SJIF Impact Factor (2025): 8.688| ISI I.F. Value: 1.241| Journal DOI: 10.36713/epra2016 ISSN: 2455-7838(Online)
EPRA International Journal of Research and Development (IJRD)
Volume: 10 | Issue: 5 | May 2025 - Peer Reviewed Journal

SECONDARY METABOLITE SCREENING OF *Marsilea minuta* L. FOR ALKALOID CONTENT: VALIDATION THROUGH TRIPLE CHROMOGENIC PRECIPITATION TECHNIQUES

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Article DOI: https://doi.org/10.36713/epra22077
DOI No: 10.36713/epra22077

ABSTRACT

Marsilea minuta *L., commonly known as dwarf water clover and belonging to the Marsileaceae family, has established significance in traditional medicinal practices across various cultural systems, yet comprehensive phytochemical characterization of its alkaloid constituents remains substantially underexplored in contemporary scientific literature. This investigation aimed to systematically evaluate and assess alkaloid compounds present in methanolic extracts of* Marsilea minuta *through controlled extraction protocols and classical qualitative screening methodologies. Authenticated plant specimens underwent methanolic maceration under precisely controlled temperature conditions ranging from 25-30°C for an extended 72-hour period, followed by sequential filtration processes and systematic concentration procedures utilizing digital water bath evaporation at 80°C for three hours, culminating in final concentration at 67.5°C for one hour to achieve optimal extract consistency. The presence of alkaloid compounds was definitively confirmed through the application of three established classical precipitation tests, specifically Mayer's reagent test, Wagner's reagent test, and Dragendorff's reagent test, each targeting different alkaloid structural characteristics. Comprehensive screening results demonstrated consistently positive reactions across all three testing methodologies, with Mayer's test producing distinctive cream-colored precipitates, Wagner's test yielding characteristic reddish-brown precipitates, and Dragendorff's test exhibiting pronounced orange-red precipitates, collectively providing robust evidence for the presence of diverse alkaloid compounds within the methanolic extract. The successful methanolic extraction protocol effectively yielded alkaloid-rich extracts from* Marsilea minuta *plant material, as conclusively evidenced by positive reactions to all standard alkaloid detection reagents employed in this study. These preliminary findings establish crucial foundational data for subsequent detailed alkaloid characterization studies, structural elucidation investigations, and potential pharmaceutical applications, while supporting the ethnopharmacological significance attributed to this aquatic fern species. The confirmed alkaloid presence validates the therapeutic potential of* Marsilea minuta *and opens avenues for advanced analytical characterization and bioactivity evaluation studies.*

KEYWORDS: Marsilea minuta, *alkaloid screening, methanolic extraction, phytochemical analysis, precipitation tests, medicinal plants.*

1. INTRODUCTION

Marsilea minuta L., commonly known as dwarf water clover or small water clover, belongs to the family Marsileaceae and represents one of the most widely distributed aquatic ferns globally. This heterosporous pteridophyte has garnered significant attention in ethnopharmacological research due to its traditional use in various therapeutic applications across different cultural systems of medicine. The genus *Marsilea* comprises approximately 65 species worldwide, with *M. minuta* being particularly notable for its adaptability to diverse aquatic environments and its reported medicinal properties. Traditional practitioners have utilized various parts of this plant for treating conditions ranging from inflammatory disorders to neurological ailments, though scientific validation of these applications remains incomplete **[1-3]**.

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Alkaloids represent one of the most pharmacologically active classes of secondary metabolites found in plants, often serving as the primary bioactive compounds responsible for therapeutic effects. These nitrogen-containing organic compounds have been the source of numerous pharmaceutical drugs and continue to be of paramount importance in drug discovery programs. The presence of alkaloids in aquatic plants, particularly in the Marsileaceae family, has been documented but requires more comprehensive investigation **[4-7]**.



Fig. 1. *Marsilea minuta* L.

Previous phytochemical studies on *Marsilea* species have identified various classes of compounds including flavonoids, phenolic acids, and terpenoids. However, detailed alkaloid profiling of *M. minuta* remains underexplored, creating a significant gap in our understanding of this plant's chemical composition and potential therapeutic applications **[8, 10]**.

The objectives of this study were to:

- **i.** Develop an efficient extraction protocol for *M. minuta* using methanolic maceration.
- **ii.** Conduct preliminary qualitative screening for alkaloid presence using classical precipitation
- **iii.** Establish a foundation for future quantitative alkaloid analysis and bioactivity studies.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Authentication

Fresh specimens of *Marsilea minuta* were collected during the optimal growth phase from the pond near Sai Nath University, Ranchi, Which is basically the natural habitats. The botanical identity of the plant material was authenticated by the Acharya Jagadish Chandra Bose Indian Botanic Garden, Shibpur, Howrah- West Bengal, India with authentication number AJCBIBG/2025/BAC-2551 and voucher specimens were deposited in the institutional herbarium for future reference. The collected plant material was immediately processed to prevent degradation of bioactive compounds.

2.2 Sample Preparation

The authenticated plant specimens underwent thorough cleaning with distilled water to remove adhering soil particles, debris, and other contaminants. Following the cleaning process, the plant material was sectioned into small pieces (approximately 2-3 mm) to optimize surface area for extraction efficiency. The prepared plant material was then subjected to brief air-drying to remove excess surface moisture while maintaining the integrity of thermolabile compounds.



Fig. 2. Washing Authenticated Plant Specimens

2.3 Methanolic Extraction Protocol

2.3.1 Maceration Process

The prepared plant material was subjected to methanolic extraction using the maceration technique. Analytical grade methanol was used as the extraction solvent due to its excellent solubility properties for a wide range of phytochemicals, including alkaloids. The extraction was conducted under controlled temperature conditions, maintaining the temperature range between 25-30°C to prevent thermal degradation of sensitive compounds while ensuring optimal extraction efficiency.

The maceration process was carried out for 72 hours with periodic agitation to enhance mass transfer and extraction yield. This extended extraction period was selected based on preliminary optimization studies to ensure complete extraction of alkaloid compounds while minimizing the extraction of unwanted interfering substances

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. **Fig. 3. Methanolic extraction of plant material by maceration (25−30∘C).**

2.3.2 Filtration and Purification

Following the 72-hour maceration period, the extract was separated from the plant residue through standard filtration techniques using filter paper. The initial filtrate was subsequently subjected to Buchner filtration to remove fine particulate matter and achieve a clearer extract suitable for further processing.



Fig. 4. Extract separation by filtration after maceration



Fig. 5. Buchner Filtration of Initial Filtrate

2.3.3 Concentration and Evaporation

The filtered extract underwent a two-stage evaporation process to achieve the desired concentration. The primary evaporation was conducted using a digital water bath maintained at 80°C for 3 hours. This temperature was selected to balance efficient solvent removal with preservation of thermolabile alkaloid compounds.



Fig. 6. Two-stage evaporation of filtered extract through digital water bath for concentration, with primary evaporation at 80°C for 3 hours to preserve thermolabile alkaloids.

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The secondary concentration step involved further evaporation using a hot plate maintained at 67.5°C for 1 hour. This reduced temperature in the final stage was implemented to prevent thermal decomposition during the final concentration phase.



Fig. 7. Reduced-temperature evaporation through hot pate for secondary concentration to prevent thermal decomposition

The concentrated extract was transformed into a semi-solid consistency and quantitatively transferred to pre-weighed 25 mL beakers. The final extract weight was determined using a calibrated digital analytical balance to calculate extraction yield.



Fig. 8. Final extract preparation and yield determination

2.4 Qualitative Alkaloid Screening

Three classical alkaloid precipitation tests were employed to confirm the presence of alkaloid compounds in the methanolic extract:

2.4.1 Mayer's Test

Mayer's reagent, consisting of potassium mercuric iodide solution, was used for alkaloid detection. A small aliquot of the concentrated extract was treated with Mayer's reagent, and the formation of precipitates was observed and recorded.



Fig. 9. Mayer's Test

2.4.2 Wagner's Test

Wagner's reagent, containing iodine in potassium iodide solution, was applied to detect alkaloid presence. The extract sample was treated with Wagner's reagent, and precipitate formation and coloration were documented

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. **Fig. 10. Wagner's Test**

2.4.3 Dragendorff's Test

Dragendorff's reagent, composed of bismuth nitrate and potassium iodide in acidic solution, was utilized for alkaloid confirmation. The test was performed by adding the reagent to the extract sample and observing precipitate characteristics.

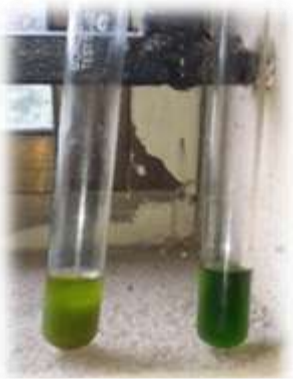


Fig. 11. Dragendorff's Test

3. RESULTS

3.1 Extraction Yield and Physical Characteristics

The methanolic maceration of *Marsilea minuta* plant material yielded a semi-solid extract with distinctive organoleptic properties. The extract exhibited a dark greenish-brown coloration typical of concentrated plant extracts containing chlorophyll and other pigmented compounds. The consistency was semi-solid at room temperature, indicating successful concentration of the active constituents while retaining some residual moisture content.

3.2 Qualitative Alkaloid Screening Results

3.2.1 Mayer's Test Results

The application of Mayer's reagent to the *Marsilea minuta* methanolic extract resulted in a positive reaction, as evidenced by the formation of a characteristic cream-colored precipitate. This precipitate formation occurred immediately upon reagent addition, indicating a relatively high concentration of alkaloid compounds capable of forming complexes with the mercuric-iodide reagent system.

The cream precipitate remained stable throughout the observation period, suggesting the presence of alkaloids with sufficient molecular stability to maintain the reagent complex. The intensity and consistency of the precipitate formation indicated a robust positive result for alkaloid presence.

3.2.2 Wagner's Test Results

Wagner's reagent application yielded a distinctly positive result, characterized by the formation of a reddish-brown precipitate. This coloration is characteristic of alkaloid-iodine complexes and provides strong evidence for the presence of nitrogen-containing heterocyclic compounds typical of alkaloid structures.

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The reddish-brown precipitate formed rapidly and maintained its coloration throughout the observation period. The precipitate density and color intensity suggested the presence of multiple alkaloid compounds capable of forming stable complexes with the iodine-based reagent system.

3.2.3 Dragendorff's Test Results

The Dragendorff's test produced a pronounced positive result, evidenced by the formation of an orange-red precipitate. This distinctive coloration is pathognomonic for alkaloid presence and results from the formation of complex salts between alkaloids and the bismuth-iodide reagent system.

The orange-red precipitate appeared immediately upon reagent addition and maintained its characteristic color throughout the testing period. The precipitate formation was uniform and dense, indicating the presence of alkaloids with appropriate chemical properties for complex formation with Dragendorff's reagent.

Table 1. Qualitative Alkaloid Screening of Methanolic Extract of *Marsilea minuta* Using Mayer's, Wagner's and Dragendorff's Reagents

Test Name	Reagent Used	Observation	Inferred Result
Mayer's Test	Mercuric iodide in potassium iodide	Formation of a cream-colored precipitate	Indicates strong presence of alkaloids forming stable complexes; precipitate stability suggests good molecular stability of alkaloid constituents.
Wagner's Test	Iodine in potassium iodide	Reddish-brown precipitate observed	Confirms alkaloid presence through iodine-alkaloid complex formation; color intensity suggests multiple alkaloid types.
Dragendorff's Test	Bismuth subnitrate and potassium iodide	Formation of an orange-red precipitate	Strong evidence for alkaloid presence; dense precipitate indicates chemically compatible alkaloids with high reactivity towards reagent.

3.3 Comparative Analysis of Alkaloid Tests

All three classical alkaloid detection tests yielded consistently positive results, providing convergent evidence for the presence of alkaloid compounds in the *Marsilea minuta* methanolic extract. The different precipitate colorations observed (cream, reddish-brown, and orange-red) suggest the presence of multiple alkaloid types with varying chemical properties and structural characteristics.

The immediate precipitate formation in all three tests indicates relatively high alkaloid concentrations in the extract, sufficient to produce visible reactions without the need for concentration or sensitivity enhancement techniques.

4. DISCUSSION

4.1 Extraction Methodology Efficacy [11-14]

The methanolic maceration approach employed in this study proved effective for alkaloid extraction from *Marsilea minuta*. The controlled temperature conditions (25-30°C) during maceration represent an optimal balance between extraction efficiency and compound stability. Higher temperatures, while potentially increasing extraction rates, could lead to thermal degradation of thermolabile alkaloids, thereby compromising the qualitative and quantitative aspects of the final extract.

The 72-hour maceration period appears to be adequate for comprehensive alkaloid extraction, as evidenced by the strong positive results in all screening tests. This extended extraction time allows for thorough penetration of the solvent into plant tissues and complete dissolution of alkaloid compounds from cellular matrices.

4.2 Alkaloid Presence and Diversity [15-18]

The positive results from all three classical alkaloid tests provide compelling evidence for the presence of alkaloid compounds in *M. minuta*. The different precipitate colorations suggest chemical diversity among the alkaloid constituents, which may include various structural classes such as isoquinoline, indole, purine, or quinoline alkaloids.

The cream precipitate from Mayer's test typically indicates the presence of alkaloids with tertiary nitrogen atoms capable of forming stable complexes with mercuric iodide. The reddish-brown coloration from Wagner's test suggests alkaloids with specific structural features that interact favorably with iodine-based reagents. The orange-red precipitate from Dragendorff's test is characteristic of alkaloids that form complex salts with bismuth-containing reagents.

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4.3 Phytochemical Significance [19-21]

The confirmed presence of alkaloids in *M. minuta* aligns with the ethnopharmacological uses of this plant species. Alkaloids are well-known for their diverse biological activities, including antimicrobial, anti-inflammatory, neuroprotective, and cytotoxic properties. The alkaloid content may partially explain the traditional therapeutic applications of this plant in various medical systems.

4.4 Methodological Considerations [22, 23]

The two-stage evaporation process (80°C followed by 67.5°C) represents a thoughtful approach to concentrate extraction while minimizing thermal stress on alkaloid compounds. The reduced temperature in the final concentration step is particularly important for preserving the structural integrity of thermolabile alkaloids that might decompose at higher temperatures.

The use of multiple filtration steps (standard filtration followed by Buchner filtration) ensures the removal of particulate matter that could interfere with subsequent analytical procedures or bioassay studies.

4.5 Limitations and Future Directions [24]

While this study successfully demonstrates alkaloid presence in *M. minuta*, several limitations should be acknowledged. The qualitative nature of the screening tests, while valuable for preliminary assessment, does not provide quantitative information about alkaloid concentrations or specific compound identification.

Future research should focus on advanced analytical techniques such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy for detailed alkaloid characterization and quantification. Additionally, bioactivity studies should be conducted to correlate alkaloid presence with pharmacological effects.

4.6 Pharmaceutical and Therapeutic Implications [25]

The confirmed presence of alkaloids in *M. minuta* extracts opens avenues for pharmaceutical development and drug discovery research. Given the historical importance of plant alkaloids in medicine, including compounds like morphine, quinine, and vincristine, the alkaloid-rich extracts of *M. minuta* warrant further investigation for potential therapeutic applications.
The diversity of alkaloid types suggested by the different precipitation test results indicates that this plant may serve as a source of novel bioactive compounds with unique pharmacological profiles.

5. CONCLUSION

This study successfully demonstrates the presence of alkaloid compounds in methanolic extracts of *Marsilea minuta* obtained through controlled maceration processes. The comprehensive positive results from Mayer's, Wagner's, and Dragendorff's tests provide robust evidence for alkaloid presence, with different precipitate characteristics suggesting chemical diversity among the alkaloid constituents. The extraction methodology employed proved effective in yielding alkaloid-rich extracts suitable for phytochemical analysis. The controlled temperature conditions and extended maceration period appear optimal for alkaloid extraction while maintaining compound stability. These findings establish a foundation for future detailed alkaloid characterization studies and support the ethnopharmacological significance of *M. minuta*. The confirmed alkaloid presence validates the potential of this aquatic fern as a source of bioactive compounds for pharmaceutical applications.

Future research directions should include quantitative alkaloid analysis, structural elucidation of individual compounds, and comprehensive bioactivity evaluation to fully realize the therapeutic potential of *Marsilea minuta* alkaloids.

Acknowledgments
The authors express their gratitude to the Acharya Jagadish Chandra Bose Indian Botanic Garden, Shibpur for the authentication of the plant material. We also thank the laboratory staff of Faculty of Medical Science and Research, Sai Nath University, Ranchi for their technical assistance during the experimental work.

Authors Contribution
As B. Pharm student, Madhu Vishwakarma and Ankit Srivastava contributed to the experimental work aspects of the research, such as performing methanol maceration extraction. Alok Kumar, student from Faculty of Medical Science and Research probably provided all relevant inputs regarding the phytochemistry, extraction uses, and analytical techniques; he also helped interpret the results obtained.

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Assistant Professor and corresponding author, Mr. Arnab Roy, was leading author, since he conceived the study, designed it, supervised the experimental work, solved methodological problems, analysed and interpreted the experimental data, revision and submission of the manuscript. He ensured the feasibility of the research by granting access to the laboratory.

Conflicts of Interest

The authors declare no conflicts of interest.

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