RESEARCH ARTICLE

Azolla filiculoides: A Promising Feedstock with Rapid Growth and High Nutritional Value

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DOI: 10.18811/ijpen.v10i04.20

Abstract

Azolla filiculoides, a highly adaptable aquatic fern, demonstrates significant potential across various fields due to its unique properties and applications. Cultivated in a pond in Mittlakalte village near Davangere, Karnataka, India, our study aimed to evaluate its uses and benefits. Sourced from Krishi Vigyan Kendra, Davangere, the culture adapted quickly to the pond environment, thriving at temperatures of 20 to 30°C. Molecular characterization using 18S RNA primers confirmed the species as *A. filiculoides*, with sequencing results available in GenBank under accession number PP472463. Our analysis examined key parameters, including dry matter (4.56%) and high moisture content (95.44%), indicating its water-rich nature. Significant findings included high total ash (7.71%), acid-insoluble ash (6.43%), and crude protein (22.37%), suggesting its value as a mineral and protein source. Additionally, ether extract (3.00%) and crude fiber (9.69%) highlighted its potential as a lipid and fiber source, while nitrogen-free extractives (31.64%) emphasized its carbohydrate content. Notable mineral levels, particularly calcium (6.73%) and phosphorus (1.05%), enhance its nutritional profile for diverse applications. The presence of lignin (3.5%) presents new opportunities for bioenergy, livestock feed, and environmental remediation. Overall, our study underscores *A. filiculoides*' biochemical richness, supporting its potential for agriculture, aquaculture, and environmental management.

Key words: *Azolla filiculoids*, nutrient analysis, animal feed.

Highlights:

- *Azolla filiculoides* fixes atmospheric nitrogen through its symbiosis with Anabaena azollae, enhancing soil fertility and reducing chemical fertilizer use.
- The plant's high moisture content (95.44%) underscores its water-rich nature and potential for hydration applications.
- With 22.37% crude protein, *A. filiculoides* serves as a valuable protein source for animal feed and nutrition.
- Notable mineral levels, including total ash (7.71%), calcium (6.73%), and phosphorus (1.05%), support its use in agriculture and aquaculture.
- The 31.64% nitrogen-free extract indicates high carbohydrate content, while 3.5% lignin offers potential in bioenergy and environmental remediation.

International Journal of Plant and Environment (2024); **ISSN:** 2454-1117 (Print), 2455-202X (Online)

Introduction

Azolla, also known as the "green gold mine," is a small, floating aquatic fern in the family Azollaceae, found worldwide in freshwater and brackish habitats. Its tiny, fern-like fronds, typically a few millimeters in size, exhibit reddish or green hues influenced by species and environmental factors (Yadav *et al.,* 2023). *Azolla* includes species like *A. filiculoides*, *A. pinnata*, and *A. caroliniana*, each adapted to diverse habitats (Kumar *et al.,* 2016). *Azolla* is valued as a sustainable livestock feed for animals such as cattle, poultry, and fish (Hossiny *et al.,* 2008; Chatterjee *et al.,* 2013; Leterme *et al.,* 2009) and has applications in biogas production, bioremediation, hydrogen fuel generation, water purification, and weed management (Das *et al.,* 1994; Sood *et al.,* 2012; Golzary *et al.,* 2021). Nutritionally, *Azolla* is rich in protein with essential amino acids like lysine, along with minerals (iron, calcium, magnesium, potassium, phosphorus, manganese) and vitamins, including vitamin A, beta-carotene, and B12 (Mathur *et al.,* 2013; Parashuramulu *et al.,* 2013; Cherryl *et al.,* 2014; Henry *et al.,* 2017). It has negligible carbohydrate and fat content, high digestibility due to low lignin, and rapid growth with nitrogen-fixing abilities, which is especially useful in rice paddies in Southeast Asia (Costarelli *et al.,* 2021; Hill, 2022). Medicinally, *Azolla* shows hepatoprotective, anti-inflammatory, antioxidant, and anti-apoptotic properties, suggesting its potential for treating hepatotoxicity (Chaudhary *et al.,* 2020). It is also used in cough medicines (Raja *et al.,* 2012).

Azolla filiculoides, also known as false fern or red azolla, is an aquatic fern that forms a dense, moss-like layer on water surfaces, shifting from green in the shade to reddish-brown under direct sunlight. It thrives in warm, low-calcium water with organic matter, demonstrating resilience at altitudes up to 5000 meters. This adaptability allows *A. filiculoides* to adjust growth and pigment synthesis in response to environmental factors, enhancing photosynthesis under low light while tolerating cold, though high light and cold stress may induce flavonoid accumulation for photoprotection (Cannavò *et al.,* 2023). In

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How to cite this article: Raikar, S.P., Umadevi, K.M., Roopa M.C. and Kallimani, S. (2024). *Azolla filiculoides:* A Promising Feedstock with Rapid Growth and High Nutritional Value. International Journal of Plant and Environment. 10(4), 186-192.

Submitted: 22/05/2024 **Accepted:** 27/11/2024 **Published:** 31/12/2024

the UK, it has been controlled as an invasive species using the biocontrol agent *Stenopelmus rufinasus*, reducing management costs (Corin *et al.,* 2022).

In agriculture, fertilization with guinea pig manure has been shown to improve the growth and nutritional value of *A. filiculoides* for livestock feed (Mancilla Castro and Pérez Roman, 2022). Its phytoremediation potential includes biodegradation of phenanthrene at low concentrations (Kosesakal and Seyhan, 2022), while its lignin-free cell wall structure makes it suitable for bioactive compound extraction and biorefinery applications (Carballo-Sanchez *et al.,* 2022). The high protein and ether extract content further support its role as a ruminant feed alternative (Nurul *et al.,* 2019). Studies also demonstrate that it enhances growth and phytochemical responses in jojoba plants when used as a nutrient source (Atteya *et al.,* 2022). Genetic diversity research in Uganda has identified distinct *Azolla* species, including *A. filiculoides*, for agricultural and environmental applications (Lydia *et al.,* 2023). In environmental remediation, *A. filiculoides* effectively removes hazardous substances like hydrazine from water (Eimoori *et al.,* 2020) and contains metal-tolerant microbiome strains, aiding survival in heavy-metal-contaminated environments (Banach *et al.,* 2020). Its efficacy in wastewater treatment is shown through significant removal of nitrogen, phosphorus, and chemical oxygen demand, reducing eutrophication risks (Taghilou *et al.,* 2021).

Chemical analysis of *A. filiculoides* reveals nutritional and bioactive properties valuable for various applications. High protein, amino acids, vitamins, minerals, and bioactives make it a potential supplement for livestock and human nutrition. Additionally, certain compounds contribute to its role in bioremediation, facilitating pollutant removal and water purification. Thus, understanding its chemical content is crucial for leveraging *A. filiculoides* as a resource in food, health, and environmental sustainability.

Materials and Methods

Site Selection and Pond Preparation for A. filiculoides Cultivation

The chosen site for cultivating *A. filiculoides* was strategically located within Mr. Raghavendra Bhat's coconut plantation in Mittlakatte, Davangere district. This site was selected for its proximity to essential amenities, facilitating easy maintenance and monitoring. The pond floor was thoroughly prepared by leveling and clearing any obstructions, ensuring an optimal environment for *A. filiculoides* growth. Water levels were consistently maintained, and shading was provided to reduce evaporation and preserve water levels, creating ideal growth conditions.

Pond Construction and Cultivation Setup

Three cement tanks, each measuring 6x3x2 feet, were constructed for *A. filiculoides* cultivation. A nutrient-rich mixture of fine soil, cow dung, and vermicompost was spread across the pond floor and moistened to form a slurry. After filling the ponds to three-fourths capacity, a fresh culture of *A. filiculoides* from Krishi Vigyan Kendra, Davangere, was introduced. Water temperature and pH were regularly monitored, and a green net provided shade and protected against contamination. Every six months, the ponds were emptied and replenished with a fresh culture to maintain optimal growth conditions. (Table 1)

Table 1: Primers used in the PCR

Primer	*Sequence*	*Annealing Temperature*
18S F	5'- CGGACGGGTGAGTAACGCGTGA-3'	53^0C
18SR	5'-GACTACAGGGGTATCTAATCCCTTT-3'	57^0C

Harvesting and Storage of *A. filiculoides*

Under favorable conditions, *A. filiculoides* reaches harvest readiness within two weeks. Harvesting is done with a mesh tray, followed by thorough washing to remove any residual cow dung odor. The harvested *A. filiculoides* is sun-dried for 2-3 days and packed in airtight bags for storage.

Molecular Identification of *A. filiculoides* using 18s rRNA method

The molecular identification of *A. filiculoides* using the 18S rRNA was achieved by isolating DNA from *A. filiculoides* samples and then using reverse transcription polymerase chain reaction (RT-PCR) to amplify the 18S rRNA gene. The PCR products were then sequenced and compared to known sequences in databases to determine the species of *A. filiculoides* present. The Following is the detailed procedure followed.

DNA Isolation by CTAB Method

A small amount of *A. filiculoides* was mixed with CTAB extraction buffer, vortexed, and incubated at 60°C for 30 minutes. After centrifugation, the supernatant was mixed with chloroform/isoamyl alcohol, vortexed, and centrifuged. The aqueous phase was transferred, precipitated with cold isopropanol, and incubated at -20°C. The resulting DNA pellet was washed with ethanol, dried, and dissolved in TE buffer, followed by RNase treatment to remove RNA contamination before quantification.

Column Purification of DNA (Kit Method)

DNA was mixed with binding buffer and applied to a column in portions, spinning after each transfer. After two wash steps, a dry spin was followed by elution with buffer to obtain purified DNA for quantification.

PCR Conditions

PCR began with initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30 seconds), and elongation (72°C for 1-minute), ending with a final elongation at 72°C for 10 minutes.

Preparation of PCR Reaction Mixture

The 25 µL PCR mix included a DNA template, reaction buffer, $MgCl_2$, dNTPs, primers, Taq polymerase, and molecular-grade water. Primers targeting the 18S rRNA gene were used.

Gel Purification Protocol

The desired DNA band was excised, dissolved in buffer at 55°C, mixed with isopropanol, and transferred to a column. After washing and drying steps, DNA was eluted with buffer.

Sanger Sequencing PCR

Sanger PCR began with denaturation at 95°C, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing (50°C for 30 seconds), and termination (60°C for 4 minutes), then held at 4°C.

Post Sequencing and PCR Purification

EDTA and ethanol were added to each well, vortexed, and centrifuged for DNA precipitation. After decanting and air-drying, HiDiFormamide was added, and the samples were denatured before sequencing.

Data analysis

The sequencing files obtained were in. AB1 format, which could be conveniently viewed using various software applications such as FinchTV, BioEdit, ChromasLite, and SeqScanner. The quality of the acquired sequence data was assessed by examining the Electropherogram peaks. Further analysis of the sequencing data was conducted using the BLAST server or other servers associated with specific databases to identify and characterize the sequences obtained.

Chemical analysis

The chemical analysis was conducted following the AOAC (2007) standard methods.

Dry Matter (DM) and Moisture Content

Collected samples were weighed dried in a hot air oven at 100°C until a constant weight was achieved, then re-weighed to calculate moisture and DM content.

Total Ash (TA)

An accurately weighed sample was decarbonized by heating, then ashed in a muffle furnace at 550 to 600°C for 2 to 3 hours. The final weight of the ash provided the total ash content.

Acid Insoluble Ash (AIA)

The total ash was treated with dilute HCl, boiled, filtered, and ignited in a muffle furnace at 550 to 600°C. The weight of the remaining residue indicated acid-insoluble ash.

Crude Protein

Using the Kjeldahl method, nitrogen content was estimated by digesting the sample in sulfuric acid with a digestion mixture, followed by distillation and titration. Nitrogen values were converted to protein content.

Ether Extract (Crude Fat)

Fat was extracted from the dried sample using petroleum ether in a Soxhlet apparatus. The residue was weighed to determine crude fat content.

Crude Fiber

The fat-free sample was sequentially digested with sulfuric acid and sodium hydroxide, filtered, dried, and ashed. The weight difference gave the crude fiber content.

Neutral Detergent Fiber (NDF)

The sample was refluxed with neutral detergent solution, filtered, washed, and dried to determine NDF, providing an estimate of cell wall components.

Calcium

Acid extract of total ash was treated with ammonium oxalate, forming a precipitate which was titrated to determine calcium content.

Phosphorus

The acid extract was treated with ammonium molybdate to form a yellow precipitate, which was dissolved in NaOH and titrated to estimate phosphorus.

Lignin

Acid detergent lignin (ADL) was prepared by treating the sample with 72% sulfuric acid, then dried and ashed to determine lignin content.

Results

Cultivating *A. filiculoides* in Pond Environments

In a remarkable display of rapid biomass accumulation, the ponds witnessed a profuse proliferation of *A. filiculoides* within a mere 2 to 3-week timeframe. The *A. filiculoides*, characterized by its diminutive yet prolific growth habit, densely colonized the water surface, forming a verdant mat. This accelerated expansion underscores the inherent vigor and adaptability of *A. filiculoides* species, particularly under favorable environmental conditions. The exponential growth of *A. filiculoides* not only imparted a visually striking aspect to the aquatic landscape but also played a pivotal role in nutrient cycling and water quality maintenance. (Figure 1A to 1F) illustrates the sequential stages involved in *A. filiculoides* cultivation, outlining the sequential stages from pond preparation to the readiness for harvesting.

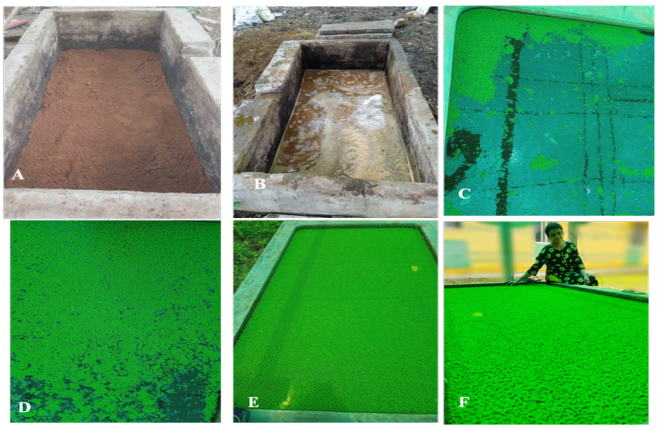

Fig. 1: Various stages of *A. filiculoides* production. A = The pond is prepared for *A. filiculoides* cultivation; B = Cow dung slurry is uniformly distributed across the pond surface to provide nutrients for *A. filiculoides* growth; C = Initial inoculation of *A. filiculoides* into the prepared pond to initiate growth; D = The progress of *A. filiculoides* growth is observed one week after inoculation; E = *A. filiculoides* proliferation is shown 15 days post-inoculation, displaying significant Multiplication; F = Mature *A. filiculoides* is ready for harvesting, indicating the completion of the growth cycle.

Molecular Identification of *A. filiculoides* through 18S rRNA Analysis

The molecular analysis of *A. filiculoides* involved the use of 18S rRNA primers, resulting in the amplification of a 500 base pair fragment (Fig. 2). Subsequent sequencing allowed for a comparative analysis on the NCBI platform, where homology searches against existing sequences were conducted. This comparison unveiled a significant similarity of 95.95% with known sequences, affirming the taxonomic identity of the amplified fragment. Following validation, the sequence was officially submitted to the NCBI database and assigned the accession number PP472463.

Chemical analysis

Analysis of the compositional parameters of *A. filiculoides* provides valuable insights into its nutritional and chemical constituents. With a moisture content of 95.44% and a corresponding dry matter content of 4.56%, the plant's hydration status is evident. Notably, its ash content is substantial, with total ash and acid insoluble ash levels measuring 7.71 and 6.43%, respectively, indicating its mineral composition and insoluble mineral fractions. *A. filiculoides* exhibits a noteworthy protein content of 22.37%, suggesting its potential as a proteinaceous resource. Additionally, its lipid content, represented by 3.00% ether extract, denotes a moderate presence of lipids. The plant's crude fiber content, quantified at 9.69%, underscores its significance as a source of dietary fiber. Essential minerals, including calcium (6.73%) and phosphorus (1.05%), contribute to its nutritional value, particularly in terms of bone health and metabolic functions. Furthermore, lignin, a structural component, constitutes 3.5% of its composition, enhancing its structural integrity. This systematic analysis delineates the intricate nutritional profile and chemical composition of *A. filiculoides*, highlighting its potential versatility in various scientific and industrial applications. Overall, the comprehensive chemical analysis of *A. filiculoides* underscores its potential as a versatile feed ingredient and highlights its suitability for various applications in agriculture, aquaculture, and environmental remediation efforts. These findings provide valuable insights into harnessing the nutritional and functional benefits of *A. filiculoides* for sustainable livestock production and ecosystem management (Table 2).

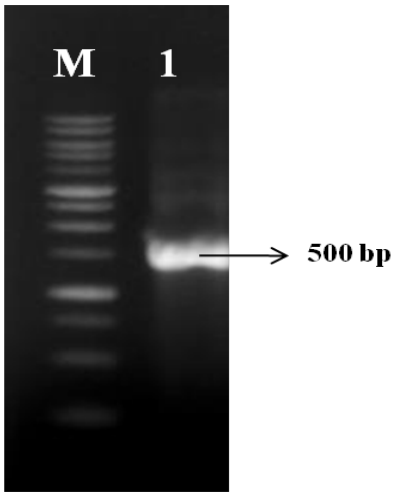

Fig. 2: PCR amplification of the 18s gene from *A. filiculoides*, visualized on a 1.2% agarose gel. Lane M, Marker; Lane 1: The distinct band observed corresponding to a 500 bp amplicon, confirms the amplification of the targeted gene fragment in *A. filiculoides.*

Table 2: Biochemical composition of *A. filiculoides*: Nutritional and mineral analysis

Parameters	*A. filiculoides*
Dry matter	04.56 %
Moisture content	95.44%
Total ash	7.71%
Acid insoluble ash	6.43%
Crude protein	22.37%
Ether extract	3.00%
Crude fibre	9.69%
Nitrogen free extractives	31.64%
Calcium	6.73%
Phosphorus	1.05%
Lignin	3.5%

Discussion

This study's molecular identification of *A. filiculoides* using 18S rRNA primers and sequencing aligns with previous research on molecular characterization. Banach *et al.,* (2020) emphasized molecular methods to detect genetic variations in *Azolla* species in Uganda, supporting molecular approaches in assessing taxonomic identity and genetic diversity, a need highlighted by Kumar *et al.,* (2021) due to morphological overlaps within *Azolla*. This research complements findings on *A. filiculoides*' symbiosis with nitrogen-fixing *Nostoc azollae*, furthered by gene expression studies (Lydia *et al.,* 2023) and endophytic bacterial diversity research, which suggest roles in host fitness and productivity (Vries *et al.,* 2018). Eily *et al.,* (2019) explored its microbiome's potential for biotechnological applications, particularly in bioremediation.

In terms of dry matter, this study's finding of 4.56% differs from higher DM values in controlled settings, such as 7.92% crude lipids under elevated CO_2 for bioenergy (Carballo-Sanchez *et al.,* 2022). For animal feed, Brouwer *et al.,* (2016) recorded DM productivity of 90 to 97.2 kg ha^{-1} d^{-1} and protein yields of 176–208 g kg^{-1} DW, with CO_2-augmented growth increasing biomass yield to 48.3 t ha^{-1} yr^{-1}. DM is vital for biosorption in environmental applications, as shown in gold uptake from wastewater (Brouwer *et al.,* 2018), while studies highlight its role in heavy metal remediation and biodiesel lipid extraction (Zazoli *et al.,* 2014; Asbchin *et al.,* 2012). Seasonal variations further underscore DM's importance in diverse applications (Golzary *et al.,* 2021).

The observed moisture content of 95.44% in *A. filiculoides* aligns with values typical of aquatic plants, slightly higher than the 91.77 to 92.25% reported by Sreenath *et al.,* (2015), affirming its water-rich adaptation. Studies on biofertilizers (Hanafey *et al.,* 2021), optimal growth (Adzman *et al.,* 2021; Golzary *et al.,* 2021),

and phytoremediation (Banach *et al.,* 2020; Kösesakal *et al.,* 2016; Rezooqi *et al.,* 2021) support the benefits of high moisture for growth and resilience, aiding its aquaculture role (Umali *et al.,* 2006; Mancilla Castro & Pérez Román, 2022). The ash content of 7.71% reflects *A. filiculoides*' mineral richness, with variations across studies (Sreenath *et al.,* 2015; Anitha *et al.,* 2016; Gupta *et al.,* 2018; Kumari *et al.,* 2018; Sharma *et al.,* 2021). Studies on *A. pinnata* (Roy *et al.,* 2016) and work by Golzary *et al.,* (2021) and Brouwer *et al.,* (2016) show that growth conditions and lipid content affect mineral composition. Phytoremediation studies (Rezooqi *et al.,* 2021; Balarak *et al.,* 2016; Zazoli *et al.,* 2014) suggest mineral accumulation could also impact ash levels.

The crude protein content of 22.37% in *A. filiculoides* aligns with high values reported in *Azolla* studies, where Sreenath *et al.,* (2015) found lower protein levels (3.9–5.2%), while Parashuramulu *et al.,* (2013) observed a similar 21.37%. Swain *et al.,* (2022) and Brouwer (2018) support its suitability for poultry feed and rapid growth conditions, respectively, with additional aquaculture feed validation by Shiomi & Kitoh (2001) and Costa *et al.,* (1999). The ether extract content of 3.00% aligns with ruminant feed studies (Kamaruddin *et al.,* 2019; Parashuramulu *et al.,* 2013), while higher lipid values for biofuel (Brouwer *et al.,* 2016) suggest environmental impact on lipid content.

The nitrogen-free extract of 31.64% indicates a high carbohydrate level, supporting its nutritional value (Kosesakal *et al.,* 2016; Anitha *et al.,* 2016) and energy potential for biodiesel (Brouwer *et al.,* 2016). A calcium content of 6.73% reinforces *A. filiculoides*' role as a calcium-rich feed, consistent with studies by Kamaruddin *et al.,* (2019) and Espinoza & Gutiérrez (2003) on mineral variation across ecotypes. Adaptability across environmental conditions is further supported by Sanchez-Viveros *et al.,* (2016) and Rezooqi *et al.,* (2021).

The phosphorus content of 1.05% supports *A. filiculoides*' role in phosphorus removal, with efficiencies of up to 66% noted by Taghilou *et al.,* (2021) and Hosseini *et al.,* (2021) and 44% removal in nutrient solutions reported by Golzary *et al.,* (2018), demonstrating its bioremediation potential, as further validated by Temmink *et al.,* (2018). Studies on phosphorus mobilization in iron-rich soils (Zhen-li, 2007; Gremmen, 2016) affirm its value in nutrient recycling. The 3.5% lignin content observed here contrasts with previous reports of lignin absence (Lucas & Duckett, 1980; Carballo-Sanchez *et al.,* 2022) and may reflect analytical variations. Low lignin levels facilitate biofuel production (Kathirvelan *et al.,* 2015) and enhance digestibility for livestock feed (Costa *et al.,* 1999). Structural properties make *A. filiculoides* effective in pollutant removal, as shown by Moris *et al.,* (2022) and Umali *et al.,* (2006). The nutrient content analysis conducted by Muruganayaki *et al.,* and Miranda *et al.,2020* reported phosphorus concentrations ranging between 0.5 and 1.9 g.kg^-1 in *A. filiculoides*, aligning with our findings and providing additional validation to the observed phosphorus content (Muruganayaki *et al.,* 2019; Miranda *et al.,* 2020).

Conclusion

Our comprehensive analysis of *A. filiculoides* sheds light on its remarkable potential across various scientific and industrial domains. Through molecular identification using 18S rRNA analysis, we reaffirmed the taxonomic identity of *A. filiculoides*, contributing to the broader understanding of its genetic diversity and symbiotic relationships. Chemical analysis revealed the intricate nutritional profile and chemical composition of *A. filiculoides*, highlighting its versatility as a feed ingredient and its suitability for applications in agriculture, aquaculture, and environmental remediation. The rapid biomass accumulation of *A. filiculoides* underscores its inherent vigor and adaptability, emphasizing its role in nutrient cycling and water quality maintenance. Our findings on compositional parameters such as moisture content, ash content, protein content, lipid content, crude fiber content, and mineral composition provide valuable insights into its nutritional value and functional properties. Moreover, the presence of lignin in *A. filiculoides* challenges previous assumptions and warrants further investigation into its implications for bioenergy production, livestock feed digestibility, and environmental remediation applications. Comparison with existing research highlights the variability of *A. filiculoides*' dry matter content and moisture content across different growth conditions and applications. Despite this variability, the plant's nutritional richness and adaptability underscore its potential for diverse uses, from biofuel production to wastewater treatment.

Acknowledgments

The authors would like to express our sincere gratitude to the Department of Studies in Environmental Science at Davangere University, Shivagangothri, Davangere, Karnataka, India, for providing us with the invaluable opportunity and support necessary to conduct the research presented in this manuscript. Secondly, we extend our heartfelt thanks to Mr. Raghavendra B. Bhat of Mittalkakke, District Davangere, Karnataka, for generously providing us with the necessary space to cultivate our *A. filiculoides*. His support and cooperation were crucial for our successful growth and study, enabling us to carry out our research effectively. We greatly appreciate his contribution and assistance, which have been invaluable to our work.

Contribution of Authors

All authors have made significant contributions to the research and preparation of this manuscript. Mrs. Shilpa, P. Raikar, carried out the research under the mentorship and supervision of Dr. Sharadadevi Kallimani. Umadevi, K.M., and Roopa, M. C. provided valuable assistance in the manuscript's preparation, ensuring the quality and coherence of the final document.

Conflict of interest

All the authors declare that they have no conflict of interest.

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