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Research

Pharmacognostical, Phytochemical Evaluation of *Marsilea quadrifolia* L. Leaves

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Abstract

Marsilea quadrifolia L., commonly known as water clover or pepperwort, is an aquatic fern of significant ecological, medicinal, and nutritional importance. Widely used in Ayurvedic medicine for over 3,000 years, it is traditionally employed in the treatment of skin diseases, bronchitis, diabetes, mental disorders, eye conditions, diarrhea, and cough. In addition to its therapeutic uses, it serves as a leafy green vegetable in several Asian countries. Botanically characterized by heterospory- a key evolutionary trait leading to seed development *M. quadrifolia* exhibits both sexual and vegetative reproduction, thriving in shallow, nutrient-rich aquatic habitats. Ecologically, it plays a vital role in wetland restoration and nutrient cycling, particularly in the phytoremediation of nitrogen-enriched waters. Although introduced to North America and widespread globally, the species is now classified as "Vulnerable" in the European Union Red List due to habitat loss, eutrophication, and modern agricultural practices. Despite its broad application in traditional medicine, limited studies exist on the phytochemical composition of the plant. The current investigation aims to identify and quantify key phytocompounds such as phenolics, flavonoids, alkaloids, tannins, and saponins in the leaves and stems of *M. quadrifolia*. Further research is essential to validate its medicinal properties and support conservation efforts.

Keywords: *Marsilea quadrifolia* L., Pharmacognosy, Phytochemical screening, Flavonoids, Tannins, Alkaloids, Saponins, GC-MS analysis.

INTRODUCTION [1]

Marsilea quadrifolia L., commonly known as water clover or pepperwort, is an aquatic fern of considerable ecological, nutritional, and medicinal significance. It has been widely used in Ayurvedic medicine for more than 3,000 years for the treatment of various ailments, including skin diseases, bronchitis, diabetes, mental disorders, diarrhea, and cough. In addition to its therapeutic applications, the plant is consumed as a leafy vegetable in several Asian countries, thereby serving both nutritional and medicinal purposes.

Botanically, *M.quadrifolia* is characterized by heterospory a significant evolutionary trait leading toward seed development. The plant reproduces through both sexual and vegetative means and thrives in shallow, nutrient-rich aquatic habitats. Its leaves are typically quadrifoliate, with dichotomous venation, and possess mild astringent taste. Taxonomically, the species belongs to the family Marsileaceae and is classified under the division Pteridophyta, which encompasses ferns.

Pharmacognostically, the plant exhibits unique morphological and anatomical features, including thin, silky leaves with dichotomously branching veins, petioles covered in fine hairs, and fibrous roots. Histological studies reveal dorsiventral leaf arrangement, collateral vascular bundles, and the presence of both diacytic and anisocytic stomata. Powder microscopy further highlights diagnostic features such as epidermal fragments, stomata, vessels with spiral thickenings, and starch-containing parenchyma cells.

Phytochemical evaluations of *M.quadrifolia* have identified a diverse range of bioactive constituents, including alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, proteins, and carbohydrates. These compounds contribute to its wide spectrum of pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, and neuroprotective effects. Despite its extensive traditional use, there are limited detailed studies validating its phytochemical profile and pharmacognostic characteristics.

Ethnobotanically, the plant has been cultivated and used for generations in traditional medicine, particularly in India, where it is known as Sunishannaka. Tribal communities of the Western Ghats, Anamalai Hills, and other regions have incorporated it into their healing practices for centuries. Ecologically, *M.quadrifolia* plays an important role in wetland restoration and nutrient cycling, especially through phytoremediation of nitrogen-rich waters. However, due to habitat degradation, eutrophication, and agricultural expansion, the species is now listed as "Vulnerable" in the European Union Red List.

Considering its ecological, ethnomedicinal, and pharmacological relevance, the present study aims to provide a detailed pharmacognostical and phytochemical evaluation of *Marsilea quadrifolia* L. leaves. The work focuses on identifying and quantifying major secondary metabolites such as phenolics, flavonoids, alkaloids, tannins, and saponins through various pharmacognostic, chromatographic, and GC-MS techniques, thereby validating its traditional applications and highlighting its potential for future therapeutic development.

MORPHOLOGICAL CHARACTER

Fresh leaflet are green coloured; compound, quadrifoliate with 4 leaflets; leaflets obdeltoid, somewhat wedge shaped; glabrous, apex retuse, base connate, margin entire, venation dichotomously branched; measuring 0.8 to 2 cm long and 6 to 1.5 cm broad; young leaf shows circinate venation; petiole long, slender, measuring 5 to 11 cm long; odour nil and mild astringent taste.

Marsilea quadrifolia **(European water -clover) Four clover Leaves.**

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

PHARMACOGNOSTICAL PROFILE
Morphological Characteristics

It is found that *Marsilea quadrifolia* has a unique flavor and no smell. The texture of the leaves is thin and silky. The leaves are approximately 1.9–2.2 cm wide and 2.1–2.5 cm long. The apex of the leaf seems rounded. Leaf veins are dichotomously branching and have entire edges. The leaves are oblong and have a large petiole. Petiole: greenish-colored, cylindrical, 6–16 cm long, coated in white hairs, fibrous fracture, astringent flavor. Stem that is cylindrical, greenish in color, fragmented into fibrous fibers, and has a bitter taste. The root was discovered to be dark, cylindrical, fibrous fractured, astringent-tasting, and 0.4–1.1 mm wide. Additionally, it was discovered to exist both within and at nodes. Our results corroborate a prior study on the thorough Pharmacognostical evaluation of *Marsilea quadrifolia* L.

Histological And Powder Evaluation

The transverse section of the leaves displays the dorsiventral arrangement. The epidermal cells in the top and lower epidermis are essentially the same. One layer of the uppermost epidermis is covered by the cuticle. Spongy parenchyma follows a few layers (three to four) of palisade-parenchyma cells immediately beneath the epidermis. A vascular bundle surrounded by an endodermis bundle is present in the mesophyll area. The vascular bundle is collateral and closed, and the phloem envelops the xylem on the bottom side. Both the diacytic and anisocytic forms of stomata are found in the epidermis. From the mesophyll region to the lower epidermis, airspace stretches.

Phytoconstituents

A phytochemical examination of *M. quadrifolia* leaf and stem extracts showed the presence of phenolic compounds, tannins, saponins, flavonoids, steroids, Terpenoids, alkaloids, proteins, and carbohydrates. The phytocompounds composition of *M.quadrifolia* leaf and stem extracts, including total phenolic compounds, flavonoids, alkaloids, tannins, and saponins, was also ascertained.[Zhang et al. (2016)]

Ethnobotany Use

M.quadrifolia has been farmed for food and for ethno botanical value for many generations **(Ripa et al., 2009)**. The Vaidyas, or practitioners of Ayurveda, in India have been using it in traditional Ayurvedic therapy for thousands of years **(Joy et al., 2019)**. It is specifically used by the Indian tribal communities of the Western Ghats, Anamailais Hills, Malasars, Pulaiyars, Kadars, and Malaimalasars (Ramachandran, 2007). Here, the plant is called Sunishannaka.

PHARMACOGNOSTICAL STUDIES
Fresh leaves were subjected to Pharmacognostical studies includes organoleptic and morphological studies.

MORPHOLOGICAL STUDIES OF *Marsilea quadrifolia Linn*
External feature of leaves was documented using Nikon D-5600 Digital camera.
MICROSCOPICAL STUDIES OF *Marsilea quadrifolia Linn*
Transverse Section

Leaves were preserved in fixative FAA for more than 48 hr. The preserved specimens were cut into thin transverse section using a sharp blade and the sections were stained with safranin. Transverse sections were photographed using Nikon ECLIPSE E200 trinocular microscope attached with Zeiss Axio Cam Erc5s digital camera under bright field light. Magnifications were indicated by scale bar.

Determination Of Leaf Constant (C.K.Kokate., 2002)

The fresh leaf samples were boiled with saturated cholral hydrate solution and slides prepared. Vein islets, vein termination, epidermal number, Stomatal number, Stomatal index and palisade ratio were determined.

Preparation Of Leaf Powder

The leaves were collected and shade dried powdered was sieved in sieve no. 60 and stored in a well closed Container.

Powder Microscopy (Wallis Te, 1965)

A pinch of the powdered leaf was mounted on a microscopic slide with a drop of 50% glycerol after treating with saturated chloral hydrate for clearing and potassium iodide solution for testing starch grains. Characters were observed using Nikon ECLIPSE E200 trinocular microscope attached with Zeiss ERc5s digital **camera** under bright field light. Photomicrographs of diagnostic characters were captured and documented.

MATERIALS AND METHODS

Plant Material Collection and Preparation

Fresh leaves of Marsilea quadrifolia L. were collected, shade-dried, and coarsely powdered. The powdered drug was sieved through mesh no.60 and stored in an airtight container for further pharmacognostical, physicochemical, and phytochemical studies.

Pharmacognostical Studies

Pharmacognostical evaluation included organoleptic, macroscopic, and microscopic analyses.

Macroscopy was carried out by recording morphological features of the leaves such as size, shape, color, margin, venation, and surface characteristics.

Microscopy involved transverse sections of leaves and petioles preserved in FAA solution. The sections were stained with safranin and observed under a Nikon Eclipse E200 trinocular microscope with a Zeiss Axio Cam Erc5s digital camera. Diagnostic features such as epidermal cells, stomata, vascular bundles, and parenchyma were documented.

Powder microscopy was performed by mounting powdered drug in 50% glycerol after treatment with chloral hydrate, and diagnostic characters such as epidermal cells, stomata, trichomes, vessels, and starch grains were studied.

Quantitative Microscopy

The important identifying characteristic of leaf constants like Stomatal Number, Stomatal Index, Vein-islet number, Vein termination number and palisade ratio were found out.

Clear the piece of the leaf (middle part) by boiling with chloral hydrate solution or alternatively with chlorinated soda. Peel out upper and lower epidermis separately by means of forceps. Keep it on slide and mount in glycerin water. Arrange a camera lucida and drawing board for making the drawings to scale. Draw a square of 1mm by means of stage micrometer. Place the slide with cleared leaf (epidermis) on the stage. Trace the epidermis cell and stomata. Count the number of stomata present in the area of 1 sq. mm. Include the cell if at least half of its area lies within the square. Record the result for each of the ten fields and calculate the average number of stomata.

DETERMINATION OFVEIN-ISLETS NUMBER AND VEIN TERMINATION NUMBER

A vein-islet is the small area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islets per square millimetre of a leaf surface. It is determined by counting the number of vein islets in area of 4 sq. mm. of the central part of the leaf between the midrib and the margin.

Procedure

Clear a piece of the leaf by boiling in choral hydrate solution for about thirty minutes. Arrange camera lucida and drawing board for making drawings to scale. Place stage micrometer on the microscope and using 16 mm objectives, draw a line equivalent to 1mm as seen through the microscope. Construct a square on this line. Move the paper so that the square is seen in the eye piece, in the centre of the field. Place the slide with the cleared leaf (epidermis on the stage). Trace off the veins which are included within the square, completing the outlines of those islets which overlap two adjacent sides of the square. Count the number of vein islets in the square millimetre. Where the islets are intersected by the sides of the square, include those on two adjacent sides and exclude those islets on the other sides. (To obtain a critical result for a leaf, 4 sq. mm. should be used, preferably in one large area of 4 sq. mm). Find the average number of vein islets from the four adjoining squares, to get the

values for one sq. mm.

VEIN TERMINATION NUMBER

Veinlet termination number is defined as the number of veinlet termination per sq. mm of the leaf surface, midway between midrib of the leaf and its margin. A vein terminationis the ultimate free termination of veinlet.

Procedure

Clear a piece of the leaf by boiling in choral hydrate solution for about thirty minutes. Arrange camera lucida and drawing board for making drawings to scale. Place stage micrometer on the microscope and using 16 mm objectives, draw a line equivalent to 1mm asseen through the microscope. Construct a square on this line. Move the paper so that the square is seen in the eye piece, in the centre of the field. Place the slide with the cleared leaf (epidermis on the stage). Trace off the veins which are included within the square, completing the outlines of those islets which overlap two adjacent sides of the square. Count the number of veinlet terminations present within the square. Find the average number of veinlet termination number from the four adjoining squares, to get the values for one sq. mm.

PALISADE RATIO

A piece of the leaf is boiled in chloral hydrate and is placed under microscope. Camera lucida and drawing board were arranged and the outline of four cells of the epidermiswas traced using 4 mm objective. Then, palisade layer is focused down and sufficient cells for covering the tracing of the epidermal cells were traced off. The outline of those palisade cells which were intersected by the epidermal walls was completed. The palisade cells under the four epidermal cells (including cells which are more than half and excluding cells which are less than half within the area of epidermal cells) are counted. The determination for five groups of four epidermal cells from different part of the leaf was repeated. The average number of cells beneath epidermal cells is calculated known as palisade ratio.

PREPARATION OF LEAF POWDER

The leaves are collected and shade dried. It was powdered in a mixer. The coarse powder was sieved in sieve no. 60 and was stored in a well closed container.

QUALITATIVE PHYSIO-CHEMICAL PARAMETERS

The powder was subjected to physiochemical parameters such as loss on drying, Foreign matter, ash value, and extractive value with different solvents in increasing order of polarity.

DETERMINATION OF FOREIGN MATTER

Medicinal plant materials should be entirely free from visible signs of contamination by mould or insects, stones, other animal contamination, including animal excreta and unwanted parts of the plants.

Procedure

Weigh a sample of plant material, spread it in a thin layer and sort the foreign matters into a groups either by visual inspection using magnifying lens (6x or 10x), or with the helpof a suitable sieve, according to the requirements for the specific plant material. Shift the remainder of the sample through a No. 250 sieve to remove the mineral admixtures. Weighthe portions of this sorted foreign matter within 0.05 g.

DETERMINATION OF LOSS ON DRYING

An accurately weighed 10gm of coarsely powdered drug was placed in a tarred evaporating dish. Then the dish was dried at 105 ºC for 5 hours and weighed. The drying and weighing was continued at one-hour intervals until the difference between the successive weighing is not more than 0.23%.The loss on drying was calculated with reference to the amount of powder taken.

DETERMINATION OF ASH VALUE (Kadam etal., 2012)

The ash remaining after complete ignition of the medicinal plant materials is determined by three different methods known as Total ash, Acid insoluble ash and water- soluble ash.

Total Ash Value

Total ash is designed to measure the total amount of ash remaining after incineration. This includes both physiological ash which is derived from plant tissue itself and non- physiological ash which is the residue of the extraneous matter adhering to the plant surface.

Procedure

Incinerate about 2 to 3 g accurately weighed, of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450ºC until free from carbon, cool and weigh. If a carbon free ash cannot be obtained in this way, exhaust the charred mass with hot water, collect the residue on an ash less filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature not exceeding 450 ºC. Calculate the percentage of ash obtain after incineration.

Acid-In soluble ash

It is the residue obtained after boiling the total ash with dilute hydrochloric acid and igniting the remaining insoluble matter. This measures the amount of silica present, especially as and siliceous earth.

Procedure

The total ash was boiled with 25 ml of 2 N HCl for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot Water, ignited and weighed, then calculated the percentage of acid insoluble ash with reference to the air dried drug.

Water-soluble ash

It is the difference in weight between the total ash and the residue after treatment of the total ash with water.

Procedure

The total ash was boiled with 25 ml. of water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450C. The weight of insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried drug.

DETERMINATION OF MOISTURE CONTENT (Marina vera Zambrano et al., 2019)

Moisture content determination is important, not only to know excess water, but also in conjunction with suitable temperature moisture will lead to the activation of enzymes and gives suitable conditions to the proliferation of living organism. As most vegetable drugs contain all the essential food requirements for mould, insects and mites, deterioration can be very rapid, once infestation has taken place. Various methods for moisture determination are loss on drying, separation and measurement of moisture, chemical methods, electrometric methods, and spectroscopic methods as per IP. 10gm of powder was weighed and placed it in a moisture content apparatus. Temperature was adjusted to 100-1100c till weight get constantand collected in desiccators and weighed. The loss of weight was regarded as a measure of moisture content as per IP.

DETERMINATION OF EXTRACTIVE VALUE (Pawar etal., 2015)

PROCEDURE

About 5 g of the air dried coarsely powdered drug was macerated with 50 ml of ethanol in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowed to stand for 18 hours. Thereafter filtered rapidly, taking precaution against loss of solvent. Then evaporate 25 ml of the filtrate to dryness in a tared flat bottomed shallow dish dry at 105 ºC and weighed. Similar procedure was adopted for the determination of petroleum ether,Hydro- alcoholic, chloroform, Ethanol, ethyl acetate and water.

EXTRACTION

PREPARATION OF ETHANOLIC EXTRACT OF *Marsilea quadrifolia* Linn (EEMQ)

PROCEDURE

The leaves were collected, shade dried and coarsely powdered, passed through sieve no 60. The powdered leaves were defatted with petroleum ether and then defatted marc was extracted with ethanol by soxhlet technique, was concentrated to dryness and stored in a closed container for further use. The ethanolic extract of *Marsilea quadrifolia* L.(EEMQ) is subjected to qualitative and quantitative analysis. Qualitative analysis includes phytochemical screening of primary and secondary metabolites such as flavonoids, carbohydrates, alkaloids, glycosides, sterols, tannin, protein, amino acids, coumarin, anthocyanin, terpenoids, phenolic content, saponins, Gum and Mucilage and High performance thin layer chromatography of the extract were determined. Quantitative analysis includes estimation of total tannin content, total Gallic acid content, total flavonoid contents in terms of total tannic acid equivalent, total Gallic acid equivalent, total flavonoids equivalent (quercetin) and extract were determined.

PRELIMINARY PHYTOCHEMICAL SCREENING

Ethanolic extract of *Marsilea quadrifolia Linn* (Leaf) is subjected to qualitative chemical analysis. The various chemical tests were performed on this extract for the identification of flavonoids, phenolic compounds, Tannins, alkaloids, glycosides, carbohydrates, proteins, amino acids, sterols, terpenoids, Anthocyanins, Quinones, Gums and Mucilage as per **(Harborne *etal.*,1998).**

CHROMATOGRAPHIC STUDIES

The extract was subjected to chromatographic studies by means of TLC and HPTLC, the chromatogram was developed and recorded using quercetin.

EVALUATION OF EEMQ BY TLC

DETECTION OF FLAVANOID BY TLC METHOD

The extract was subjected to chromatographic studies by means of TLC chromatogram was developed and recorded using Quercetin. Evaluation of EEMQ by TLC Sample is prepared by taking 5mg of extract and dissolved in the solvent (1:1). Then on the silica gel coated TLC plate, 10µl of sample is placed and the migration is carried out using suitable solvents for each desired chemical group.

Stationary phase: Silica gel

Mobile phase: (Ethyl acetate: Formic acid: Glacial acetic acid: Water) (10:1.1:1.1:2.6).

The color of the compound can be detected by under iodine chamber and see the under UV chamber. The color of the spots is recorded.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

The standardization of a crude drug is an integral part of establishing its correct identity. The results of HPTLC investigation could serve as a basis for proper identification of the plant. In the present study, chromatographic fingerprint profile of Ethanolic Extract of *Marsilea quadrifolia* was studied by HPTLC.

Sample preparation and Application

1 mg/ml concentration of the extract was prepared in ethanol of chromatographic grade and then filtered by whatman filter paper No. 1. It was then sonicated and centrifuged. Prepared sample of extract in serial dilutions (10,20,30,40 µl each) were applied on TLC aluminium sheets silica gel 60 F 254 (Merck) with band length of 8 mm using Linomat 5 sample applicator set at a speed of 100 nl/sec.

Developing Solvent System

A number of solvent systems were tried, for the selected extract for better resolution and maximum number of spots, but the satisfactory resolution was obtained in the solvent Toluene: Ethyl acetate: Formic acid (5:4:0.5 v/v/v) .

Development of Chromatogram

The chromatograms were developed in twin trough glass chamber saturated with solvent system of Toluene: Ethyl acetate: Formic acid (5:4:0.5 v/v/v) for 20 minutes up to thedistance of 70 mm.

Scanning and Detection of spots

The air dried plates were viewed in ultraviolet light. Spots were visible at 366 nm and then visualized in visible light range 400-600nm. Scanning was performed by CAMAG HPTLC Densitometer (Scanner 4) in absorbance mode at 366 nm, using deuterium and tungsten lamp with slit dimension 6.0 X 0.45 micro. The Rf value and colour of the resolved bands were noted.

GAS CHROMATOGRAPHY-MASS SPECTROSCOPY ANALYSIS

EEMQ was analyzed by GC-MS

Make	:	SHIMADZU
GC model	:	2010 PLUS
Mass Spectrometer	:	QP2020
Software	:	GCMS solution
Library year	:	NIST-2017

INSTRUMENTATION ACQUISITION PARAMETERS

Oven: Initial temp 50°C for 3 min, ramp 15°C/min to 180 °C hold 5 min and increase 10°C/min to 280□ C hold for 4 min.

Total Run Time: 30 min
Auto Injection=280°C, Volume=1 µL, Split=10:5,
Flow Rate: 1 mL/mint
Carrier Gas=He,
Column=SH-Rxi-5Sil MS (30.0m, 0.25mmID, 0.25µm df)

MASS CONDITION (EI)

Solvent Delay=3.00 min Transfer
Temp=290°C Source Temp=230°C
Scan: 50 to 600 Da

PROCEDURE

The shimadzu GCMS QP 2020 was used in the analysis employed a fused silica column, packed with SH-Rxi-5Sil MS (30 m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 ml/min. The injector temperature was set at 280°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 50 °C (3 min); followed by 180 °C at the rate of 15 °C min−1 The mass detector conditions were: transfer line temperature 290 °C; ion source temperature 230 °C; and ionization mode electron impact at 70 eV, a scan time 0.2 sec and scan interval of 0.1 sec.
The fragments from 50 to 600 Da. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS NIST (2017) library.

RESULTS AND DISCUSSIONS

MACROSCOPIC STUDIES ON *Marsilea quadrifolia* L.

Macroscopy of *Marsilea quadrifolia* (L.)

Fresh leaves of *Marsilea quadrifolia* (L.) was subjected to organoleptic and Macroscopical studies and the results were presented in **Table 1 and2.**

Table 1: Organoleptic characters of *Marsilea quadrifolia* (L.)

S.No	Organoleptic Characters	Observation
1.	Colour	Yellowish Green
2.	Odour	Characteristic
3.	Taste	Astringent

Table 2: Macroscopical studies of *Marsilea quadrifolia* L.

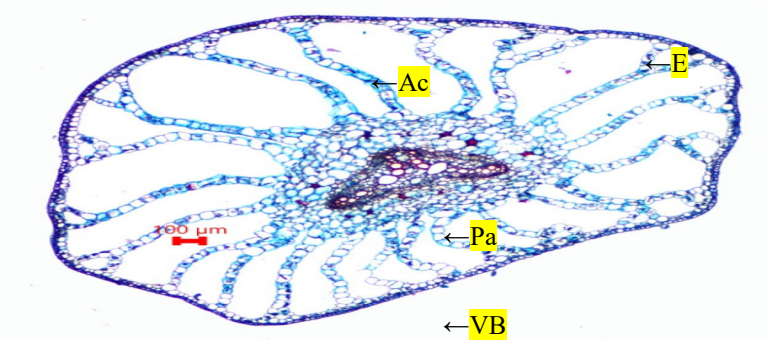
S.No	Characters	Observation
1.	SIZE	LENGTH:2.0cm,WIDTH:1.5cm
2.	SHAPE	OBOVATE
3.	TEXTURE	SHORT
4.	FRACTURE	EASY

5.	Surface	Smooth And Glabrous
6.	Arrangement	Simple
7.	Apex	Obtuse,Retuse
8.	Base	Cordate
9.	Petiole	Long
10.	Margin	Entire/ Dentate
11.	Venation	Radiating,Dichotomously Branced

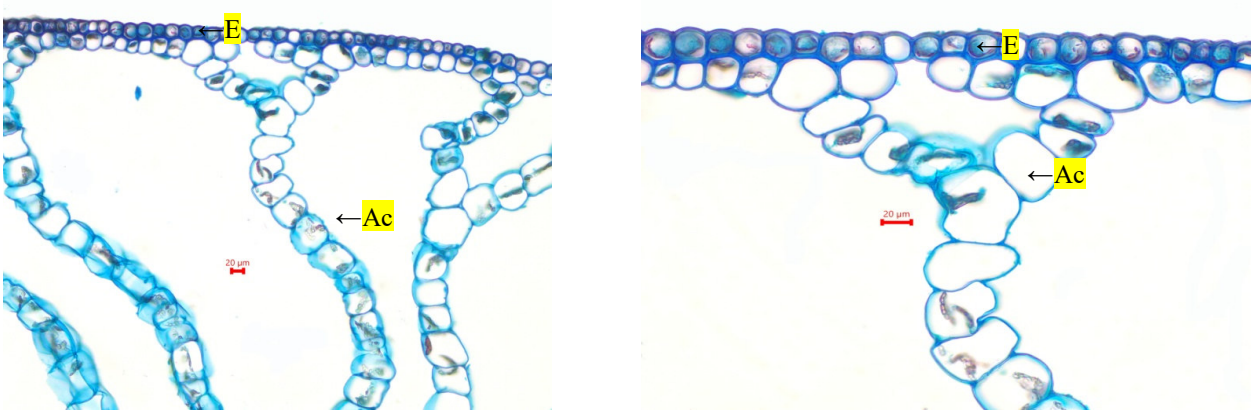
Fresh leaflet are green coloured; compound, quadrifoliate with 4 leaflets; leaflets obdeltoid, somewhat wedge shaped; glabrous, apex retuse, base connate, margin entire, venation dichotomously branched; measuring 0.8 to 2 cm long and 6 to 1.5 cm broad; young leaf shows circinate venation; petiole long, slender, measuring 5 to 11 cm long; characteristic odour and mild astringent taste.

HISTOLOGICAL STUDIES
QUALITATIVE MICROSCOPY
Petiole

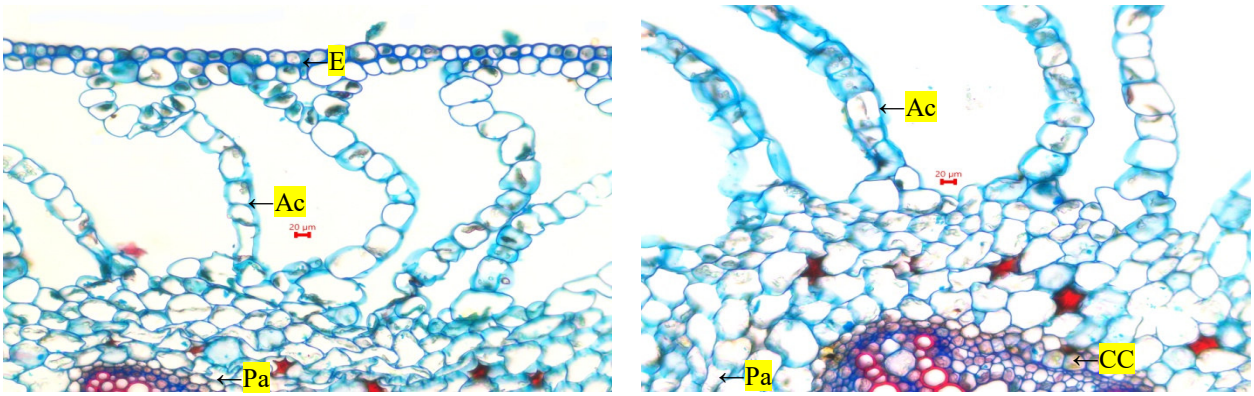
TS of petiole is irregularly circular shaped; outer layer is single layered epidermis; cortex is broad with large aerenchyma cells followed by 3 to 4 layers of parenchymatous cells; center portion of section is occupied by concentric, amphicribal vascular bundle covered by single layer of parenchymatous pericycle; some cell contents and starch grains are found scattered in parenchyma cells (Fig.1).



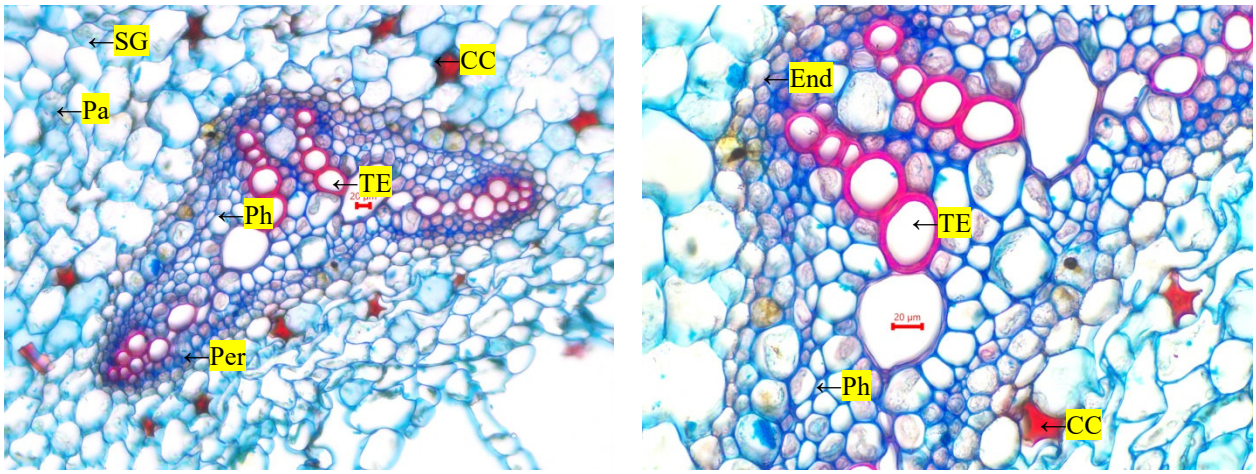
TS of petiole



Upper portion enlarged



Enlarged view of cortex



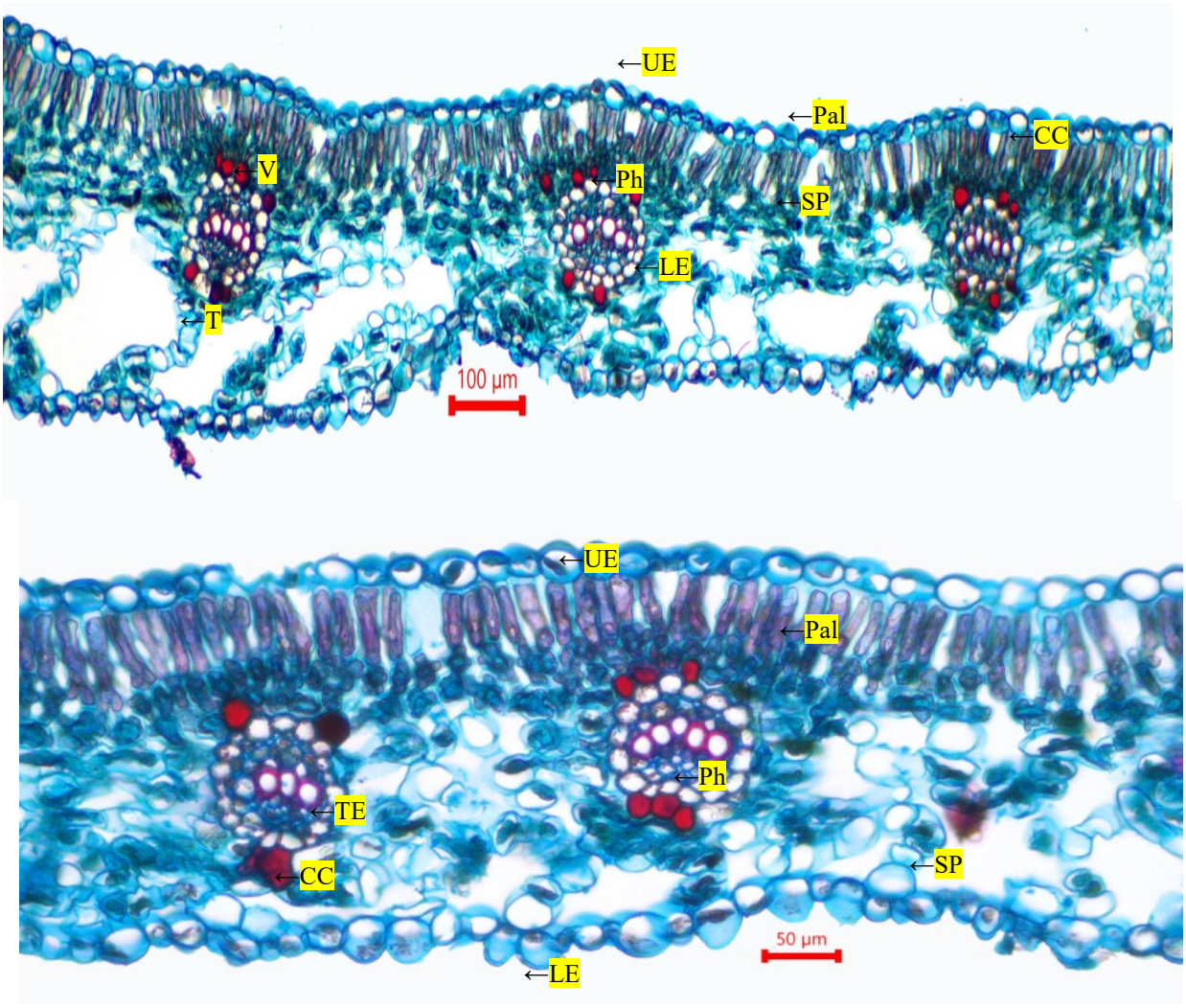
Enlarged view of vascular region

Ac - aerenchyma; **CC** - cell contents; **E** - epidermis; **End** - endodermis; **Pa** - parenchyma; **Per** - pericycle; **Ph** - phloem; **SG** - starch grain; **TE** - tracheary element; **VB** - vascular bundle

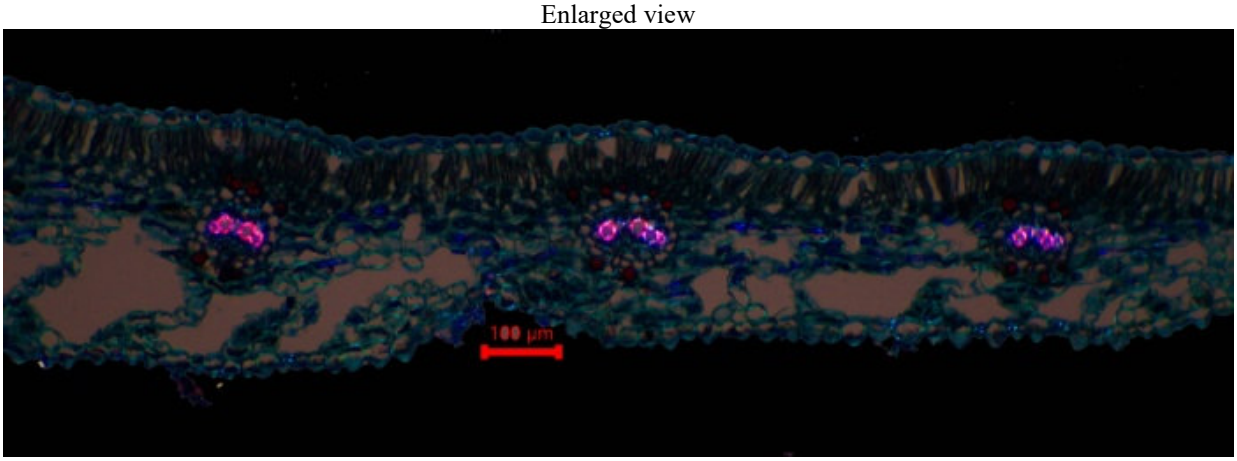
Fig 1: TS of *Marsilea quadrifolia* petiole

Leaflet

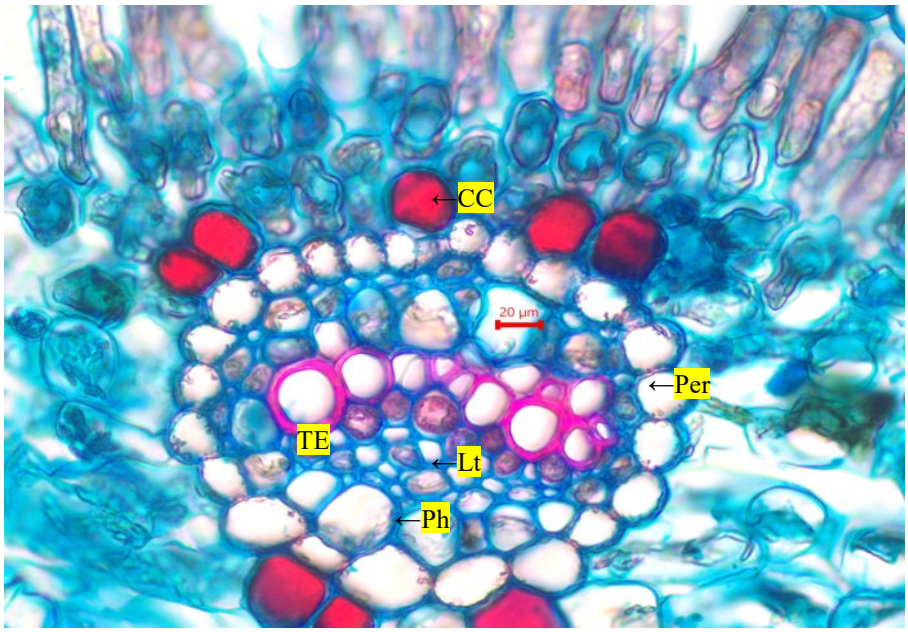
TS of leaflet is flat and ribbon shaped; upper and lower epidermi are single layered and covered by thin cuticle and bears few covering trichomes; mesophyll tissue is differentiated into upper single row of compactly arranged palisade cells and lower 3 to 4 layers of loosely arranged spongy parenchyma cells; several vascular bundles can be seen traversing through the mesophyll tissue; vascular bundles are concentric and amphicribal covered by single layer of parenchymatous pericycle; some cell contents are found scattered near the vascular tissue; some latex tubes are found in phloem (Fig. 2).



Enlarged view



TS of leaf under polarized field



Enlarged view of vascular region

CC - cell contents; **LE** - lower epidermis; **Lt** - latex tube; **Pa** - parenchyma; **Pal** - palisade cells; **Per** - pericycle; **Ph** - phloem; **SP** - spongy parenchyma; **T** - trichome; TE - tracheary element; **UE** - upper epidermis.

Fig 2: TS of *Marsilea quadrifolia* leaflet passing through veins

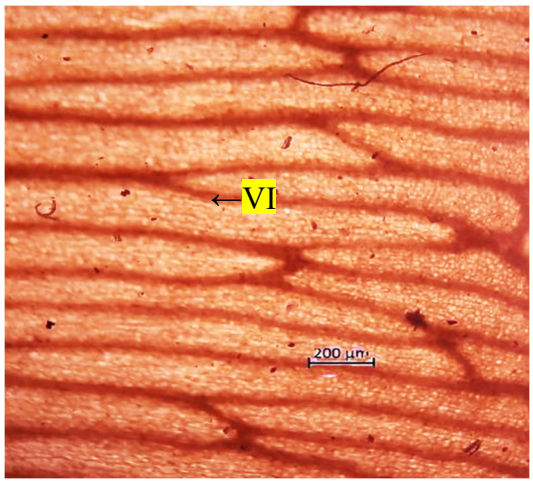
QUANTITATIVE MICROSCOPY

The quantitative parameters obtained during microscopic observation of epidermal peelings of leaf were recorded (Table 3). The leaf shows sunken stomata (Fig. 3).

Table 3: Quantitative microscopy of *Marsilea quadrifolia* leaflet

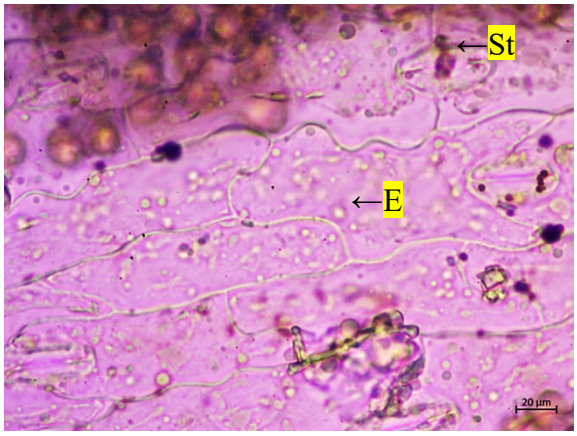
Parameters	Upper epidermis (/mm^2)	Lower epidermis (/mm^2)
Epidermal number	200 – 210	275 – 280
Stomatal number	55 – 60	85 – 90
Stomatal index	21.5 – 22	23.6 - 24.3
Palisade ratio	7 – 10	
Vein islets	12 – 15	
Vein terminations	-	

Upper epidermis Lower epidermis



Dichotomous venation

E - epidermis; **St -** stomata; **VI -** vein islets



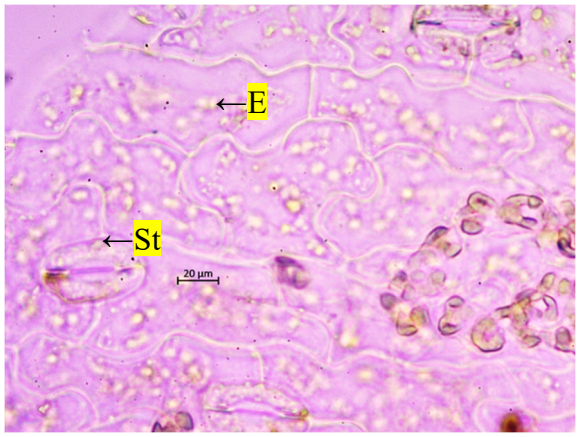
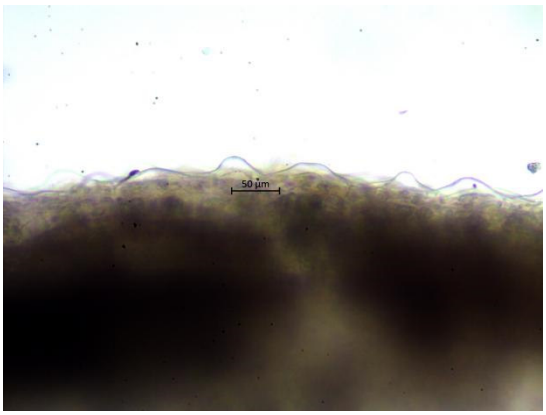
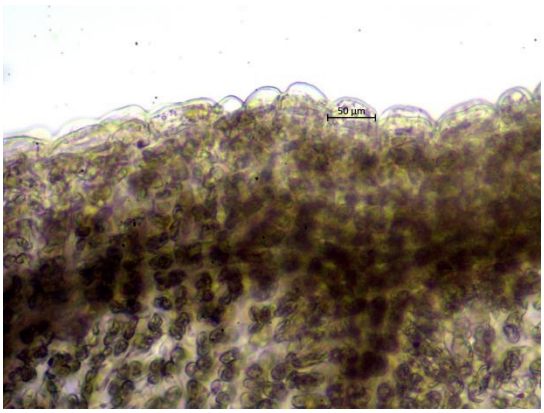


Fig 3: Quantitative microscopy of *Marsilea quadrifolia* leaflet

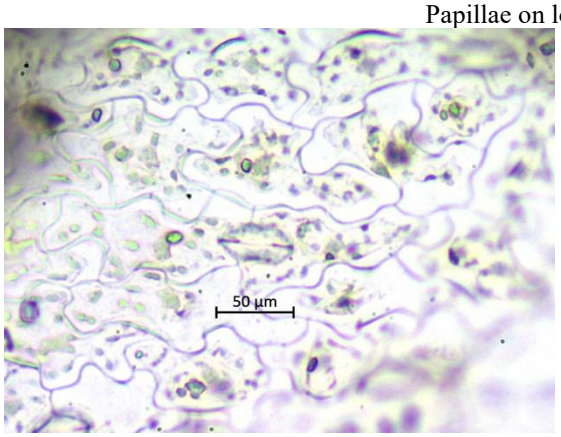
POWDER MICROSCOPY

The powder is pale yellowish green coloured with no odour and mild astringent taste; shows the characters like lower pappilate epidermis, fragment of upper epidermis with stomata; fragment of petiole epidermis in surface view, spongy parenchyma cells, aerenchyma cells from petiole, vessels with simple pitted and spiral thickenings, parenchyma cells with starch grains from petiole cortex (Fig. 4).

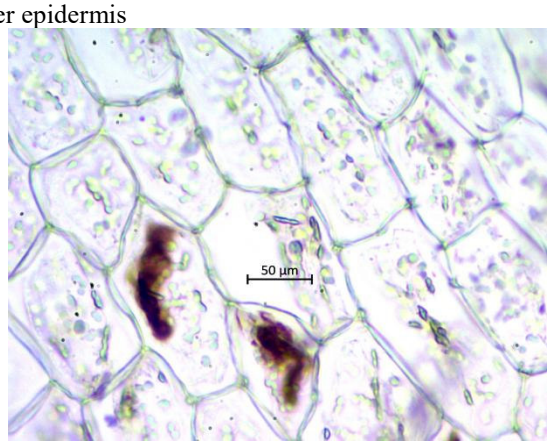




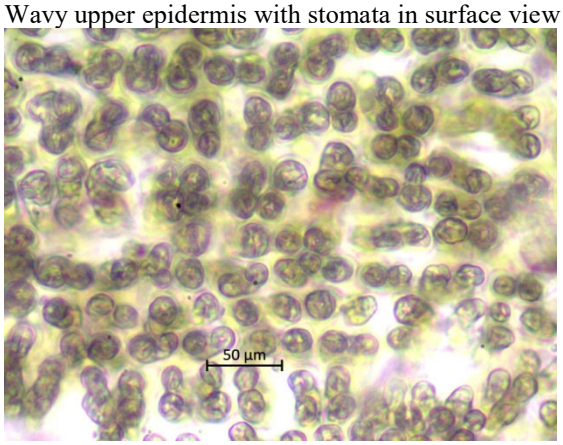
Papillae on lower epidermis



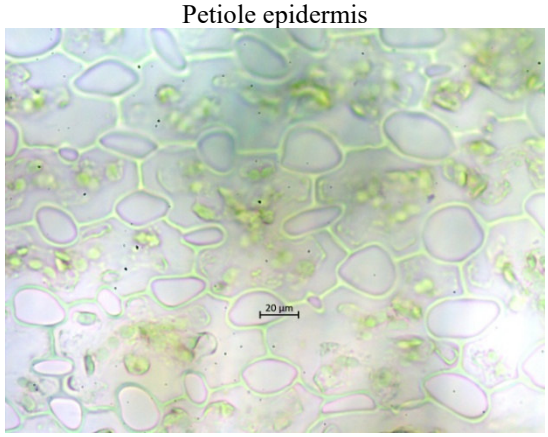
Wavy upper epidermis with stomata in surface view



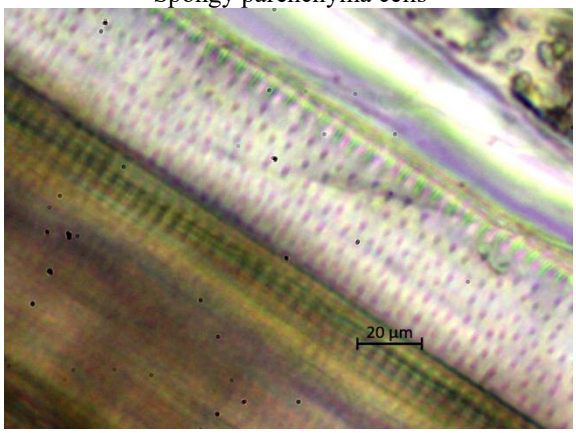
Petiole epidermis



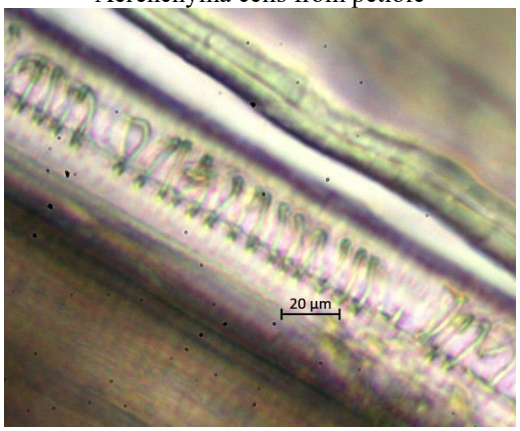
Spongy parenchyma cells



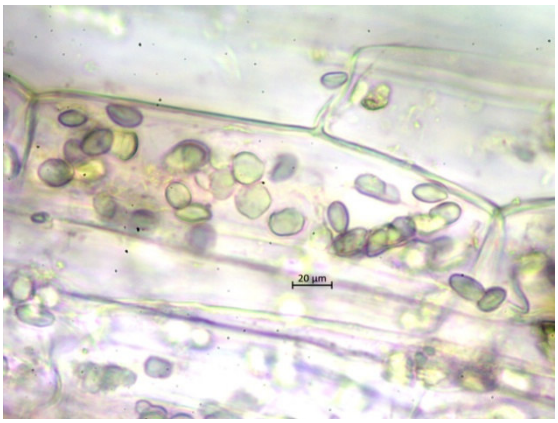
Aerenchyma cells from petiole



Pitted vessels



Spiral vessels



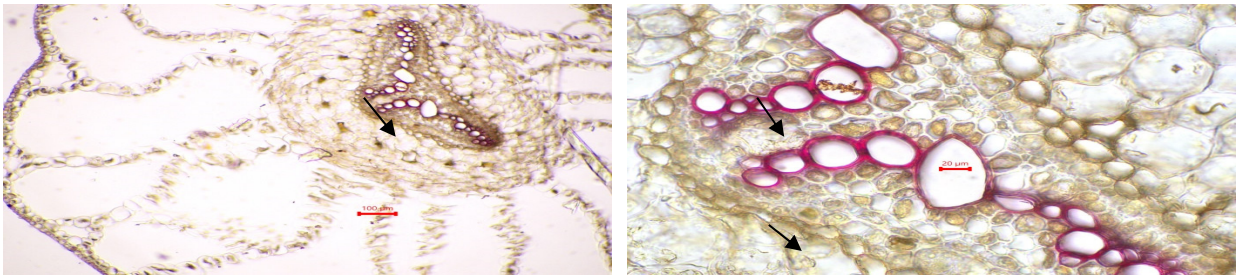
Parenchyma with starch grains

Fig 4: Powder microscopy of *Marsilea quadrifolia* leaf

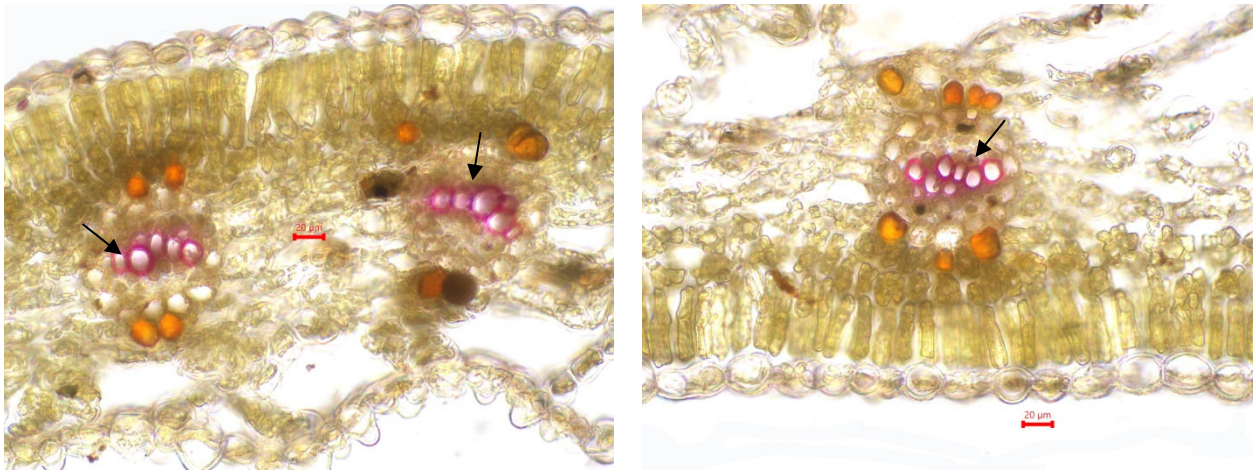
HISTOCHEMISTRY

Leaf

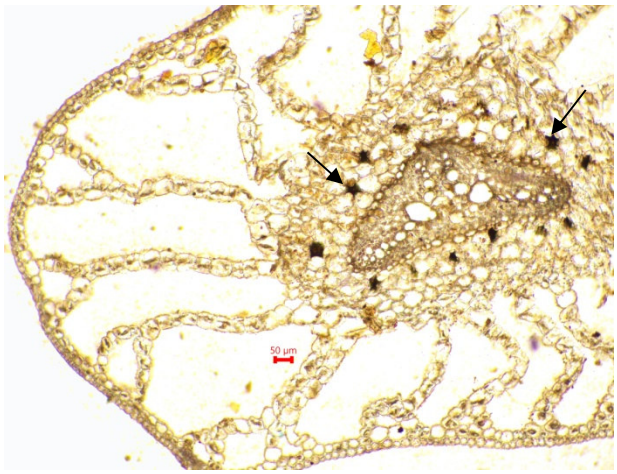
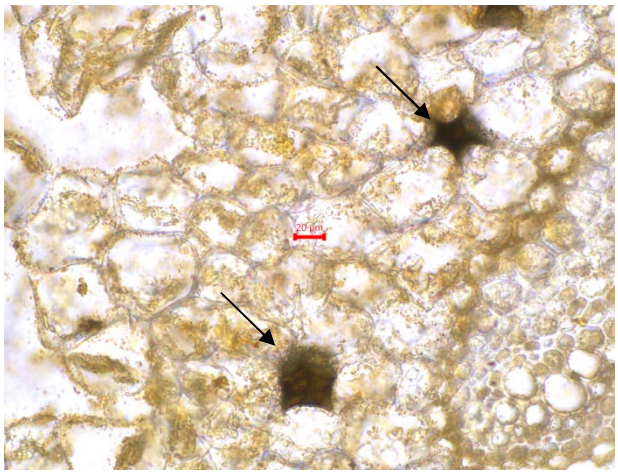
Alkaloids, phenolic compounds and resins detected in leaf and petiole; lignin present in xylem of both petiole and lamina; starch grains observed in petiole; cutin found in petiole epidermis; mucilage and oil globules absent in leaf (Fig. 5).



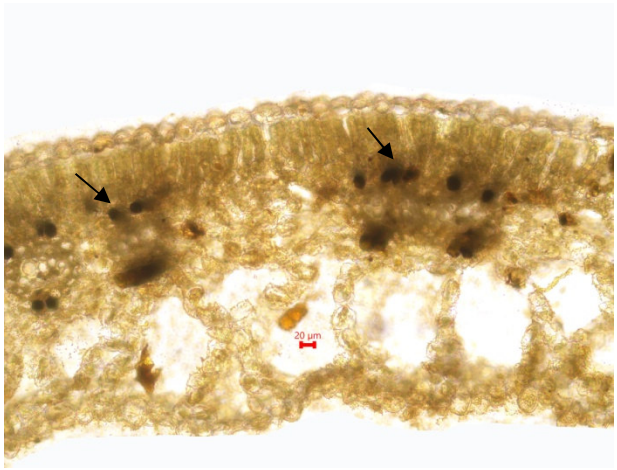
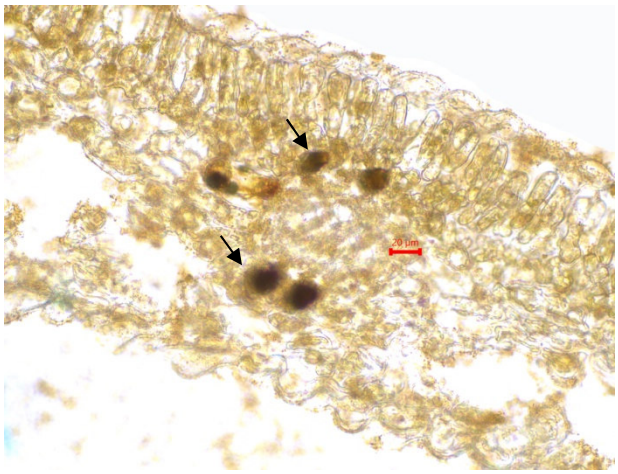
Lignin from petiole



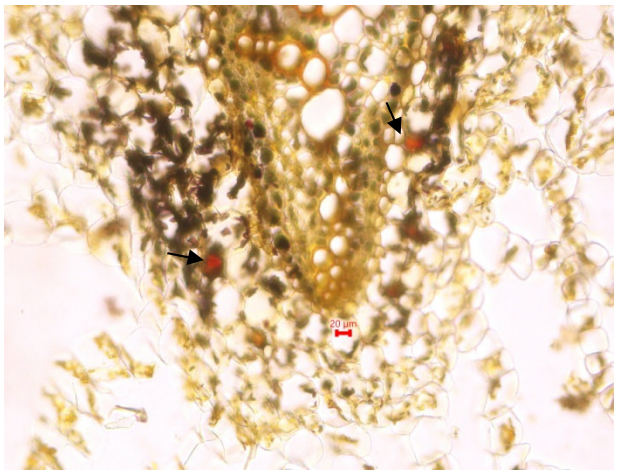
Lignin from lamina

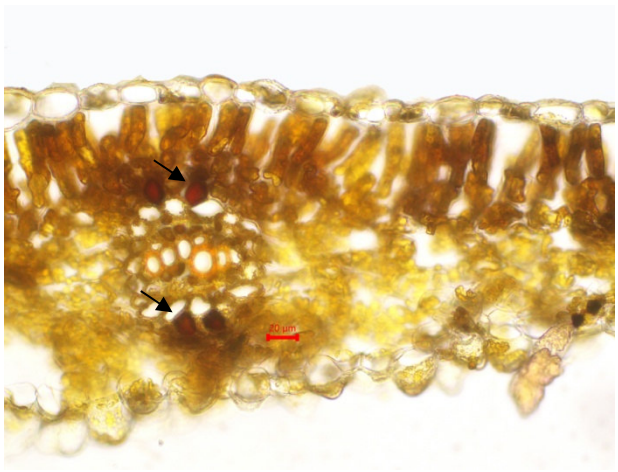
Phenolic compound in petiole

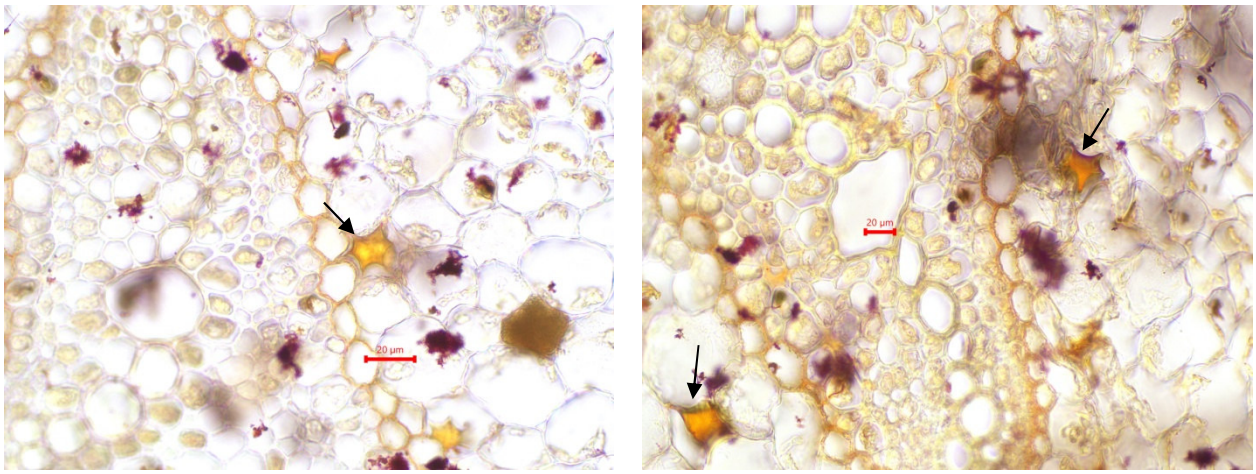
Phenolic compound in lamina



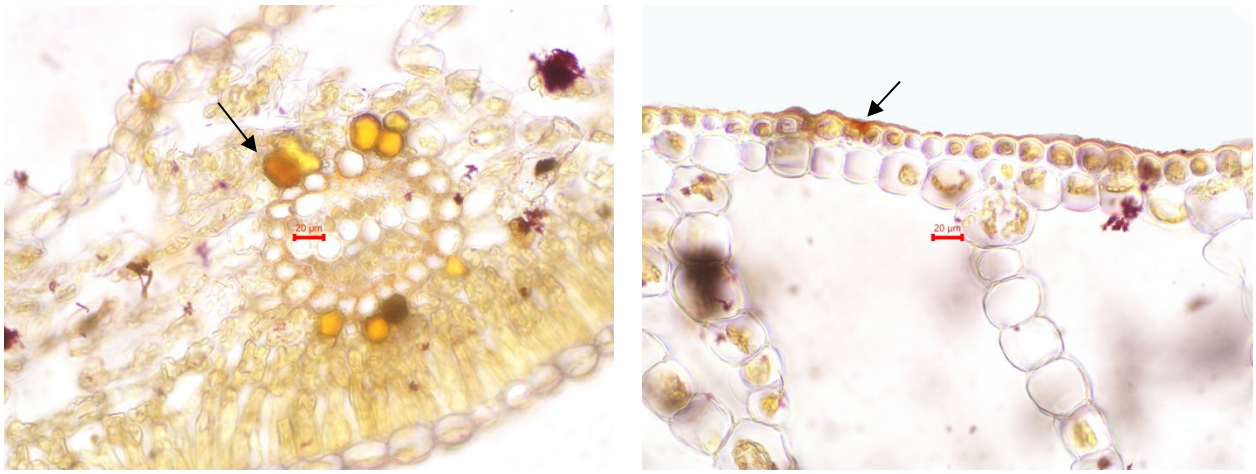
Alkaloid and starch in petiole



Alkaloid in leaf



Resin in petiole



Resin in leaf

Cutin in petiole epidermis

Fig 5: Histochemistry of *Marsilea quadrifolia* leaf

PHYSICOCHEMICAL STUDIES

PHYSICOCHEMICAL EVALUATION OF LEAVES OF *Marsilea quadrifolia* Linn

The powdered drug was evaluated for loss on drying, foreign matter, ash values and extractive values.

Table 4: Physiochemical parameters of *Marsilea quadrifolia* L.

S.No	Physio-chemical parameters	Reports %w/w
1	Swelling Index	1.461ml
2	Loss on drying	12.99w/w
3	Extractive value	
3.1	Petroleum ether extractive	10.5±0.289%w/w
3.2	Ethyl acetate extractive	5.13±0.185%w/w
3.3	Chloroform extractive	14.17±0.219%w/w
3.4	Aqueous extractive	28±0.289%w/w
3.5	Ethanolic extractive	35.8±0.440%w/w
3.6	Hydro alcoholic extractive	30.16±0.440%w/w
4	Ash value	

4.1	Total ash	6.67±0.166%w/w
4.2	Water soluble ash	2.43±0.093%w/w
4.3	Acid insoluble ash	0.3±0.029%w/w

S.NO	NAME OF THE TEST	OBSERVATION	INFERENCE
1	**Test for alkaloids** **a) Mayer's test**	Cream colour precipitateis formed	Presence of alkaloids
	b) Dragendroff's test	Orange brown precipitate is formed	Presence of alkaloids
	c) Wagner's test	Reddish brown precipitate is formed	Presence of alkaloids
	d) Hager's test	Formation of yellow precipitate	Presence of Alkaloids
2	**Test for carbohydrates** **a) Molish's test**	Appearance of purple colour	Presence of Carbohydrates
	b) Fehling's test	Formation of reddish brown precipitate	Presence of reducing sugar
	c) Benedict's test	No Formation of red precipitate	Presence of reducing sugar
3	**Test for glycosides-Anthraquinone** **a) Borntrager's test**	pink colour is formed in the ammonical layer	Presence of anthraquinone glycoside.[O-glycoside]
	b) Modified borntrager's test	pink colour is formed in the ammonical layer	Presence of anthraquinone Glycoside.[C-glycoside]
4	**Test for cardiac glycosides(for deoxysugar)** **a) Keller Killiani test**	No Reddish brown colour ring at the junction	Absence of cardiac glycoside
	b) Legal's test	No Blood red colour	Absence of cardiac glycoside
	Test for cyanogenetic glycosides	No brick red colour on paper	Absence of Cyanogenetic Glycosides
5	**Test for coumarin glycosides**	No violet colour formation on addition of ferric chloride	Absence of coumarin glycosides
6	**Test for sterols** **a) Salkowski's test**	A red colour in chloroform layer	presence of sterols
	b) Liberman burchard's test	Bluish green colour in the upper layer	Presence of sterols
7	**Test for flavonoids** **a) Shinoda's test**	Appearance of red colour	Presence of flavonoids
	b) Alkali test	Appearance of reddish- brown precipitate	Presence of flavonoids
	c) Lead acetate test	Formation of white precipitate	Presence of flavonoids
	d) Acid test	Appearance of yellow colour	Presence of flavonoids
8	**Test for proteins** **a) Millon's Test**	Red colour is formed on heating	Presence of protein
	b) Biuret Test	Violet colour is formed	Presence of protein
9	**Test for aminoacids** **a) Ninhydrin test**	Violet colour is Formed	Presence of amino acids
	b) With nitric acid	Yellow colour is Formed	Presence of aminoacids
10	**Test for terpenoids** **Noller's test**	Pink colour solutionappeared	Presence of terpenoids
11	**Test for triterpenoids**	Reddish brown colour isformed	Presence of triterpenoids
12	**Test for saponins** **Foam test**	Frothing occurs	Presence of saponins
13	**Test for Phenols** **Ferric chloride test**	Appearance of bluish black colour	Presence of Phenols
	Lead acetate test	Formation of precipitate	Presence of Phenols
14	**Test for Tannins**	White precipitate	Presence of Tannins

Gelatin test		
Braymer's test	Appearance of blue colour	Presence of Tannins

Physicochemical parameters such as loss on drying, total ash value, acid insoluble ash, water soluble ash and extractive value can be used as reliable aid for detecting adulteration. These were simple but reliable standards, that play an important role in preventing the possible steps of adulteration. Physico-Chemical parameters for the leaves was determined, calculated and the results showed that leaf powder has devoid of foreign matter and the loss on drying was found to be 12.99% w/w.

Then extractive value of the powder with different solvents were determined which showed that pet.ether extractive value is 10.5 % w/w ethyl acetate extractive value is 5.13%w/w, Chloroform extractive value is 14.17%w/w, Ethanolic extractive value is 35.8%w/w, Aqueous extractive value is 28%w/w, and hydro alcoholic extractive value was found to be 30.16%w/w respectively.

PHYTOCHEMICAL STUDIES
QUALITATIVE ANALYSIS PRELIMINARY PHYTOCHEMICAL STUDIES

Table 5: Preliminary phytochemical studies

S.No	NAME OF THE TEST	OBSERVATION	INFERENCE
1	**Test for alkaloids**		
	a) Mayer's test	Cream colour precipitate is formed	Presence of alkaloids
	b) Dragendroff's test	Orange brown precipitate is formed	Presence of alkaloids
	c) Wagner's test	Reddish brown precipitate is formed	Presence of alkaloids
	d) Hager's test	Formation of yellow precipitate	Presence of Alkaloids
2	**Test for carbohydrates**		
	a) Molish's test	Appearance of purple colour	Presence of carbohydrates
	b) Fehling's test	Formation of reddish brown precipitate	Presence of reducing sugar
	c) Benedict's test	No Formation of red precipitate	Presence of reducing sugar
3	**Test for glycosides-Anthraquinone**		
	a) Borntrager's test	pink colour is formed in the Ammonical layer	Presence of anthraquinone ycoside
	b) Modified borntrager's test	No pink colour in ammoniacal layer	Absence of anthraquinone Glycoside
4	**Test for cardiac glycosides (for deoxysugar)**		
	a) Keller Killiani test	No Reddish brown colour ring at the junction	Absence of cardiac glycoside
	b) Legal's test	No Blood red colour	Absence of cardiac glycoside
	Test for cyanogenetic glycosides	No brick red colour on paper	Absence of Cyanogenetic Glycosides
5	**Test for coumarin glycosides**	No violet colour formation on addition of ferric chloride	Absence of coumarin glycosides
6	**Test for sterols**		
	a) Salkowski's test	A red colour in chloroform layer	presence of sterols
	b) Liberman burchard's test	Bluish green colour in the upper layer	Presence of sterols
7	**Test for flavonoids**		
	a) Shinoda's test	Appearance of red colour	Presence of flavonoids
	b) Alkali test	Appearance of reddish- brown precipitate	Presence of flavonoids
	c) Lead acetate test	Formation of white precipitate	Presence of flavonoids
	d) Acid test	Appearance of yellow colour	Presence of flavonoids
8	**Test for proteins**		
	a) Millon's Test	Red colour is formed on heating	Presence of protein
	b) Biuret Test	Violet colour is formed	Presence of protein
9	**Test for aminoacids**	Violet colour is	Presence of amino acids

	a) Ninhydrin test	Formed	
	b) With nitric acid	Yellow colour is Formed	Presence of aminoacids
10	**Test for terpenoids Noller's test**	Pink colour solutionappeared	Presence of terpenoids
11	**Test for triterpenoids**	Reddish brown colour isformed	Presence of triterpenoids
12	**Test for saponins Foam test**	Frothing occurs	Presence of saponins
13	**Test for Phenols Ferric chloride test**	Appearance of bluish black colour	Presence of Phenols
	Lead acetate test	Formation of precipitate	Presence of Phenols
14	**Test for Tannins Gelatin test**	White precipitate	Presence of Tannins
	Braymer's test	Appearance of blue colour	Presence of Tannins

Preliminary phytochemical screening is a valuable step in the detection of the bioactive principles present in the medicinal plants which leads to drug discovery and development. Since medicinal plants have been used in the treatment of different ailments, the therapeutic activity of the plant is related to the secondary metabolites present in the plant.

CHROMATOGRAPHY PROFILE

IDENTIFICATION OF FLAVONOID

TLC is a rather simple but relatively popular method used in the analysis of secondary metabolites. Here the TLC was done by using silica gel G as a stationary phase, Toluene: Ethyl acetate: Acetic acid: Methanol (2.5:7:0.25:0.25) as mobile phase EEMQ and quercetin were used as sample and standard respectively. The Rf value was calculated and the results are presented.

Thin layer chromatography of quercetin and EEMQ

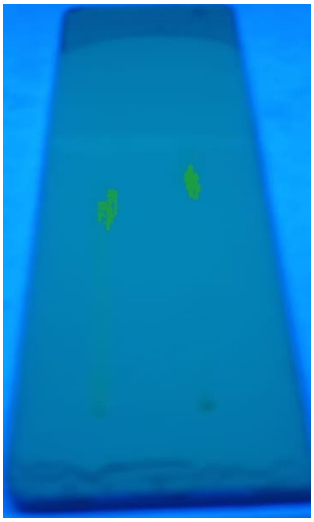


Table 6: Thin layer chromatography of EEMQ

Mobile phase	Detector	Name of the sample	Rf value
Toluene: Ethyl acetate: Acetic acid: Methanol (2.5:7:0.25:0.25)	Iodine vapour	EEMQ	0.73
		Quercetin	0.74

REPORT

EEMQ exhibited a spot at the Rf value of 0.73 and standard quercetin showed a spot at the Rf value of 0.74.This study revealed the presence of Quercetin in EEMQ

Flavonoid was identified in EEMQ by TLC method .

The Rf value of flavonoid in EEMQ was **0.73** (Kancharla bhanukiran et al., 2022)

DETERMINATION OF QUERCETIN BY HPTLC METHOD IN EEMQ

Track 1-6 represent Quercetin and
Track 7-12 represent EEMQ

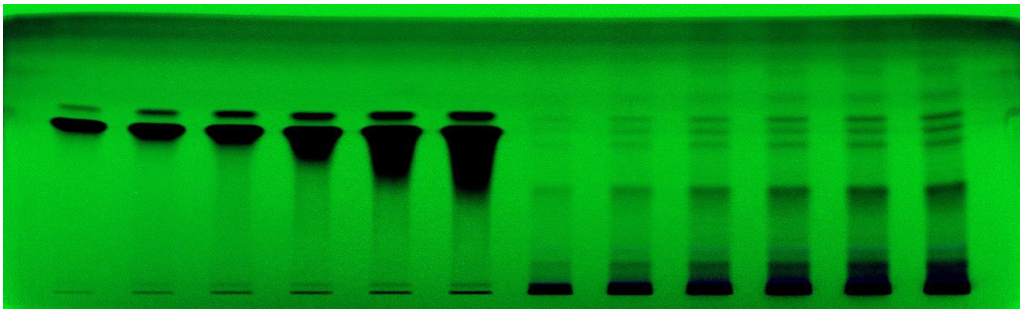


Fig 6: AT 254 nm

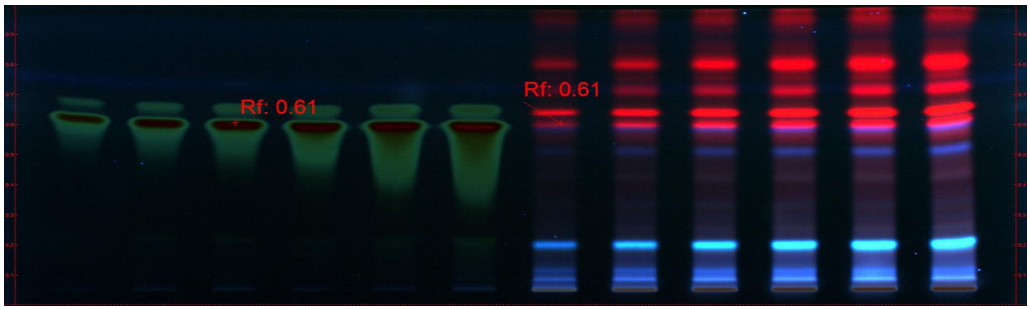


Fig 7: AT 366 nm

HPTLC CHROMATOGRAPHY RESULTS OF STANDARD QUERCETIN AND EEMQ

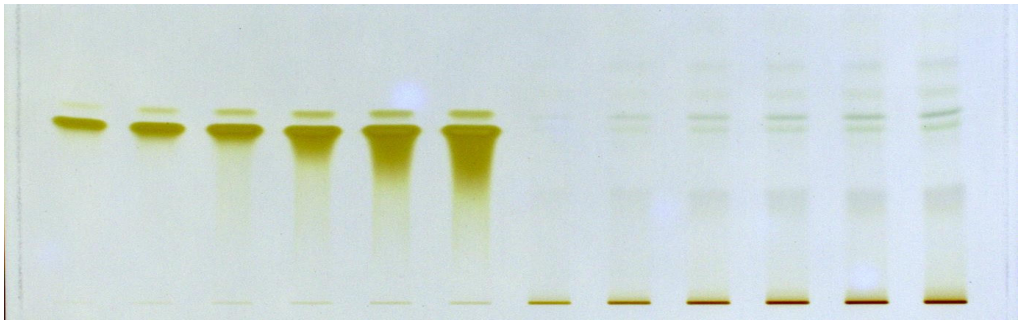


Fig 8: WHITE LIGHT

Table 7: HPTLC results standard quercetin and EEMQ

S.No.	RF (STD. Quercetin)	RF (EEMQ)	Colour OF THE BAND
1	-	0.1	Fluorescent blue
2	0.2	0.2	Fluorescent blue
3	-	0.5	Fluorescent blue
4	0.61	0.61	Yellow
5	0.68	0.68	Fluorescent red
6	-	0.75	Fluorescent red
7	-	0.8	Fluorescent red

GAS CHROMATOGRAPHY MASS SPECTROSCOPY ANALYSIS OF EEMQ

The result of GC-MS analysis of ethanolic extract of *Marsilea quadrifolia* L. was tabulated in Table 8 and its major compounds were tabulated in Table 9 & Fig 9.

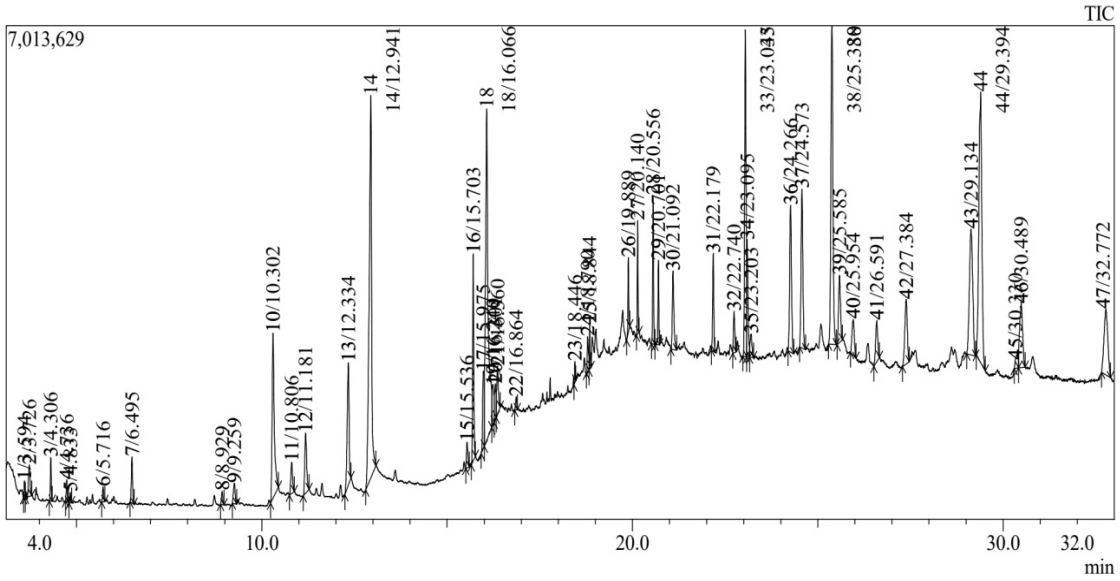


Fig 9: GC-MS analysis of ethanolic extract of *Marsilea quadrifolia* L.

Table 8: GC-MS analysis of ethanolic extract of *Marsilea quadrifolia* L.

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Similarity	CAS#	Name
1	3.594	348617	0.16	235099	0.38	1.48	0	0-00-0	Benzoic acid, 4-propyl-, hexadecyl ester
2	3.726	1390013	0.64	447598	0.73	3.11	0	0-00-0	Cycloheptasiloxane, tetradecamethyl-
3	4.306	1052043	0.48	615571	1.00	1.71	0	0-00-0	Cycloheptasiloxane, tetradecamethyl-
4	4.736	538206	0.25	310021	0.50	1.74	0	0-00-0	Naphthalene, 1-bromo-
5	4.833	255474	0.12	144693	0.24	1.77	0	0-00-0	2,4-Di-tert-butylphenol
6	5.716	420882	0.19	211258	0.34	1.99	0	0-00-0	3-[[2-(1H-1,3-benzimidazol-2-yl)ethyl]amino

7	6.495	1535432	0.71	652917	1.06	2.35	0	0-00-0	Cyclooctasiloxane, hexadecamethyl-
8	8.929	431356	0.20	190510	0.31	2.26	0	0-00-0	Loliolide
9	9.259	925257	0.43	286725	0.47	3.23	0	0-00-0	Cyclononasiloxane, octadecamethyl-
10	10.302	9446120	4.34	2341328	3.81	4.03	0	0-00-0	Neophytadiene
11	10.806	1449471	0.67	441912	0.72	3.28	0	0-00-0	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
12	11.181	2996194	1.38	854690	1.39	3.51	0	0-00-0	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
13	12.334	6054548	2.78	1736835	2.82	3.49	0	0-00-0	7,10,13-Hexadecatrienoic acid, (Z,Z,Z)-
14	12.941	25031353	11.50	5430647	8.83	4.61	0	0-00-0	n-Hexadecanoic acid
15	15.536	965560	0.44	362408	0.59	2.66	0	0-00-0	,15-Octadecatrienoic acid, methyl ester, (Z,Z
16	15.703	6387153	2.94	2943756	4.79	2.17	0	0-00-0	Phytol
17	15.975	3076178	1.41	1097371	1.78	2.80	0	0-00-0	nylmethyl-2,8,9-trioxa-5-aza-1-sila-bicyclo[3
18	16.066	23060557	10.60	4707525	7.66	4.90	0	0-00-0	cis,cis,cis-7,10,13-Hexadecatrienal
19	16.240	1866963	0.86	549961	0.89	3.39	0	0-00-0	
20	16.301	1383461	0.64	497901	0.81	2.78	0	0-00-0	Linoleic acid ethyl ester
21	16.360	2109459	0.97	677297	1.10	3.11	0	0-00-0	2,15-Octadecatrienoic acid, ethyl ester, (Z,Z,
22	16.864	356529	0.16	184508	0.30	1.93	0	0-00-0	t-4-enoylamide, 2-methyl-N-(2-butyl)-N-pen
23	18.446	393309	0.18	302358	0.49	1.30	0	0-00-0	Phytyl, 2-methylbutanoate
24	18.790	1095139	0.50	494970	0.81	2.21	0	0-00-0	Dipropylene glycol, 2TMS (isomer 1)
25	18.844	928588	0.43	649507	1.06	1.43	0	0-00-0	Heneicosane
26	19.889	1623888	0.75	1062276	1.73	1.53	0	0-00-0	Hexatriacontane
27	20.140	2347820	1.08	1610795	2.62	1.46	0	0-00-0	Benzenedicarboxylic acid, bis(2-ethylhexyl) e
28	20.556	3422187	1.57	2110750	3.43	1.62	0	0-00-0	Squalene
29	20.701	2002013	0.92	1202432	1.96	1.66	0	0-00-	Nonacosanal

								0	
30	21.092	3313467	1.52	1116318	1.82	2.97	0	0-00-0	Heptatriacontane
31	22.179	3011607	1.38	1386989	2.26	2.17	0	0-00-0	Nonacosanal
32	22.740	1431201	0.66	585651	0.95	2.44	0	0-00-0	1-Hexacosanol
33	23.045	12605898	5.79	4641111	7.55	2.72	0	0-00-0	Vitamin E
34	23.095	3927812	1.81	1656272	2.69	2.37	0	0-00-0	.alpha.-Tocopherolquinone
35	23.203	1143714	0.53	332087	0.54	3.44	0	0-00-0	Perhydro-2-oxo-3-stearoylfuran
36	24.266	7099698	3.26	2083034	3.39	3.41	0	0-00-0	Ergost-5-en-3-ol, (3.beta.)-
37	24.573	7636469	3.51	2259591	3.68	3.38	0	0-00-0	Stigmasterol
38	25.380	17536614	8.06	4524544	7.36	3.88	0	0-00-0	.gamma.-Sitosterol
39	25.585	3475056	1.60	954964	1.55	3.64	0	0-00-0	Stigmasta-5,24(28)-dien-3-ol, (3.beta.,24Z)-
40	25.954	2146828	0.99	502773	0.82	4.27	0	0-00-0	
41	26.591	2275084	1.05	582749	0.95	3.90	0	0-00-0	9,19-Cyclolanost-25-ene-3,24-diol
42	27.384	4278824	1.97	914695	1.49	4.68	0	0-00-0	.gamma.-Sitostenone
43	29.134	10970297	5.04	1784512	2.90	6.15	0	0-00-0	E,E,Z-1,3,12-Nonadecatriene-5,14-diol
44	29.394	21079517	9.69	3839348	6.24	5.49	0	0-00-0	Phytyl tetradecanoate
45	30.330	1070998	0.49	120641	0.20	8.88	0	0-00-0	
46	30.489	5141888	2.36	907244	1.48	5.67	0	0-00-0	Stigmastane-3,6-dione, (5.alpha.)-
47	32.772	6551767	3.01	935307	1.52	7.00	0	0-00-0	Phytyl stearate
		217590509	100.00	61481449	100.00				

Table 9: Some of the Phytoconstituents and their Biological activity

S.No	Compound	Molecular Weight	Molecular Formula	Biological Activity
1.	Squalene	410.73	C30H50	Spermatogenis, anti-oxidant.
2.	Heptatriacontane	436.85	C31H64	Aphrodisiac activity, anti-tumor.
3.	Vitamin E	430.71	C29H50O2	Spermatogenis, anti-inflammatory.
4.	gamma.-Sitosterol	414.71	C29H50O	Spermatogenis, sperm maturation.
5.	Phytyl tetradecanoate	506.9	C34H66O2	Anti-oxidant, rich in Flavonoids
6.	alpha.-Tocopherolquinone	446.7	C29H50O3	Enhances spermatogonial stemcell proliferation.

- ❖ The Present work is an attempt to compile Pharmacognostical, phytochemical work on *Marsilea quadrifolia* L.
- ❖ The morphological and anatomical features observed in the transverse section (TS) of *Marsilea quadrifolia* L. were found to be consistent with the characteristic traits of the genus and species of this plant. This is the first documented report of such observations.
- ❖ Microscopical studies shows that the Stomata is mainly of sunken stomata on both upper and epidermis.
- ❖ The quantitative constants were determined for the leaves of this plant and these parameters are considered as significant one especially for the evaluation of this plant.The physicochemical properties determined for the leaf material revealed that the physical constants are within the prescribed limit.
- ❖ Phytochemical screening of EEMQ revealed the presence of alkaloids, flavonoids, sterols, terpenoids, triterpenoids, saponins, tannins.
- ❖ The GC-MS chromatogram of the ethanolic extract of *Marsilea quadrifolia* L. indicates the presence of bioactive compounds such which possess various biological properties such as anticonvulsant, anti-tumor, antiviral , antimicrobial activity, anti-inflammatory, analgesic , antifungal, sedative, and hypnotic properties respectively.

CONCLUSION

The study provides the first detailed Pharmacognostical and phytochemical profile of *Marsilea quadrifolia* L. The plant showed distinctive morphological and anatomical features consistent with its genus, including sunken stomata and characteristic leaf constants. Physicochemical evaluations confirmed that its parameters were within standard limits, supporting its authenticity. Phytochemical screening revealed the presence of alkaloids, flavonoids, sterols, terpenoids, triterpenoids, saponins, and tannins, while GC-MS analysis detected several bioactive compounds with reported antioxidant, anti-inflammatory, antimicrobial, antitumor, and neuroprotective properties. These findings justify the plant's long-standing use in traditional medicine and suggest its potential as a source for future drug development. Moreover, the results underscore the necessity for conservation strategies to protect this vulnerable species while advancing further pharmacological studies to validate its therapeutic benefits

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