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Analytical profile and *In Vitro* antimicrobial activity on leaves of *Marsilea minuta* Linn. (Marsileaceae)

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Abstract
To establish the fingerprint profile of *Marsilea minuta* Linn. using thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC) technique and to investigate the antibacterial potentials of leaves of *Marsilea minuta* Linn. The TLC study was carried out in two solvent systems, which showed different R_f value and HPTLC study was carried out using methanol extract, compared with reference standard (β-sitosterol). The antibacterial activities of extracts of *Marsilea minuta* were tested against some medicinally important bacterial strains. The antimicrobial activity was determined in the extract using agar disc diffusion method. These plants can be used to discover bioactive natural products that may serve as route in the development of new pharmaceuticals research activities.

Keywords: *Marsilea minuta* Linn, TLC, HPTLC, β-sitosterol, antimicrobial activity

1. Introduction
Marsilea minuta Linn. is an aquatic leptosporangiate fern (pteridophyte) and grows in marshy and shady places by the side of tanks and rivers and also in the rice fields of tropical India [1]. The rhizome is aerial, slender, creeping below the surface of the soil. Roots are borne at the nodes. The young leaves are circinate, four leaflets terminate the petiole, and the leaflets are folded together, till maturity [2]. The leaves are employed as nervine tonic in epilepsy and insomnia and also act as anti-venom drug [3].
Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plants species used in herbal medicines and further exploration plant antimicrobials needs to occur. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials [4].
In this study, the antimicrobial property of the plant leaves extracts against some strains of microorganisms was evaluated and the fingerprint profile of *M. minuta* using TLC and HPTLC technique has been established. Thus, the phytochemical with sufficient antimicrobial efficacy proved that the plant may be adapted for treatment of some infections caused by microorganisms. The study will also help in future for identifying this plant for future research.

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

2.2. TLC Profile [5]
2.2.1. Preparation of Test Sample
The coarsely powdered dried *M. minuta* Linn. leaves (40g) were successively extracted on small scale with petroleum ether (300ml) at room temperature for 48 hours, using percolator apparatus and then plant material was dried at room temperature. The dried plant material was extracted with chloroform (300ml) at 40 ^{0}C for 48 hrs and then it was dried at room temperature. Again the same process was repeated for methanol at room temperature.

2.2.2. Procedure
Laboratory made TLC plates prepared from silica gel G (0.25 mm thickness, E. Merck) were used for qualitative work after activation for thirty minutes at 110 ^{0}C. Final chromatograms were taken on precoated silica plates (Alumina base,0.2 mm thickness, E. Merck) after choosing appropriate solvent systems. About 10µl of these extracts were applied on precoated plates for developing final chromatograms.

2.2.3. Developments of Plates
The plates were developed in CAMAG TLC jars saturated with the solvent systems. Different solvent systems were used for different extracts and the plates were developed to 8 cm, allowed to air dry. The plates were observed visibly and under UV light (366 nm).

2.2.4. Calculation of Retardation factor (R_f) value

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Table 1: Mobile phase solvent system employed for pet ether and MeOH extract

S.No.	Solvent system	Proportion
1.	Toluene: ethyl acetate	9:1
2.	Toluene : acetone	9:1

2.3. Qualitative Hptlc Study [6]
2.3.1 TLC Plates
Precoated silica gel 60 F_{254} TLC plates (E. Merck) of uniform thickness of 0.2 mm.

2.3.2. Solvent System
Toluene: Acetone (9:1).

2.3.3. Standard Solution
Dissolve 5 mg β-sitosterol in 5 ml of chloroform.

2.3.4. Test Solution
Extract about 5g of freshly powdered drug with 50 ml methanol by shaking for 30 min at 50 ^{0}C in conical flask. Filter and concentrate the filtrate under vacuum to about 2 ml.

2.3.5. Procedure
Apply separately about 10µl each of test and reference solution on a precoated silica gel 60 F_{254} TLC plate (E. Merck) of uniform thickness of 0.2 mm. Develop the plate in the solvent system to a distance of 8 cm and dry in a current of hot air.

2.3.6. Visualization
Spray the air dried plate with conc. H_2SO_4: Methanol (1:1) and heat at 105 ^{0}C for 5 min. The R_f value and colour of band in the reference and test solution was observed.

2.4. Anti-Microbial Activity [7]
Anti-microbial Methods
The test was done from government approved Santi Scan Centre, Uttarpara, Hooghly. The disc diffusion method was used for testing antimicrobial activity. Aseptically 10 ml of sample was transferred to 100 ml lactose broth/soyabean casein digest broth medium and media was incubated at 37 ^{0}C for 24 hrs. The flask was examined for growth and the content was mixed by gentle shaking. 1 ml of the enriched culture was pipetted into the tubes containing 10 ml Mac Conkey's broth and incubated at 35 ^{0}C for 26 hrs. Concomitantly streaking on the surface Mac Conkey's agar medium was done using a loopful of enriched culture and the plates were incubated at 37 ^{0}C for 24 hrs.

3. Result and Discussion
3.1. TLC Profile
Many solvent systems were tried as given in Table to develop most appropriate solvent system for all the extracts separately for developing TLC profile. The solvent system showing optimum resolution was selected and their results are shown below.

3.1.1. TLC profile for petroleum ether extract:
Solvent system: Toluene: Ethyl acetate (9:1)
Distance travelled by solvent = 8.0 cm

Table 2: TLC profile for petroleum ether extract of *M. minuta* Linn.

Distance travelled by solute (cm)	R_f value	Colour of spots at UV (366 nm)	Distance travelled by solute (cm)	R_f value	Colour of spots at visible light
3.1	0.3924	Red	0.7	0.088	Deep green
3.5	0.4430	Blue	1.4	0.177	Deep green
4.4	0.5569	Red	2.0	0.2531	Yellowish green
-	-	-	2.6	0.3291	Green
-	-	-	3.1	0.3924	Light green
-	-	-	3.8	0.4810	Yellowish green
-	-	-	4.3	0.5443	Yellow
-	-	-	4.5	0.5696	Yellowish green

The solvent system, Toluene: Ethyl acetate (9:1) gave the best resolution and maximum number of resolved components for petroleum ether extract. Different components were separated as shown in the images. The R_f values of separated constituents ranged from 0.5696 to 0.088.

3.1.2. TLC profile for chloroform extract
Solvent system: Toluene: Ethyl acetate (9:1)
Distance travelled by solvent = 8.2 cm

Table 3: TLC profile for Chloroform extract of *M. minuta* Linn.

Distance travelled by solute (cm)	R_f value	Colour of spots at visible light	Distance travelled by solute (cm)	R_f value	Colour of spots at UV (366 nm)
0.8	0.0975	Yellowish green	3.5	0.4268	Blue
2.0	0.2439	Yellow	3.9	0.4756	Red
2.4	0.2926	Light yellow	6.5	0.7926	Blue
3.3	0.4024	Light yellow	-	-	-
3.9	0.4756	Light brown	-	-	-

The solvent system, Toluene: Ethyl acetate (9:1) gave the best resolution and maximum number of resolved components for chloroform extract of *M. minuta* Linn. leaves. Different components were separated as showed in images. The R_f values of separated constituents were ranged from 0.0975 to 0.4756.

3.1.3. TLC profile for methanol extract:
Solvent system: Toluene: Ethyl acetate (9:1)
Distance travelled by solvent=7.9 cm

Table 4: TLC profile for methanol extract of *M.minuta* Linn.

Distance travelled by solute (cm)	R_f value	Colour of spots at visible light	Distance travelled by solute (cm)	R_f value	Colour of spots at UV (366 nm)
0.6	0.0759	Pale green	2.8	0.3544	Red
1.3	0.1645	Green	3.1	0.3924	Red
2.7	0.3417	Fluorescent green	3.6	0.4556	Blue
3.2	0.4050	Light green	3.8	0.4810	Red
3.8	0.4810	Green	4.4	0.5569	Red
4.2	0.5316	Light green	-	-	-
4.6	0.5822	Green	-	-	-

The solvent system, Toluene: Ethyl acetate (9:1) gave the best resolution and maximum number of resolved components for methanol extract of *M.minuta* Linn leaves. Different components were separated as showed in the images. The R_f values of separated constituents were ranged from 0.0759 to 0.5822.

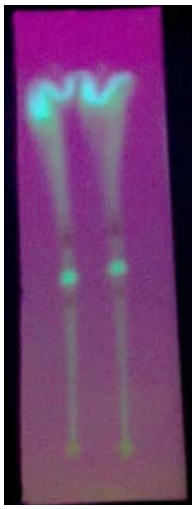


Fig 1: Pet ether Extract at 366 nm

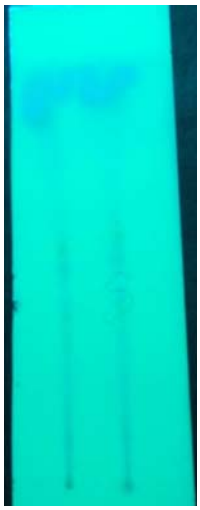


Fig 2: Pet ether Extract at Visible light

Fig 3: $CHCl_3$ Extract at 366 nm

Fig 4: $CHCl_3$ Extract at visible light

Fig 5: MeOH Extract at 366 nm

Fig 6: MeOH Extract at visible light

3.1.4 TLC profile for Pet ether, MeOH ext. and β-sitosterol

Solvent system: Toluene: Ethyl acetate (9:1)
Distance travelled by solvent = 6.3 cm
Spraying reagent=Liebermann burchard reagent

Table 5: TLC profile for Pet ether, MeOH ext. and β-sitosterol

Pet ether extract		β-sitosterol		MeOH extract	
Distance travelled by solute (cm)	**R_f value**	**Distance travelled by solute (cm)**	**R_f value**	**Distance travelled by solute (cm)**	**R_f value**
1.8	0.285	2.3	0.365	2.3	0.365
2.5	0.396	-	-	2.9	0.460
2.8	0.444	-	-	3.4	0.539
3.6	0.571	-	-	3.7	0.587
4.2	0.666	-	-	4.7	0.746
4.5	0.714	-	-	-	-
4.9	0.777	-	-	-	-

Fig 7: TLC profile at visible light

Fig 8: TLC profile at 366 nm

3.1.5. TLC profile for Petroleum ether extract

Solvent System: Toluene: Acetone (9 : 1)
Distance travelled by Solvent = 8.0 cm

Table 6: TLC profile for Petroleum ether extract of *M. minuta* Linn.

Distance travelled by solute (cm)	R_f value	Colour of spots at visible light	Distance travelled by solute (cm)	R_f value	Colour of spots at UV (366 nm)
0.7	0.08	Yellow	3.6	0.45	Red
1.0	0.125	Yellow	3.8	0.475	Red
3.4	0.425	Green	4.0	0.5	Blue
3.9	0.4875	Yellow	4.9	0.6125	Red
4.9	0.6125	Yellowish green	5.7	0.7125	Red
5.5	0.6875	Yellowish green	-	-	-
6.8	0.85	Yellow	-	-	-
7.0	0.875	Yellow	-	-	-

3.1.6. TLC profile for chloroform extract

Solvent system: Toluene: Acetone (9 : 1)
Distance travelled by solvent = 8.0 cm

Table 7: TLC profile for chloroform extract of *M. minuta* Linn.

Distance travelled by solute (cm)	R_f value	Colour of spots at UV (366 nm)
4.3	0.5375	Blue

3.1.7 TLC profile for methanol extract

Solvent system: Toluene : Acetone (9.1)
Distance travelled by solvent = 8.0 cm.

Table 8: TLC profile for methanol extract of *M. minuta* Linn.

Distance travelled by solute (cm)	Rf value	Colour of spots at visible light	Distance travelled by solute (cm)	Rf value	Colour of spots at UV (366 nm)
0.6	0.07	Green	3.5	0.4375	Red β
0.93	0.1125	Green	4.0	0.5	Red
1.6	0.2	Yellow green	4.3	0.5375	Red
3.6	0.45	Yellow green	5.1	0.6375	Blue
3.8	0.475	Green	5.8	0.725	Red
4.3	0.5375	Yellowish green	-	-	-
4.9	0.6125	Yellowish green	-	-	-
5.1	0.6375	Yellow	-	-	-
5.7	0.7125	Green	-	-	-
6.0	0.75	Green	-	-	-

3.1.8 TLC profile for Pet ether, MeOH extract and β-sitosterol

Solvent system: Toluene: Acetone
Distanced travelled by solvent = 5.5 cm
Spraying agent: Liebermann Burchard reagent
Standard used: β-sitosterol

Table 9: TLC Profile for Pet ether and MeOH ext. when compared with β-sitosterol

Pet ether Extract		β-Sitosterol		MeOH extract	
Distance travelled by solute	Rf value	Distance travelled by solute	Rf value	Distance travelled by solute	Rf value
2.5	0.454	2.9	0.527	2.9	0.527
3.0	0.545	-	-	3.5	0.636
3.5	0.636	-	-	4.2	0.763
4.0	0.727	-	-	4.5	0.818
4.3	0.781	-	-	-	-
5.0	0.90	-	-	-	-

Fig 9: Pet ether Extract at 366 nm

Fig 10: Pet ether Extract at visible light

Fig 11: $CHCl_3$ Extract at 366 nm

Fig 12: $CHCl_3$ Extract at visible light

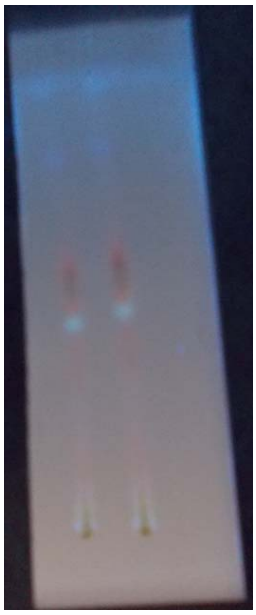


Fig 13: MeOH extract at 366 nm

Fig 14: MeOH extract at visible light

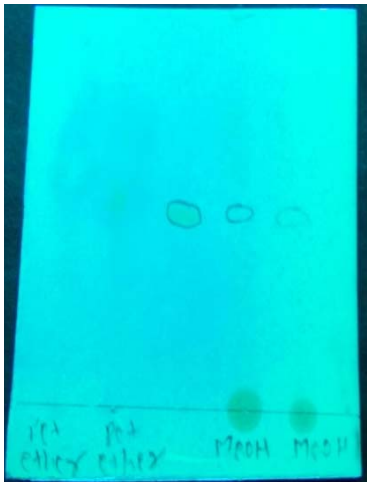


Fig 15: TLC profile at visible light

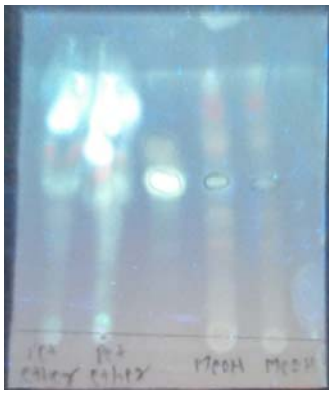


Fig 16: TLC profile at 366 nm

3.2. HPTLC Study

3.2.1. Evaluation

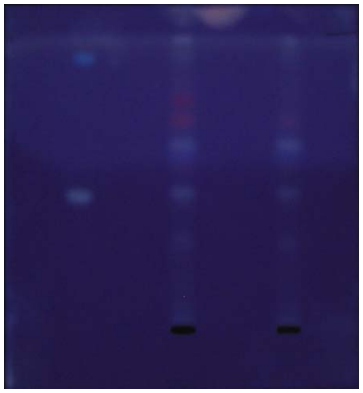
No band corresponding to β-sitosterol is visible in the reference and test solution at visible light was observed. Basically β-sitosterol gives purple colour spot. But off blue colour band corresponding to β-sitosterol was visible in the reference and test solution at 366 nm. Where Standard (Std) = β-sitosterol; MeOH = Methanol Extract.

Table 10: HPTLC details of MeOH ext. of *M. minuta* Linn. leaves.

Plant	Solvent system	Wavelength (nm)	No. of spots (R_f values)	Colour of the band
M. minuta Linn.	Toluene : Acetone (9:1)	Visible light	-	No colour band
		at 366	1 (0.473)	Off Blue

Std MeOH MeOH

Fig 17: HPTLC profile at visible light



Std MeOH MeOH

Fig 18: HPTLC profile at 366 nm

3.3. Antimicrobial Activity

Table 11: Results of antimicrobial activity

Sample	Microorganisms	Results
Methanolic extract of leaf of *M minuta* Linn.	*Escherichia coli*	+ + ve
	Pseudomonas aeruginosa	+ ve
	Staphylococcus aureus	+ ve

In case of antibacterial activity against *Staphylococcus aureus* (G +ve), Methanol extract of leaves showed moderate activity. In case of antibacterial activity against *Escherichia coli* (G –ve), Methanol extract of leaves showed best activity. In case of antibacterial activity against *Pseudomonas aeruginosa* (G –ve), Methanol extract of leaves showed moderate activity.

4. Conclusion

Although modern medicines may be available, due to socio-economical, cultural and historical reasons, herbal medicines have maintained their importance. Plants have been used as a traditional medicine to cure several ailments. *M.minuta* Linn. is used traditionally for medical purposes. The present investigations were aimed at standardization, developing TLC profiling and biological evaluation of the leaves of *M. minuta* Linn. The pharmacological activity was evaluated using in vitro models and emphasis was laid to evaluate antimicrobial activity.

In the antimicrobial activity, methanol extract of leaves bears best activity against gram positive bacteria *Staphylococcus aureus* as well as gram –ve bacteria *Escherichia coli* and *Pseudomonas aeruginosa*.

From the above, it can be concluded that the crude extracts of the plant *M. minuta* Linn. is giving positive results for antimicrobial activity. The sophisticated techniques of standardization using TLC and HPTLC give qualitative information about the main active constituents or marker compounds present in the crude drug. Further work can be done by isolating the components which are responsible for the activity which can lead to future drug development. In future toxicological and clinical studies can also be performed to assure quality and purity.

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