract and Ascorbic acid

3.4.2. Hydrogen Peroxide Scavenging Activity

Table 3.4.2: Percentage scavenging of various extracts of *M. minuta* Linn. by Hydrogen Peroxide Method

Conc. (µg/ml)	% Inhibition Methanol Extract	% Inhibition Ascorbic acid
20	36.01	42.68
40	53.48	59.99
60	58.64	65.90
80	61.26	68.72
100	72.70	74.88

Values are the average of triplicate experiments and represented as Mean. Hydrogen peroxide is an important reactive oxygen species because of its ability to penetrate biological membranes. However, it may be toxic if converted to hydroxyl radical in the cell. Scavenging of H₂O₂ by MEMM (Methanolic Extract of *M. minuta*) may be attributed to their phenolics, which could donate electrons to H₂O₂, thus neutralizing it to water. The H₂O₂ scavenging capacity of MEMM may be attributed to the structural features of their active components, which determine their electron donating abilities. The results from the present study revealed that the samples showed concentration effect with highest H₂O₂ scavenging was at 100 µg/ml.

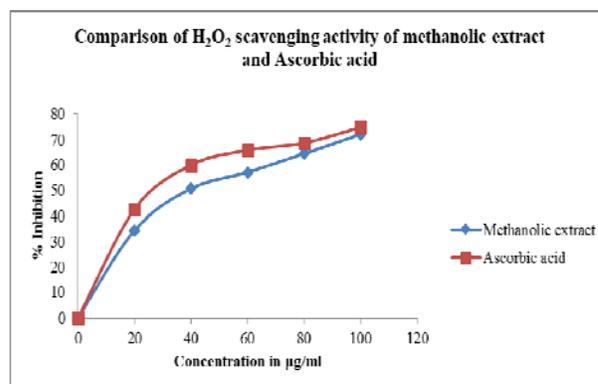


Fig 3.4.2: Graphical representation of H₂O₂ Scavenging activity of methanolic extract and Ascorbic acid

4. Conclusion

M. minuta Linn. is used traditionally for medical purpose. The Pharmacognostical studies on the plant viz. morphological, powder microscopy and quantitative microscopy and determination of physicochemical constants provides suitable standards for identification of plant material and for the isolation of components in future course of time. The present study suggests that methanol extract of *M. minuta* leaves possess potent antioxidant activity. Therefore it is suggested that *M. minuta* could be a potential source of natural antioxidant that could have great importance as therapeutic agent in preventing or slowing down the progress of ageing and age associated oxidative stress related degenerative diseases. Further work can be done by isolating the components which are responsible for this activity which can lead to future drug development. In future toxicological and clinical studies can also be performed to assure quality and purity.

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