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The secret of wetland: Marsilea quadrfolia as a promising green (wild) edible for health, hidden hunger and longevity from semiarid region

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Abstract

The future food security and hidden hunger are the major challenges which lead the developing countries for the exploration of wild edibles. Wild edibles have also been explored for their nutritional values which can potentially contribute to solving the issues such as malnutrition and livelihood in developing and rural communities of various parts of the world. This is first study to evaluate the nutritional values and phytochemical screening of semiarid populations of *Marsilea* spp. The nutritional analysis reveals carbohydrate (0.63 %), protein (4.20 %), fat (5.0 %), dietary fiber (8.6 %) and mineral content. the study also attempts various antioxidant assays such as DPPH, FRAP, ABTS, CUPRAC and PMA. The results show *Marsilea* is a very rich source of nutrition and minerals. It may help in livelihood development in developing and poor countries. This fern can also be utilized for fortification and developing the cultivation practice of *Marsilea*.

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1. Introduction

The population explosion, loss of agricultural productivity, biodiversity conservation for human welfare and food security for future human population is a major global challenge (Tscharntke et al., 2012). Sustainable development goal two targets eradication of all forms hunger and malnutrition. Human society also needs alternative food which preserves the land for nature.

The synthesis and accumulation of phytochemicals depends on various factors such as genetic material, ecological conditions or environmental settings. Stress such as salt, temperature can have a larger impact on phytochemical accumulation and synthesis.

The semi-arid zone: the stress enhances the accumulation of phytochemicals in cytosol, it has been reported semi-arid zone carry maximum antioxidant potential

The utilization of wild edibles is potential enough for the conservation of habitat and plays a significant role as a genetic resource (Rana et al., 2012). The wild edibles contribute to sustainable livelihood for local communities (Hazarika and Singh, 2018), enhancing the ecosystem services of habitat (Schulp, et al., (2014), gastro tourism (Lukovic et al., 2023), provisional ecosystem services in urban forest (Hurley et al., 2018). The wild edible plants have also been utilized for their medicinal importance such as treatment of obesity

and related disorders (Marrelli et al., 2020), antioxidant (Romojaro et al., 2013).

The practice of utilizing ferns as food is from a very ancient time in local indigenous communities. Various species of genus marselia is utilized for in food such as Marselia vitata, *Marsilea quadrifolia* L. (Joshi and Joshi, 2009). Study also have been reported for their ethnomedicinal importance. The extract of *Marsilea quadrifolia* is evaluated against Alzheimer's disease, anti-inflammatory, sleep disorder. The indigenous community of Nepal, India collects *Marsilea quadrifolia* L. shoots for food. This study aimed to evaluate the nutritional values and phytochemical screening of semiarid populations of *Marsilea* spp.

2. Material and methods

2.1 Plant material and sample preparation

Marsilea quadrifolia was collected from Nalsarovar, situated near Sanand village in Ahmedabad, Gujarat in 2021-22. The aerial parts (leaf and petiole) were collected and taken to the laboratory. The leaves were washed with tap water and dried using bloating paper. After wiping away all the moisture, the leaves were shade dried at room temperature (30˚- 40˚ C). The dried leaves were ground and stored in airtight bags at room temperature to evaluate proximate analysis, mineral content and antioxidant capacity.

2.2 Proximate analysis

The proximate composition of plants including protein content, fat content, dietary fiber and minerals were estimated from the dried sample while moisture content was determined from fresh sample.

2.2.1 Determination of Carbohydrate Content

The carbohydrate content was estimated by subtracting percentage values of moisture, protein content, ash content and crude fat from 100 (Suksathan et al., 2021).

Carbohydrate content (%) = 100 – [Moisture (%) + Protein (%) + Ash (%) + Fat (%)]

2.2.2 Determination of Protein content

The protein content was determined by Kjeldahl's method (Suksathan et al., 2021).

The protein estimation was calculated using the formula given below:

Protein (%) = [(A – B) x N x 1.4 x 6.25] / W

Where A = volume of 0.1 N HCl used in sample titration

B = volume of 0.1 N HCl used in blank titration

N = normality of HCl

W = weight of sample

2.2.3 Determination of fat content

Two gram moisture free plant sample was extracted with petroleum ether (40-60˚C) in a Soxhlet apparatus for about 6-8 hours. The extraction was filtered with Whatman filter paper and the filtrate was evaporated using a water bath at 100 ˚C in a pre-weighed beaker. The increased weight of beaker represents crude fat content. The crude fat percentage is calculated by using formula given below (AOAC, 2000).

Crude fat (%) = (Weight of fat) / (Weight of plant sample taken) x 100

2.2.4 Determination of dietary fiber

The moisture and fat free sample (2 g) was boiled in 200 ml of 0.128 M H_2SO_4 for 30 minutes on a hot plate with continuous shaking. The mixture was filtered through muslin cloth to drain acid solution. The sample was washed with hot water and transferred to the flask. Then, 200 ml of 0.313 M NaOH was poured into a flask and boiled for 30 minutes with continuous shaking. The residue was filtered with muslin cloth, washed with hot water and collected in a clean and dried crucible. The residue was dried at 230˚C in hot air oven for 2 hours and weighed. The residue sample was incinerated in a muffle furnace at 550˚C for 3-4 hours, cooled in a desiccator and reweighed (AOAC, 2000).

Dietary fiber content (%) = (Weight of dietary fiber) / (Weight of sample) x 100

2.2.5 Moisture content

The moisture content was estimated by drying fresh sample (2 g) weighed in petri-dish in hot air oven at 60˚C. The petridish was reweighed after drying. Moisture content was calculated by given formula (AOAC, 1990).

Moisture content (%) = (Difference in weight of sample) / (Weight of sample) x 100

2.2.6 Minerals content

The dried and ground plant sample (1 g) was taken in porcelain crucible to ash the samples in muffle furnace for four hours at 450 ˚C till white/greyish residue was obtained. The residue was digested in 5 ml HNO_3 and diluted up to 25 ml in volumetric flask. The mineral content was determined using Atomic Absorption Spectroscopy (Dospatliev & Ivanova, 2017).

2.3 Phytochemical analysis

2.3.1 Sample extraction

The dried samples were extracted in methanol and water. The sample was extracted in methanol using soxhlet while aqueous extracts were prepared by boiling the sample in water for 5-6 minutes. The extracts were filtered using filter paper in a petriplate. Extracts were concentrated by evaporating solvents. Aqueous extracts were concentrated by heating petriplates and methanol was dried at room temperature. These methanolic extracts and aqueous extracts were used to perform the following analyses.

2.3.2 Identification of fatty acids using gas-chromatography-mass spectroscopy (GCMS)

GCMS technique was employed for the identification of phytochemicals present in the fat content obtained from the leaf sample using method given by Elangovan et al., 2015. The analysis was carried out using Perkin Elmer system (GC Clarus 680, USA) equipped with auto injector. Detection was operated in electron impact mode with ionization energy of 70 eV with helium as a carrier gas at a constant flow of 1 ml/min. The injection volume of 1 µL of sample was employed with split ratio 10:1 and injector temperature of 240˚C. The oven temperature was increased 10˚C/min from 60˚C to 300˚C/min for 6 minutes.

The unknown compounds in spectrum obtained were identified by comparing with spectra of known compounds stored in the library.

2.3.3 Phytochemical screening

The extracts were analyzed for phenols and flavonoids qualitatively (Shaikh & Patil, 2020).

1. Phenols

Ferric chloride test: The extract (1 ml) was pipetted into the test tube and few drops of 5% ferric chloride solution were added. A dark green or bluish black colour indicated the presence of phenols.

2. Flavonoids

Alkaline reagent test: the extract (1 ml) was pipetted into a test tube and 2% of NaOH solution (2 ml) was added. An intense color develops which disappears on addition of few drops of dil. HCl afterwards. It indicated the presence of flavonoids.

2.4 Total phenolic content (TPC) and Total flavonoid content (TFC)

Total phenolic and total flavonoid content were estimated according to the methods described (Kamtekar et al., 2014). The total phenolic content was estimated by Folin-Ciocalteu's assay. It was expressed as mg of gallic acid equivalents per g of extract of sample (mg GAE/ g extract) using gallic acid as standard (0.2-1.0 µg/µl). The extracts (1 ml) were pipetted into a test tube and 5 ml of distilled water was added followed by addition of of Folin-Ciocalteu's reagent (0.5 ml). The solution was incubated for 5 minutes at room temperature. Then, 20% of Na_2CO_3 (1.5 ml) was added and final volume was made up to 10 ml with distilled water. After 10 minutes of incubation at room temperature, the absorbance was measured at 750 nm by using a spectrophotometer (Shimadzu UV-1800 UV/Vis).

The total flavonoid content was expressed as mg of Quercetin equivalents per g extract of sample (mg QE/ g extract) using Quercetin as standard (0.2-1.0 µg/µl). The extracts (1 ml) were pipetted out into a test tube and distilled water (4 ml) was added. Afterwards, 5% of $NaNO_2$ solution (0.3 ml) was added to the mixture and then the mixture incubated for 5 minutes. Then, 10% of $AlCl_3$ solution (0.3 ml) was added followed by addition of 1 M NaOH solution (2 ml) to the mixture. Finally, distilled water were added to the mixture to make a final volume of 10 ml and shaken. The absorbance was measured at 510 nm by using a spectrophotometer (Shimadzu UV-1800 UV/Vis).

2.5 Antioxidant Capacity

2.5.1 DPPH

The extract (2-10 µl) was diluted by methanol in test tubes, then 3 ml of DPPH (2.2-diphenyl-1-picrylhydrazyl) freshly prepared in methanol was added. The mixture was incubated for 30 min. at the room temperature in the dark. The absorbance

was measured at 517 nm (UV-visible spectrophotometer, Shimadzu UV 1800). Scavenging activity was calculated using following equation:

% Scavenging activity = [1 – (absorbance of sample/ absorbance of control)] x 100

The antioxidant activity of the extract is expressed as IC50. IC50 is the concentration of plant extract (mg/ml) required for 50% inhibition of DPPH radicals. Each value was determined from a regression equation.

2.5.2 FRAP

FRAP reagent was prepared by mixing acetate buffer, TPTZ solution and ferric chloride solution in 10:1:1 proportion. The solution was placed in water bath at 37˚C for 10 minutes. Acetate buffer (300 mM, pH 3.6) was prepared by adding sodium acetate trihydrate to glacial acetic acid and then it was diluted with distill water to obtain the required concentration. The pH was adjusted using pH meter. TPTZ solution (10 mM) was prepared by adding TPTZ to HCl (40 mM) and dissolved at 50˚C in water bath. Ferric chloride solution (20 mM) was prepared by dissolving ferric chloride in distill water. The plant extract (100-1000 µl) was diluted by methanol in test tubes and 3 ml of FRAP reagent is added. The mixture is incubated for 30 minutes. The absorbance is taken at 593 nm. Frap activity was calculated as ferrous equivalent in mM. A standard curve for different concentrations of Fe2+ was plotted to measure antioxidant potential equivalent to Fe2+.

The antioxidant activity of the extract is expressed as EC50. EC50 is the concentration of plant extract (mM) required for 50% reduction of Fe2+ ions. Each value was determined from a regression equation obtained.

2.5.3 ABTS

ABTS (2,2' azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) reagent was prepared by mixing ABTS solution and potassium persulfate solution in equal proportions. ABTS solution was prepared by dissolving ABTS in distilled water to the concentration of 7 mM and potassium persulfate solution was prepared by dissolving potassium persulfate ($K_2S_2O_8$) in distilled water to concentration of 2.45 mM. The reagent was incubated for 12-16 hours in dark room before use. The stock solution was diluted with methanol to obtain working solution of ABTS with absorbance 0.700 at 734 nm with spectrophotometer (UV-visible spectrophotometer, Shimadzu UV 1800). The plant extract (25-200 µl) was diluted by methanol in test tubes and 3 ml of working solution of ABTS is added. The mixture is incubated for 6 minutes. The absorbance is taken at 730 nm. Percentage Inhibition activity is calculated by given equation.

% Inhibition = [1 – (absorbance of sample/ absorbance of control)] x 100

A standard curve for different concentrations of trolox was plotted to measure antioxidant capacity of plant extract equivalent to trolox.

The antioxidant activity of the extract is expressed as IC50. IC50 is the concentration of plant extract (mg/ml) required for 50% inhibition of ABTS radical cation. Each value was determined from a regression equation.

2.5.4 CUPRAC

The copper (II) chloride solution (10^{-2} M) was prepared by dissolving dehydrate copper salt in distill water. Ammonium acetate buffer (1 M at pH = 7) was prepared by dissolving ammonium acetate (NH_4Ac) in distill water. Neocuproine solution (7.5 x 10^{-3} M) was prepared by dissolved and diluted with 96% ethanol. The plant extract (200 µl) is taken in test tubes, later one ml of Copper chloride solution, Neocuproine and ammonium acetate buffer were added. After mixing well, the mixture was incubated for 30 minutes. The absorbance was taken at 450 nm. The cuprac activity is expressed as trolox equivalents (mg TE/ g extract).

A standard curve for different concentrations of trolox was plotted to measure antioxidant capacity of plant extract equivalent to trolox.

2.5.5 PMA

Phosphomolybdate reagent was prepared by mixing 0.6 M sulfuric acid solution (a), 28 mM Sodium phosphate solution (b) and 4 mm Ammonium molybdate (c) in equal proportions. All the three solutions were dissolved in distill water to attain required concentration. The plant extract (300 µl) was diluted by respective solvents used to prepare plant extracts. 3 ml of phosphomolybdate reagent was added and incubated test tubes in water bath at 95˚C for 90 minutes. The absorbance

Table 1 Nutritional and mineral composition of *Marsilea quadrifolia*

Parameters	Daily value (DV)**	Low (5% DV)**	Good source (10-19% DV)**	Rich source (20% DV)**	*Marsilea quadrifolia*
Moisture content (g/100 g)*	-	-	-	-	82.83 ± 1.04
Ash (g/100 g)	-	-	-	-	6.66 ± 0.57
Carbohydrate (g/100 g)	275	≤ 13.75	27.5 – 52.25	55.00	0.63 ± 0.28
Protein (g/100 g)	50	≤ 2.5	5.00 – 9.5	10.00	4.20 ± 0.005
Fat (g/100 g)	65	≤ 3.25	6.5 – 12.35	13.00	5.0 ± 1.0
Dietary fibre (g/100 g)	25	≤ 1.25	2.5 – 4.75	5.00	8.6 ± 0.76
Elements:					
Copper (mg/100 g)	2	≤ 0.1	0.2 – 0.38	0.40	0.081 ± 0.002
Manganese (mg/100 g)	2	≤ 0.1	0.2 – 0.38	0.40	0.975 ± 0.043
Iron (mg/100 g)	18	≤ 0.90	1.8 – 3.42	3.60	0.766 ± 0.312
Zinc (mg/100 g)	15	≤ 0.75	1.5 – 2.85	3.00	0.485 ± 0.263
Sodium (mg/100 g)	2400	≤ 120	240 – 456	480.00	198.78 ± 1.10
Potassium (mg/100 g)	3500	≤ 175	350 – 665	700.00	4282.49 ± 1.89

Values are expressed as mean of the triplicates ± SD; *Values are measured from fresh samples; **Recommended daily intake value (Food and Drug Administration US, 2013)

was taken at 765 nm after cooling the mixture. Ascorbic acid was used as standard. The results were expressed as ascorbic acid equivalent (mg AAE/ g extract).

A standard curve was prepared at different concentrations of ascorbic acid to measure the antioxidant capacity of plant extract in equivalent to ascorbic acid.

2.6 Statistical analyses

All experiments were carried out in triplicates and values were expressed as mean ± SD. The significant differences were analyzed using one-way ANOVA with $p \leq 0.05$. Pearson correlation was also calculated. The data was analysed in SPSS (IBM SPSS Statistics ver 26).

3. Results and discussion

3.1 Proximate composition

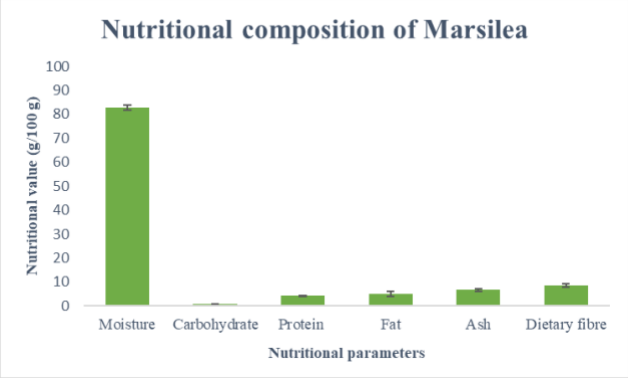
The leaves of marsilea were analysed for proximate composition. The proximate values of marsilea leaves is represented under Table 1. The proximate analysis of marsilea leaves revealed carbohydrate content, protein content, crude fat content, crude fibre and moisture content. The analysis revealed moisture content 82.83 ± 1.04 g/100 g, carbohydrate content 0.63 ± 0.28 g/100 g, protein 4.20 ± 0.005 g/100 g, crude fat 5.00 ± 1.00 g/100 g, dietary fibre 8.60 ± 0.76 g/100 and ash content 6.66 ± 0.57 g/100 g in sample.

3.2 Minerals

Mineral elements such as copper, manganese, iron, zinc, sodium and potassium are quantified. Table 1 represents mineral content in mg/100 g of the dried leaf sample. Copper is essential in keeping bones, nerves and immune system healthy. It also helps in producing erythrocytes in body (Rajeswari & Swaminathan, 2014). Manganese is crucial for the development and energy production. Moreover, it is crucial for the immune system, antioxidant defense, reproduction and controlling neurological processes (Chen et al., 2018). Iron is involved in metabolic processes like electron transport, oxygen transport and DNA synthesis (Abbaspour et al., 2014). Zinc is necessary for enzyme activity, tissue growth, foetal development, blood clotting and bone mineralization. It is essential for the structure of protein and cell membrane. It also act as transcription factor involved in controlling gene expression (Bhowmik at al., 2010).

The mineral composition of *Marsilea quadrifolia* has been compared with recommended levels of

daily value by FDA. The results suggests that *Marsilea quadrifolia* is excellent source of potassium (4282.49 mg/100 g) and good source of sodium (198.78 mg/100 g) whereas other minerals like copper (0.081 mg/100 g), manganese (0.975 mg/100 g), iron (0.766 mg/100 g), zinc (0.485 mg/100 g) were present in lower amount in the sample.



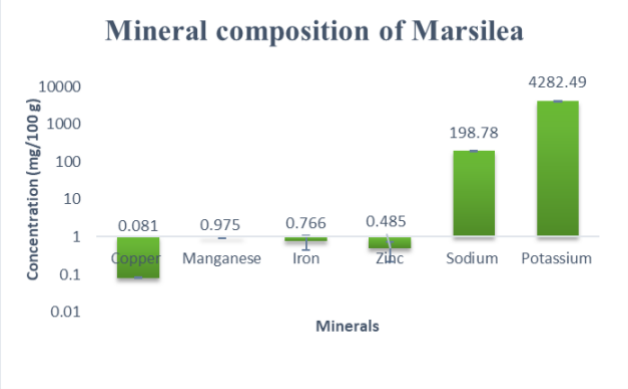


Figure 1 Nutritional profile of *Marsilea quadrifolia* A. Nutritional composition of *M. quadrifolia* B. Mineral composition of *M. quadrifolia*

3.3 Identification of fatty acids in the edible leaf by GCMS

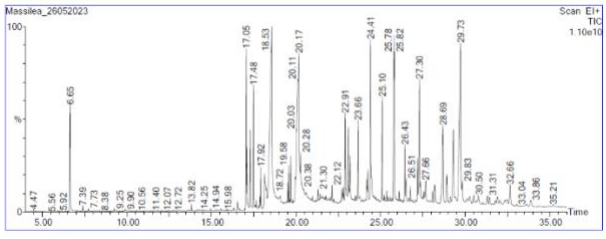


Figure 2 GCMS chromatogram of fat extraction from leaf of *Marsilea quadrifolia*

The GCMS method was successfully performed for the identification of fatty acids in lipid content of *Marsilea* leaves (Fig. 3). The active constituents found in the crude lipid extract of *M. quadrifolia* by GCMS analysis are (Table 3) Neophytadiene (2.641 %), 1,2-15,16 Diepoxyhexadecane (2.442 %), n-Hexadecanoic acid (17.106 %), 9,12,15-Octadecatrienoic acid (12.454 %), Eicosane, 2-methyl (6.509 %), 1-Hepatacosanol (2.705 %), Campesterol (2.897 %), Nonacos-1-ene (2.541 %) and ç-Sitosterol (9.414 %).

3.4 Phytochemical composition

Total phenolic content (TPC) and total flavonoid content (TFC) in leaves was analyzed in methanolic and aqueous extracts. The phenolic and flavonoid content in both the solvent extracts were compared. The results showed that methanolic extracts contained higher phenolic and flavonoid content. Total phenolic content in methanolic extract was 136.21 $\pm$ 0.006 mg GAE/g extract and 123.97 $\pm$ 0.01 mg GAE/g extract in aqueous extract. While the total flavonoid content in methanolic extract was 462.51 $\pm$ 0.099 mg QE/g extract and 2.231 $\pm$ 0.001 mg QE/g extract in aqueous extract. Phytochemical results are represented under Table 2.

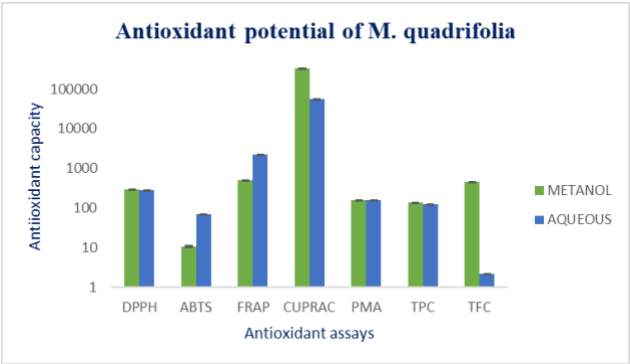


Figure 3 Antioxidant potential of *M. quadrifolia* in methanolic and aqueous solvents

3.5 Antioxidant properties

The solvents used for extraction and other factors can influence the antioxidant capacity of the plant. Hence, it is not wise to rely on one method to conclude the antioxidant properties of the plant (Alam et al., 2020). Several methods were utilized to gain better understanding of antioxidant potential of the plant extracts. In this research study, the antioxidant potential was evaluated by using DPPH, FRAP, ABTS, CUPRAC and PMA antioxidant assays.

DPPH and ABTS activity is measured as a percentage inhibitory concentration (IC50) values.

Table 2 Phytochemical composition and antioxidant properties of *Marsilea quadrifolia*

Parameters	Solvent	
	Methanol	Water
Total phenol (mg GAE/g extract)	136.21 ± 0.006	123.97 ± 0.01
Total flavonoids (mg QE/ g extract)	462.51 ± 0.099	2.231 ± 0.001
DPPH (IC50)	296.16 ± 1.90	282.79 ± 0.96
ABTS (IC50)	10.75 ± 0.68	70.54 ± 0.98
FRAP (EC50)	503.65 ± 0.79	2289.89 ± 2.37
CUPRAC (mg TE/g extract)	333473.10 ± 0.27	56645.16 ± 0.01
PMA (mg AAE/g extract)	158.15 ± 0.015	157.92 ± 0.027

Values are expressed as mean of the triplicates + SD.
IC50 (50% Inhibitory Concentration): the concentration of plant extract, which reduces the free radical about 50%
EC50 (50% Effective Concentration): the concentration of plant extract, which effectively reduces ions about 50%

The methanolic extract showed low IC50 values whereas aqueous extract showed higher IC50 values, in ABTS assay. However, no significant difference in methanolic or aqueous extract is observed in DPPH activity. FRAP activity is measured as a percentage effective concentration (EC50) value. The methanolic extract shows lower EC50 value whereas aqueous extract shows higher EC50 value. CUPRAC is expressed in equivalents of trolox. The methanolic extract showed higher antioxidant capacity (333473.10 ± 0.27 mg TE/ g extract) as compared to aqueous extract (56645.16 ± 0.01 mg TE/g extract) which was five folds lesser than methanolic extract. PMA is expressed as ascorbic acid equivalents. The assay showed no significant difference in methanolic (158.15 ± 0.015 mg AAE/ g extract) and aqueous extract (157.92 ± 0.027 mg AAE/ g extract). The antioxidant capacity of *Marsilea quadrifolia* is presented under Table 2.

3.6 Correlation between antioxidant capacity assays

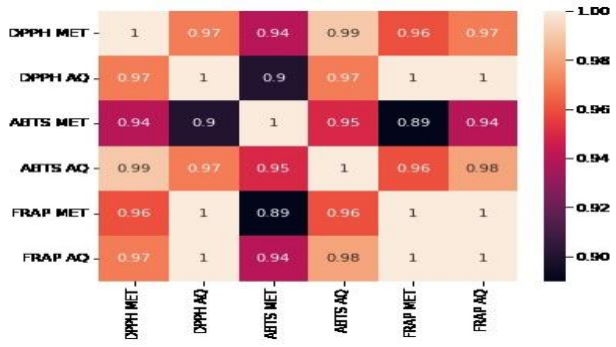


Figure 4 Correlation between antioxidant capacity assays

The Pearson correlation coefficient between DPPH, FRAP and ABTS antioxidant capacity assays in both the solvents were highly positive ranging from r = 0.89 to 0.99. The correlation results suggests that the antioxidant assays performed possess strong association.

4. Conclusion

The study shows that *Marsilea quadrifolia* is rich source of dietary fibre and is good source of protein and fat. The leaves have higher moisture content. It is also found to be significant source of minerals like potassium and sodium. The antioxidant properties and presence of phenols and flavonoids indicates that the leaves can be used to prevent malnutrition by fulfilling nutritional requirements by human beings. The study will be useful for the livelihood, food scarcity and conservation of the *Marsilea* spp. The plant can be used for the fortification with other products which have lesser mineral content in order to enhance the quality of the product.

Ethical Approval

No ethical approval was necessary for this study.

Author Contribution

All authors made substantial contributions to the conception, design, acquisition, analysis, or interpretation of data for the work. They were involved in drafting the manuscript or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all

aspects of the work, ensuring its accuracy and integrity.

Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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