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Original Article

Quality Control Standardization and *in-vitro* Antioxidant Activity of *Marsilea quadrifolia* Linn.

Kancharla Bhanukiran, Tarkeshwar Dubey, Siva Hemalatha*

ABSTRACT

Background: The species *Marsilea quadrifolia* L. belonging to the family Marsileaceae is a semi-aquatic plant widely distributed in the tropical and sub-tropical regions of India. The whole plant of *Marsilea quadrifolia* L. is extensively used for its eminent medicinal properties. **Objectives:** Despite promising therapeutic benefits, the plant seems to be less explored, and consequently, the present investigation focuses on exploring pharmacognostical parameters, quality control standardization and antioxidant studies. **Materials and Methods:** The microscopical (transverse sections of leaves, petiole, stem, and root.) studies, leaf constants (stomatal number, vein islet number, etc.), and powder microscopic examination were performed. All physicochemical evaluations, like swelling index, foaming index, etc. were accomplished as per the World health organization (WHO) guidelines. TLC profiling and HPLC standardization of ethanolic extract was also performed. *In vitro,* antioxidant activities of ethanolic extract were carried out by various methods. **Results:** The transverse sections of all parts of the plant, leaf constants, and powder microscopy were reported with clear identification. Physicochemical parameters, i.e., foaming index (<100), Loss on drying (13.99 w/w), moisture content (13.75 w/w), total ash value (13.5 w/w) were done. Ethanolic extract was standardized with reference to quercetin and quantified HPLC data was calculated to be 2.1805±0.02089 mg/g. The antioxidant potential of ethanolic extract and its IC_{50} values are reported. **Conclusion:** We have reported complete pharmacognostical standardization of *Marsilea quadrifolia L.* as per WHO guidelines. We have reported the antioxidant potential of ethanolic extract of the whole plant of *Marsilea quadrifolia* L. along with quantitative estimation of quercetin using HPLC.

Keywords: Quercetin, HPLC, Pharmacognosy, Phytopharmaceuticals, Sushni, European water clover.

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INTRODUCTION

As per the World health organization (WHO), 25% of contemporary medicines are acquired from natural origin. Almost 80% of the world population is influenced by herbal drugs for primary health care purposes,[1] especially in developing countries like India. The precise information of herbal medications is not clear at, but still, herbal medicines are of significant interest in public domain. Safety and efficacy is a challenging issue of herbal and synthetic drugs.[2]

Marsilea quadrifolia L. is a pteridophytic plant belonging to family Marsileaceae. It is a semiaquatic plant known to be European water clover. In Eastern parts of India, it is recognized by the local name Sushni. The plant grows in wet or moist soil, and it can also grow in water. The vegetative propagation takes place through the spores. The spores are released out by swelling the sporocarps. The plant has been reported to have anti-epileptic,[3] anticonvulsant,[4] anticancer, anti-bacterial,[5] antifungal,[5] and anti-inflammatory activities.[6]

Traditionally the plant is widely used in all over India, petiole and leaves are cooked in oil with salt and masalas for the treatment of headache, hypertension and sleep disorders.[7] Leaves and petiole warmed in mustard oil with garlic followed by addition of red chilies, turmeric, zeera and coriander were used for hypertension, insomnia and nerve disorders. The entire plant juice mixed with ginger can treat cough and convulsive condition of muscle and legs.[7] Juice of fresh shoots taken as a remedy for cough, respiratory problems in babies. In the form of Paste or juice of *Marsilea quadrifolia* L. applied on head to relives hypertension and sleep disorder.[7] Two drops of leaves juice give in nostril two times a day to cure migraine. For the treatment of epilepsy, 10 g fresh plant paste mixed with 100 of curd was given orally once a day in empty stomach for one month.[7] Crushed plant with honey are effectively used for treatment of diarrhea.[8] Paste prepared from roots of *Marsilea quadrifolia* L. and *Centella asiatica* is applied two times a day on the

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Figure 1: Schematic representation of pharmacognostical standardization of *Marsilea quadrifolia* L.

nipple for 7 days to improve lactation after childbirth.[9] Spores ground mixed with flour used in bread preparation and cooked leaves used as a food in Jharkhand region of India.[10] The present study is focused on establishing pharmacognostical standardization (illustration Figure 1) of *Marsilea quadrifolia* L. and its antioxidant potential of ethanolic extract.

MATERIALS AND METHODS

Collection of plant material and authentication

The whole plant of *Marsilea quadrifolia L.* were collected from the agriculture field of Banaras Hindu University in the month of August to September and authenticated (Voucher specimen no. Marsil. 2016/1) by Prof. N. K. Dubey, Department of Botany, Banaras Hindu University, Varanasi.

Pharmacognostic evaluation

Morphological, Histological and Powder drug evaluation

Macroscopic examination such as size, colour, surface characteristic, and odour was evaluated by physical observation. Collected plant fixed in formalin-aceto-alcohol solution (Formalin 5 ml, acetic acid 5 ml, and ethanol 90 ml of 70%) for further studies. The microscopic evaluation was performed by cutting freehand sections of root, stem, petiole, and leaf of fresh plant. Sections were stained with phloroglucinol and observed under microscope (Nikon E200) at 10X. For examine powder microscopy, powdered plant material was macerated with nitric acid and potassium chloride then washed with water and mounted in glycerin.[11] Leaf constants (stomatal number, stomatal index, vein islet number, and vein termination number) were determined.[12] Leaves were peeled and boiled in diluted potassium chloride solution and observed under the microscope. Stomatal index was calculated as per the given formula.

Stomatal index = S/E+S×100; Where, S= Number of stomata in a given area of leaf; E= Number of epidermal cells in a given area of the leaf (including trichomes).

Physicochemical evaluation

The physicochemical evaluation was carried out on dried powdered drugs as per WHO guidelines and Indian herbal Pharmacopeia.[13] Various physicochemical parameters for the whole plant of *M. quadrifolia* includes swelling index, foaming index, loss on drying, moisture content, extractive value, and ash value were evaluated.[14]

Preliminary phytochemical screening

Whole plant *Marsilea quadrifolia* L. was dried under the shade, powdered (150g) and extracted with 95% ethanol (400ml) by using a Soxhlet apparatus. The resulting ethanolic extract was concentrated under reduced pressure using Rota evaporator (IKA) and green residue obtained was stored at 4°C until use. Further, ethanolic extract was subjected to phytochemical screening of various classes of phytoconstituents.[15]

Quantitative estimation of phytoconstituents

Phytochemical screening of ethanolic extract of *Marsilea quadrifolia L.* suggests presence of phenols and flavonoids in ethanolic extract. So, we further decided to quantify the phenolic and flavonoid content of plant. This is the first report of quantification of phenolics and flavonoids in ethanolic extract of *Marsilea quadrifolia* L. Quantitative estimation of total phenolic and tannin content of ethanolic extract was done as per the folin-coicalteau calorimetric method using gallic acid and tannic acid respectively as a reference.[16] The total flavonoid and flavanol content was determined by using rutin as a reference compound.[17] The total alkaloid content was estimated using spectrophotometric method (470 nm) based on detection of yellow coloured precipitate formed by bromo cresol green with alkaloids.[18]

Thin-layer chromatography (TLC) analysis

TLC of ethanolic extract of *Marsilea quadrifolia* L. was performed by using precoated HPTLC silica gel 60 F_{254} as a stationary phase and hexane: ethyl acetate: chloroform (7:1:2) as mobile phase. Presence of quercetin was confirmed by TLC using reference (quercetin) under the UV light chamber at 254 and 365 nm.

Standardization of extract through HPLC

Ethanolic extract of *Marsilea quadrifolia L.* was standardized with reference to quercetin using High performance liquid chromatography (HPLC) technique. The experiment was carried out using waters 1500 series pump attached to a 2998 photodiode array detector. Data was analyzed by waters breeze software. Mobile phase, selected was acetonitrile: methanol: water (15:40:45) and stationary phase was Lichro CART *250-4 C_{18} column. Samples were filtered by using 0.45 µm membrane filter for analysis. The chromatographic conditions were as follows: flow rate 1ml/ min, detector wavelength was 368nm, injection volume 10µl. Presence of quercetin was confirmed by comparing with the standard quercetin peak and retention time. The amount of quercetin in ethanolic extract was determined using following equation (y =146949x + 230111).

In vitro antioxidant activity

In vitro, antioxidant studies for ethanolic extract of *Marsilea quadrifolia* L. were performed as per previously reported procedures. Here we report for the first time, the antioxidant potential of ethanolic extract of *Marsilea quadrifolia* L. To establish the antioxidant potential of *M. quadrifolia,* we have performed various antioxidant assays. It includes total antioxidant activity,[19] assay for reducing power, DPPH free radical scavenging activity,[20] scavenging of superoxide radical by alkaline DMSO method, scavenging of hydrogen peroxide,[21] nitric oxide scavenging assay, scavenging of hydroxyl radical by deoxyribose method.[22]

RESULTS

Pharmacognostic evaluation

Morphological characteristics

Marsilea quadrifolia L. leaves are compound and quadrifoliate. Colour of the leaves are green on upper surface and pale green on lower surface. *Marsilea quadrifolia* is found to possess no order, and characteristic taste. Texture of the leaves is thin and smooth. Length of the leaves are approximately 2.1-2.5cm and the width is 1.9-2.2cm. Apex of leaves looks rounded. The venation of leaves has dichotomously branched veins

Figure 2: *Marsilea quadrifolia L.*, A. Whole plant, B. Leaf and petiole, C. Stem and root.

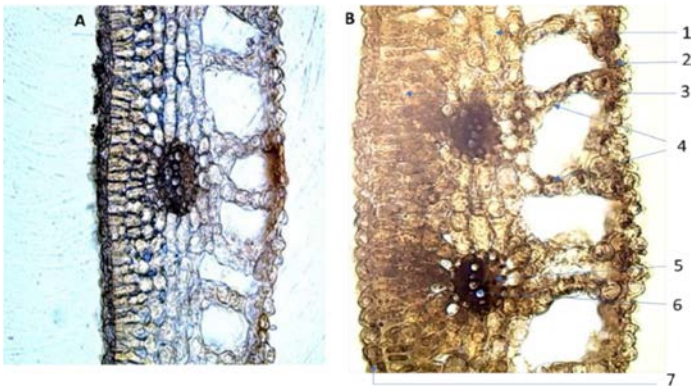

Figure 3: Transverse section of the leaf; A. Without staining, B. With staining; 1. Spongy tissue, 2. Abaxial epidermis (lower epidermis), 3. Palisade mesophyll, 4. Septa, 5. Phloem, 6. Xylem, 7. Adaxial epidermis (Upper epidermis).

and entire margins. Shape of the leaves are obovate and broad petiole (Figure 2). Petiole, cylindrical shape, 6 to 16 cm long, greenish covered with white hairs, astringent taste and fibrous fracture. Stem, cylindrical, greenish, fibrous fracture and astringent taste. Root, present at nodes and intra nodes, 0.4 to 1.1 mm width, 8 to 19 cm length, cylindrical, astringent taste, fibrous fracture and brown colour was observed. Our results are in accordance with earlier study on in-depth pharmacognostical view of *Marsilea quadrifolia L.*[23]

Histological and Powder evaluation

Transverse section of the leaves shows dorsiventral arrangement. Epidermal cells are quite similar in both the upper and lower epidermis. The upper epidermis is single layer covered by cuticle. Few layers (3-4) of palisade-parenchyma cells are present just below the epidermis followed by spongy parenchyma. Vascular bundle is present in the mesophyll region covered with a bundle of sheath (endodermis). Vascular bundle is closed and collateral and the xylem is surrounded by phloem on the lower side. Stomata is present in both the epidermis to be diacytic and anisocytic type. The mesophyll region towards the lower epidermis is filled with airspace (Figure 3).

T.S. of the petiole shows, a single layer of epidermis followed by hypodermis. Cortex region present beneath the hypodermis. The air space present in the cortex region, outer cortex region composed of aerenchyma with may septa like sheath, around 17 to 18 numbers of septa are observed. Single vascular bundle is present in triangular shape and covered with endodermis. Vascular bundle contains phloem and xylem cells, and the arrangement of xylem is v-shaped with proto and metaxylem cells, and the remaining portion is occupied by phloem cells (Figure 4).

T.S. of Stem contains single layer epidermis (parenchymatous cells). Below the epidermis is the outer cortex region number of air cavities are observed separated by air called thin septa. the cortex region is made up of parenchymatous tissues, few cells are filled with tannin content.

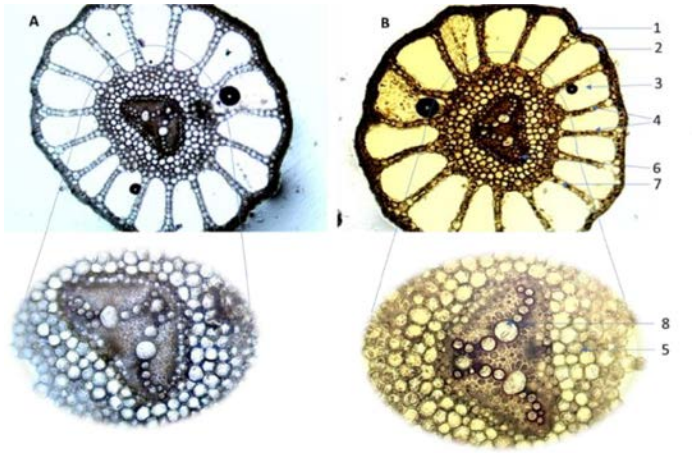

Figure 4: Transverse section of petiole; A. Without staining, B. With staining; 1. Epidermis, 2. Hypodermis, 3. Air space, 4. Septa, 5. Inner cortex, 6. Endodermis, 7. Collenchyma, 8. Metaxylem.

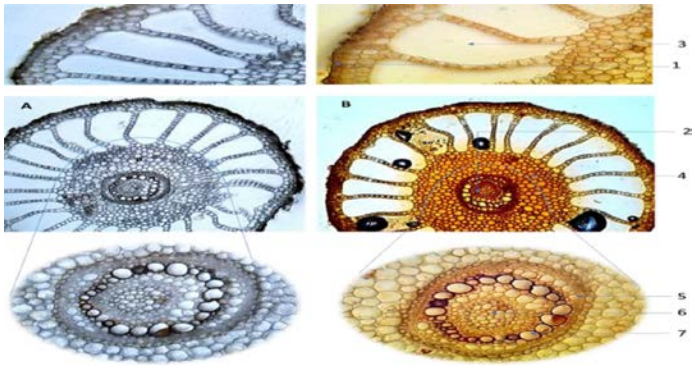

Figure 5: Transverse section of stem; A. Without staining, B. With staining; 1. Epidermis, 2. Medulla, 3. Aerenchyma, 4. Cortex, 5. Phloem, 6. Endodermis, 7. Xylem.

Vascular bundle is surrounded by endodermis and pericycle. This vascular bundle contains well-defined xylem and phloem cells. Xylem is surrounded on both sides by the phloem. The center pith is present and covered with inner endodermis (Figure 5).

T.S. of root of *Marsilea quadrifolia L.* shows the single layer epidermis covered by cuticle. The epidermis is single layered and outer cortex connected by septa with well-separated air space chamber. In the inner cortex, thick-walled sclerenchyma cells are present. The cortex is followed by the vascular bundle surrounded by endodermis. Vascular bundle contains phloem and xylem cells (metaxylem, protoxylem). Phloem is a good arrangement looking with protoxylem and metaxylem (Figure 6).

In the whole plant powder microscopy, some cells were identified such as fibers, vascular bundles, epidermal cells, phloem fibers, starch grains, and oil glands are shown in Figure 7. The leaf constants were calculated for both upper and lower epidermis, was found to be a stomatal number (upper epidermis) 32.5±0.37/mm^2, stomatal number (lower epidermis) 38.2±0.61/mm^2, stomatal index (upper epidermis) 25.8±1.26, stomatal index (lower epidermis) 28.2±1.56, and vein islet number 3.76±0.09/mm^2 are shown in Figure 8.

Physicochemical evaluation

The result of various physicochemical evaluation tests are shown in Table 1. Preliminary phytochemical analysis depicts the presence of

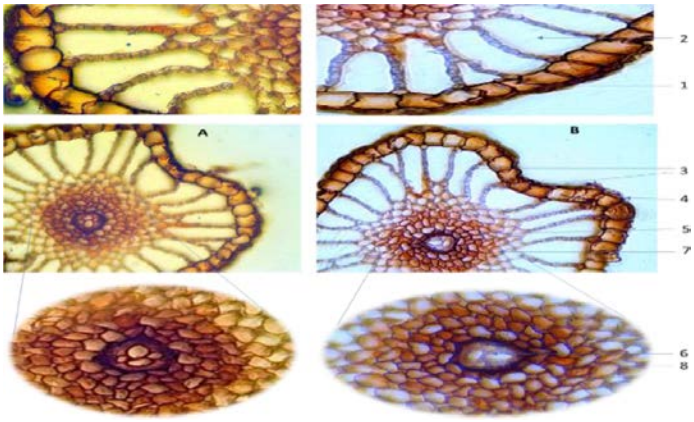

Figure 6: Transverse section of root A. Without staining, B. With staining, 1. Epidermis, 2. Air chamber, 3. Septa, 4. Cuticle, 5. Endodermis, 6. Meta xylem, 7. Cortex, 8. Proto xylem.

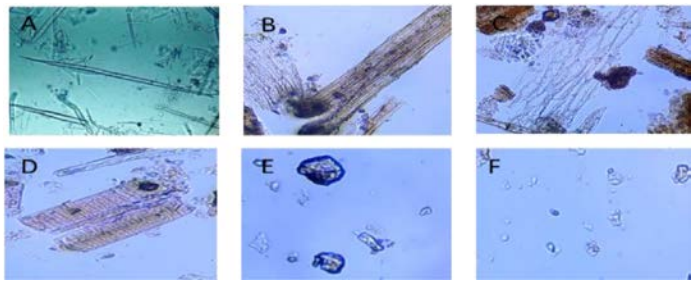

Figure 7: Powder microscopy; A. Fibers, B. Vascular bundle, C. Epidermal cells, D. Phloem fibers, E. Starch grains, F. Oil glands.

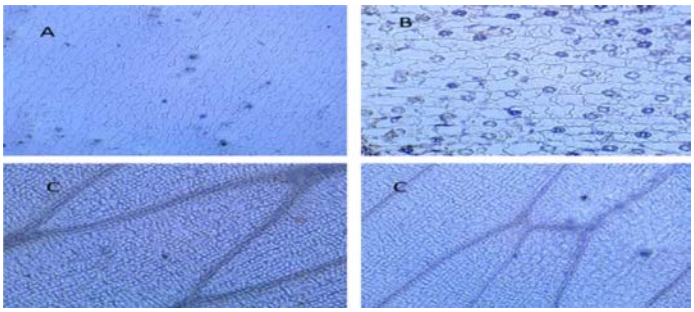

Figure 8: Leaf constants; A. Stomata of the Lower epidermis, B. Stomata of upper epidermis, C. Veinlet in young leaf.

alkaloids, phenolics, tannins, flavonoids, carbohydrates, and proteins in ethanolic extract of whole plants of *Marsilea quadrifolia* L.

Quantitative estimation of various classes of phytoconstituents

As preliminary phytochemical analysis shows the presence of phenol, flavonoids, tannins, and alkaloids in ethanolic extract. Further quantitative estimation was performed. Results of quantitative estimation confirmed the total phenolic content as 109.4459±0.52 mg/g equivalent to gallic acid whereas total tannin content was found to be 14.8019±0.049 mg/g equivalent to tannic acid. Total flavonoids and flavonol content were found to be 81.55±0.26 mg/g and 51.37±0.22 mg/g equivalent to rutin respectively. The total alkaloidal content was found to be 18.74±0.178 mg/g equivalent to atropine.

Table 1: Physicochemical parameters of powdered crude drug.

Sl. No.	Parameters	Values*
1	Swelling index	1.466ml
2	Foaming index	<100
3	Loss on drying (%)	13.99 w/w
4	Moisture content (%)	13.75% w/w
5	Water soluble extractive value (%)	17.35% w/w
6	Ethanol soluble extractive value (%)	13.5% w/w
7	Petroleum ether extractive value (%)	1.89% w/w
8	Total ash value (%)	13.5% w/w
9	Acid insoluble ash value (%)	1.615% w/w
10	Water soluble ash value (%)	2.63% w/w

*Mean value of three independent readings

Figure 9: TLC fingerprint; A. Ethanolic extract, B. Standard quercetin.

Thin-layer chromatography (TLC) analysis

TLC fingerprinting analysis (Figure 9) for extract shows seven spots with different R*f* values (0.446,0.467, 0.767, 0.790, 0.837, 0.930 and 0.976). The quercetin was confirmed to be present in the *Marsilea quadrifolia L.* by comparing the R*f* value with that of standard quercetin (R*f*. 0.798).

HPLC method of standardization

The chromatogram of ethanolic extract and standard quercetin is shown in Figure 10. A peak with retention time at 3.268 was conformed as quercetin and the calculated amount of quercetin in ethanolic extract of *Marsilea quadrifolia* L. was found to be 2.1805±0.02089 mg/g.

In vitro antioxidant potential of *Marsilea quadrifolia* L. ethanolic extract

The total antioxidant potential was determined by linear regression equation and it was expressed as number of equivalents to ascorbic acid and was found to be 108.54 ± 0.2058 µg/mL. Assay of reducing power is

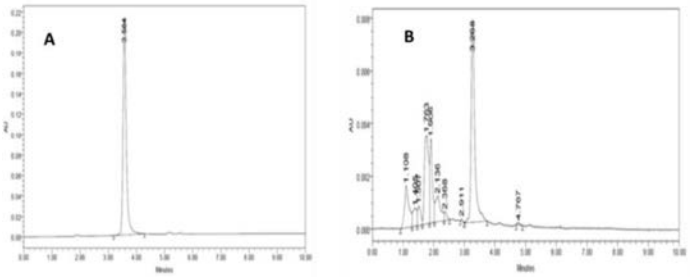

Figure 10: HPLC chromatogram; A. Standard quercetin, B. Ethanolic extract.

Table 2: *In vitro* antioxidant activities of ethanolic extract.

Drug	IC_{50} ± SEM*(µg/mL)				
	DPPH	Nitric Oxide	H_2O_2	DMSO	Deoxyribose
Standard	Ascorbic acid	Rutin	Rutin	Ascorbic acid	BHA
	46.58 ± 1.59	37.81 ± 3.57	15.83 ± 0.86	21.94 ± 2.41	31.73 ± 1.91
	Extract				
Ethanolic extract	61.726 ± 2.09	53.10 ± 3.08	25.79 ± 1.85	47.31 ± 3.39	56.80 ± 3.18

*The results are average of three determinations ± SEM

concentration dependent reaction means higher concentration indicates higher reducing power, results demonstrated the reducing potential of ethanolic extract to be 2.925±0.035 µg/mL. Capacity to reduce DPPH by donating hydrogen or electron to DPPH indicates free radical scavenging activity of ethanolic extract was showed IC_{50} of 61.72± 2.090µg/mL as compared to ascorbic acid (IC_{50}: 46.58±1.59 µg/mL). Griess reagent was used to determine the nitric oxide scavenging activity, which illustrated a similar scavenging activity of ethanolic extract (IC_{50}: 53.1033±3.080 µg/mL) reference to rutin (IC_{50}: 37.81±3.57 µg/mL). Considerably moderate scavenging potential of hydrogen peroxide by ethanolic extract of *Marsilea quadrifolia L.* was observed with an IC_{50} value of 25.796±1.856 µg/mL compared to rutin (IC_{50}: 15.83±0.86 µg/mL). In the alkaline DMSO method, IC_{50} value of 47.31 ± 3.395 µg/mL was observed in ethanolic extract whereas ascorbic acid shows IC_{50} of 21.94 ± 2.41 µg/mL (Table 2). Fenton reaction was used to assess the potential of Ethanolic extract in inhibiting the hydroxyl radical production through iron (II) dependent deoxyribose damage assay. Results reveal low scavenging activity with an IC_{50} value of 56.80±3.182 µg/mL compared to positive control Butylated hydroxyanisole (BHA) (IC_{50}: 31.73 ± 1.91 µg/mL).

DISCUSSION

Standardization of the traditional medicinal plant has become crucial to establish them as a valuable remedy and to inculcate them in modern therapy. For identification of herbal drugs, standard pharmacognostic parameters are the primary step which includes macroscopic as well as microscopic evaluation of that specific plant/plant parts. Therefore, the present study focused on standardization, which can be beneficial for proper authentication as well as identification of *Marsilea quadrifolia* L.

Physicochemical evaluation can serve as an essential tool in detecting adulteration and mishandling. Contaminants such as carbonate, oxalate, and silicate might be coming either naturally or deliberately in the plant drugs as an adulterant. These adulterants can be determined by ash values, which serve as quantitative standard. The total ash value helped in the estimation of organic and inorganic matter present in *Marsilea quadrifolia L.* The acid-insoluble ash was done for the evaluation of silica, oxalates, and earthy components mixed in plant material. The number of inorganic elements present in drugs is estimated by water-soluble ash. Extractive values can help to find out the amount of active constituents present in the plant with different solvents. Loss on drying indicates the growth of yeast, fungi, and bacteria in crude drugs. The phytochemical screening confirmed the presence of major class of chemical constituents (alkaloids, flavonoids, and tannins, etc.) in *Marsilea quadrifolia L.* The nature of constituents present in ethanolic extract gives an idea for the prediction of pharmacological activities present in herbal plants. Thin layer chromatography and HPLC standardization with reference standards can be beneficial tools for quantification of active constituents present in the extract. The antioxidant assay confirmed strong antioxidant potential of the plant extract and supports the traditional use of plant in effective management of various diseases.

CONCLUSION

The present study sets all possible standards for *Marsilea quadrifolia* L., which deciphers information regarding its identification and authentication. Also, quantification of quercetin in this plant may be helpful for better exploitation of *Marsilea quadrifolia* L. in future course of research. Presence of good generous amount of alkaloid, flavonoid and phenolic components in the plant reports the wide range of therapeutics potential of *Marsilea quadrifolia* L.. As shown by our study *in vitro* antioxidant activity of the plant supports the traditional anti-inflammatory, anticancer, antibacterial usage of *Marsilea quadrifolia* L. Further studies are required to confirm the pharmacological potential of the plant.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DMSO: Dimethyl sulfoxide; **UV:** Ultraviolet; **DPPH:** 2,2-diphenyl-1-picryl-hydrazyl-hydrate; **H_2O_2:** Hydrogen peroxide; **SEM:** Standard error of mean; **IC_{50}:** Half maximal inhibitory concentration.

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GRAPHICAL ABSTRACT

SUMMARY

Quality control standardization of the plant *Marsilea quadrifolia* was done as per World health organization (WHO) guidelines. TLC profiling and HPLC standardization of ethanolic extract *Marsilea quadrifolia* were performed. *In vitro* antioxidant activities of ethanolic extract was carried out by various methods. Results of morphological and microscopical study demonstrated various diagnostic features of the plant. Physicochemical analysis results, i.e., foaming index (<100), Loss on drying (13.99 w/w), moisture content (13.75 w/w), total ash value (13.5 w/w) were reported. Total amount of quercetin was found to be 2.1805±0.02089 mg/g in ethanolic extract of *Marsilea quadrifolia* determined through HPLC analysis which can be used as chemical/biomarker. Results of antioxidant study were indicative of significant antioxidant potential of ethanolic extract. Hence, this study sets all possible parameters for complete pharmacognostical standardization of *Marsilea quadrifolia* as per WHO guidelines.

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