Letters in Applied NanoBioScience
Platinum Open Access Journal (ISSN: 2284-6808)

Article
Volume 9, Issue 4, 2020, 1673 - 1679
https://doi.org/10.33263/LIANBS94.16731679

Azolla pinnata: Potential Phytoremediation, Antimicrobial, and Antioxidant Applications

Mabel Merlen Jacob [1,*], Magna Jom [1], Ameena Sherin [1], Binu Shahla [1]

[1] Department of Microbiology, St.Mary's College, Thrissur-20, Kerala, India
* Correspondence: merlin.mabel@gmail.com;

Scopus Author ID 57208499938

Received: 12.07.2020; Revised: 20.08.2020; Accepted: 22.08.2020; Published: 25.08.2020

Abstract: *Azolla* or the "green gold" is an aquatic nitrogen-fixing pteridophyte with a wide distribution in temperate and tropical freshwater ecosystems and paddy fields. *Azolla* is an ideal candidate for food, feed, and fodder applications. It can be utilized as a natural plant-based antimicrobial and also as a water purifier in a laboratory or industrial wastewater treatment. Its feasibility as a source for the development of health supplements was tested by analyzing the antioxidant and antimicrobial properties of the fern. The DPPH antioxidant activity of the various extracts shows the good presence of antioxidants. A fair antibacterial activity was shown against the disease, causing bacteria Staphylococcus sp. and Bacillus sp. Antioxidant and antimicrobial property of *Azolla* heightens the possibility of its use as food. The phytoremediation property of *Azolla* grown in a metal-containing sample was assessed using atomic absorption spectroscopy, and positive results indicated its prospective use in industrial or laboratory wastewater treatment. This can reduce the pollution of water bodies, like, rivers, where such water is discarded.

Keywords: Azolla; antioxidants; Staphylococcus sp.; wastewater treatment; phytoremediation property; water purifier.

1. Introduction

Azolla is an aquatic fern or small leafed floating plant, seen in quiet and slow-moving water bodies and is present in countries like Africa, Asia, and some parts of Australia. It produces maximum biomass in a relatively shorter period of time [1] and is of great applications in both developing as well as developed countries [2-4]. It is commonly called mosquito fern, duckweed fern, or water velvet. It has a symbiotic relationship with the nitrogen-fixing cyanobacterium *Anabaena Azollae*, which resides in special cavities of the dorsal leaf lobe. The endosymbiont provides sufficient nitrogen for both itself and its hosts [5]. The fern, in turn, provides a protected environment for the alga and also supplies it with a fixed carbon source. *Azolla-Anabaena* complex has great potential as a biofertilizer, because it can assimilate atmospheric nitrogen efficiently, thus increasing the productivity of rice [6] in paddy fields. *Azolla* has immense potential as an animal or poultry feed. It provides the cattle or buffalo herders with cheaper feed. It does not need extra land for cultivation as it grows well in the paddy fields, is easy to cultivate, and provides the farmer with an extra income. The dietary *Azolla* supplementation shows to have a positive effect on growth performance of fish and reduce the cost of feeding on fish meal and fish oil diet [7]. Also, it can be utilized as a feed for animals/birds [8], food for humans, water purifier, green fertilizer or vermicompost

[9], biogas [10], biolarvicide [11], and to improve soil microbial diversity [12]. *Azolla* is found especially successful in phytoremediation of water by acquiring nitrates and phosphorous [13].

In the present study, we have attempted to analyze the antimicrobial and antioxidant properties of the fern along with its capability to absorb heavy metals. The study aims to explore the vast opportunities present in the area of research and development of *Azolla* and to bring its applications possible in households.

2. Materials and Methods

2.1. Azolla cultivation.

The *Azolla pinnata* fern samples were collected from the Botany Department of St. Mary's College, after identification and authentication by the faculty. *Azolla* was grown outdoor in plastic vessels at the Department of Microbiology, St. Mary's College, Thrissur, and maintained till further treatment.

2.2. Analysis of antimicrobial activity.

2.2.1. Preparation of extract.

The *Azolla* was retrieved from the cultivation tanks and washed thoroughly with distilled water to remove all the impurities and debris. The plant was then dried in sunlight, by keeping it in the shade for two days. The dried *Azolla* was then slightly powdered using a mixer grinder. 20g of this powder was used for extraction using the soxhlet.

The antimicrobial analysis was carried out using the extracts obtained after successive extraction using the solvents ethanol and benzene.

2.2.2 Antimicrobial assay.

The antimicrobial assay was performed against the microorganisms by means of a well diffusion assay. To perform the antimicrobial activity of the leaf extract, *Staphylococcus, Bacillus,* and *Pseudomonas* cultures maintained at the Department of Microbiology, St. Mary's College, Thrissur was employed. The bacterial cultures were grown and maintained in nutrient broth and nutrient slant at 37˚C.

The Agar Well Diffusion Method of Antimicrobial Susceptibility test was used to evaluate the presence of antibacterial activities of the two solvent extracts of the *Azolla pinnata*. 24-hour old nutrient broth culture of the bacteria was used. Sterile Mueller Hinton Agar (Hi-media) plates were prepared and appropriately labeled for each bacteria. A lawn culture of the microorganisms was prepared using sterile swabs on the respective plates. Wells were cut out from the inoculated nutrient plate using a 0.1 ml sterile tip. Three wells were cut into each plate. One was used for DMSO (Dimethyl sulfoxide) as control and other two for the two different concentrations (20 µl and 30 µl) of the same plant extract. Then all the plates were kept for incubation at 37°C for 12-18 hours and observed for the formation of inhibition zones.

The nutrient agar plates were swabbed with a suspension of bacterial cultures. Following a short incubation, wells were cut on each agar plate. One of the wells was loaded with DMSO, other with ethanolic extract and ethanol was kept as control. Similarly, DMSO, acetone extract, and acetone were loaded in respective plates for acetone extract. The agar plates were incubated at 37^0C for 24 hours and checked for the development of inhibition zones.

2.3. Antioxidant assay.

2.3.1. Sample preparation.

Azolla samples were prepared at the concentration of 10mg/mL for Antioxidant assay by the DPPH method.

2.3.2. DPPH Reagent preparation.

The stock solution was prepared by dissolving 0.0025 g of DPPH in 10 ml methanol and covered with aluminum foil. The working standard was prepared by using 6 ml of the stock solution and making it up to 60 ml with methanol.

2.3.3. Evaluation of free radical scavenging activity by DPPH method.

The determination of radical scavenging activity of *Azolla* extract assayed using the DPPH assay following the modified method of Mensor *et al*. [14]. 10 µl and 20 µl of *Azolla* samples were taken in a series of test tubes and made up to 50 µl with methanol. Methanol was used as control. 2 ml of DPPH working standard was added to all test tubes, including control. The tubes were allowed to stand in the dark, at room temperature, for 20 min. The reaction was carried out in duplicates. The decrease in absorbance was measured at 515 nm on a spectrophotometer. The scavenging activity of the samples corresponds with the intensity of quenching DPPH. Lower the absorbance of the reaction mixture, higher the free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula:

$$\text{DPPH scavenging effect (\% inhibition)} = (A0 - A1)/ A0 \times 100$$

where A0 is the absorbance of the control reaction, and A1 is the absorbance of the test.

2.4. Phytoremediation property of Azolla.

Healthy mature *A. pinnata* plants obtained from the cultivation tanks were rinsed with tap water in order to remove adhering mud particles and epiphytes and used to test for the phytoremediation property.

2.4.1. Preparations of stock solutions and treatment/experiment series.

Treatment solutions of Zinc, Cadmium, and Lead were prepared by diluting the respective stock solutions of 6 ppm concentration. Salts of each of the heavy metals with 6 ppm concentrations were mixed together in 1000 ml distilled water, separated the mixture solution into two equal portions of each having a final volume of 500 ml. An additional beaker with 500 ml freshwater without adding metals was also set up for comparing the growth rate and characteristics of *Azolla* in comparison with the metal contained habitat. Healthy and matured *A. pinnata* plants were selected, rinsed with distilled water, and blotted on filter papers to remove adherent water, and 2g of the water fern were laid on the surface of one of the beaker added with metals and the other with fresh water. The third beaker with metals was kept as a control for the experiment. All of them were run for a 9 day period.

2.4.2. Sample analysis.

On the 9th day of the experiment, Water samples were filtered with Whatman No.1 filter papers and analyzed by Atomic Absorption Spectrometer for Zn, Cd, and Pb. The analysis was performed at Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

The relative growth of the plants exposed to the treatment solutions was evaluated by visual examination of the growth pattern of *Azolla* on water containing metals and water without metals. The removal percentage of metal ions by *A. pinnata* was determined by using the initial metal concentrations of the treatment and the final concentrations at the end of the experiment.

3. Results and Discussion

3.1. Analysis of antimicrobial activity.

The antimicrobial activity was negligible in the case of both the extracts. Among the two solvents used in the study, benzene extract was found more inhibitory than ethanol. The *Bacillus* and *Staphylococcus* were inhibited to a minimum by benzene than ethanol. The results are shown in Figure 1.

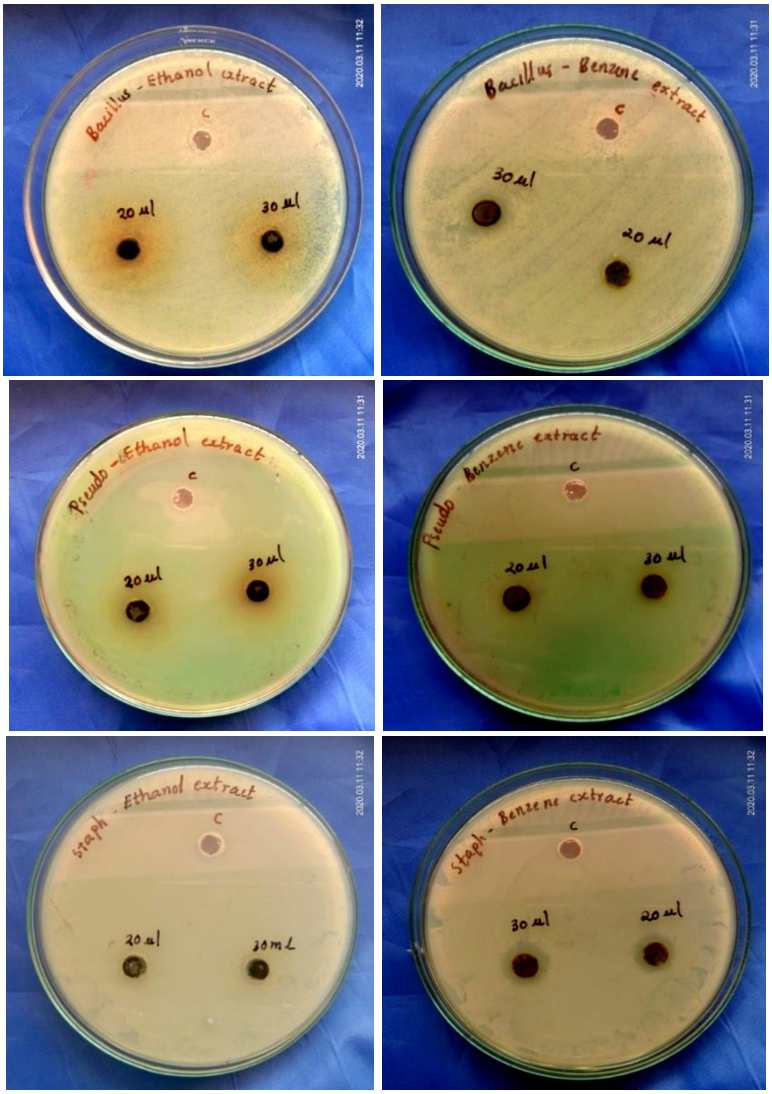

Figure 1. Antimicrobial activity of *Azolla*.

The inhibitory effect increased with that of the concentration of the extract as is seen in Table 1.

Table 1. Antimicrobial activity of *Azolla*.

Solvent	Name of organism	Zone of inhibition in mm		
		Control	20µl	30µl
Benzene	*Bacillus*	-	4	8
	Pseudomonas	-	1	3
	Staphylococcus	-	7	9
Ethanol	*Bacillus*	-	-	1
	Pseudomonas	-	1	2

3.2. Antioxidant assay.

The antioxidant activity of the *A. pinnata* benzene and ethanol extracts were tested using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method and was compared with standard ascorbic acid.

Volume	Ascorbic acid	Benzene	Ethanol	Control	Inhibition % of Ascorbic acid	Inhibition % of benzene	Inhibition % of ethanol
10 µl	0.079	0.125	0.110	0.301	74	58.47	63
20 µl	0.076	0.118	0.105	0.301	75	60.81	65

Table 2. Antioxidant activity of *Azolla* extracts.

As seen in Table 2, in comparison with the standard, *Azolla* extracts appear to be an effective scavenger of free radicals and have the potential to be used as a natural antioxidant. Higher antioxidant efficacy could be due to the higher content of total phenolics and flavonoids potentially present in the plant extract. Antioxidant activity is very important in counteracting the deleterious role of free radicals in biological food systems.

The DPPH alcohol solution is a deep purple color with an absorption peak at 517 nm, which disappears in the presence of radical scavengers in the reactive system. The scavenging capacity can be expressed as its antioxidant capability. There is the involvement of free radicals and other oxidants in the cause of oxidative stress that leads to various diseases and disorders. This led to an increasing interest in natural products as having antioxidant properties. Plants have been considered as being richer in antioxidants. In this study, the antioxidant activity of *Azolla pinnata* ethanol extract was showing more potential than benzene extract.

3.3. Phytoremediation property of Azolla.

The 9-day old samples analyzed by AAS at Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur showed positive results. The initial and final readings of metal concentration, as shown in Table 3, indicate the phytoremediation by *Azolla*.

Table 3. Phytoremediation by *Azolla.*

Sl No	Metals	Initial	Final
1	Lead (Pb)	0.59	.23
2	Cadmium (Cd)	2.33	1.89
3	Zinc	17.77	15.48

Azolla can be considered as an excellent candidate for the removal, disposal, and recovery of heavy metals. The ability to hyper accumulates heavy metals [15-17] makes them interesting research candidates, especially for the treatment of industrial effluents, heavy metal polluted water reservoirs, and sewage wastewater. The use of aquatic macrophytes, such as *Azolla* with hyperaccumulating ability, is an environmentally friendly option to restore polluted aquatic resources [18]. The prospective use in laboratory wastewater treatment is a projected aim of our studies too.

4. Conclusions

Plants are the basic source of medicines for the modern life sciences. The cheap cost, low incidences of adverse reactions when compared to modern pharmaceuticals are encouraging public and health care institutes to turn to plant medicines. *Azolla pinnata* was cultivated in tanks, and antibacterial activities were studied. A fair antibacterial activity was shown against the disease, causing bacteria *Staphylococcus* sp. and *Bacillus* sp. DPPH antioxidant activity of the various extracts shows the good presence of antioxidants, which can serve as a good source for the development of health supplements for animal health. It can be concluded that the fern has good nutritional and antibacterial activity. The heavy metal absorption was found to be promising enough to warrant an extended study and applications in the field. *Azolla* is an ideal candidate for elaborate research in food, feed, and fodder applications, as well as household cultivation and use.

Funding

This research received no external funding.

Acknowledgments

Authors would like to thank Central Instruments Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur for AAS analysis of samples.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Brouwer, P.; Bräutigam, A.; Buijs, V.A.; Tazelaar, A.O.E.; van der Werf, A.; Schlüter, U.; Reichart, G.-J.; Bolger, A.; Usadel, B.; Weber, A.P.M.; Schluepmann, H. Metabolic Adaptation, a Specialized Leaf Organ Structure and Vascular Responses to Diurnal N2 Fixation by Nostoc *Azolla*e Sustain the Astonishing Productivity of *Azolla* Ferns without Nitrogen Fertilizer. **2017**, *8*, https://doi.org/10.3389/fpls.2017.00442.
2. Acharya, P.; Mohanty, G.P.; Pradhan, C.R.; Mishra, S.K.; Beura, N.C.; Moharana, B. Exploring the effects of inclusion of dietary fresh *Azolla* on the performance of white pekin broiler ducks. *Vet World* **2015**, *8*, 1293-1299, http://dx.doi.org/10.14202/vetworld.2015.1293-1299.
3. Bharali, A.; Baruah, K.K.; Baruah, S.G.; Bhattacharyya, P. Impacts of integrated nutrient management on methane emission, global warming potential and carbon storage capacity in rice grown in a northeast India soil. *Environmental Science and Pollution Research* **2018**, *25*, 5889-5901, https://doi.org/10.1007/s11356-017-0879-0.
4. Carlozzi, P.; Padovani, G. The aquatic fern *Azolla* as a natural plant-factory for ammonia removal from fish-breeding fresh wastewater. *Environmental Science and Pollution Research* **2016**, *23*, 8749-8755, https://doi.org/10.1007/s11356-016-6120-8.
5. Peters, G.A. The *Azolla*-Anabaena *Azolla*e relationship. *Archives of Microbiology* **1975**, *103*, 113-122, https://doi.org/10.1007/BF00436337.

6. Bocchi, S.; Malgioglio, A. *Azolla-Anabaena* as a Biofertilizer for Rice Paddy Fields in the Po Valley, a Temperate Rice Area in Northern Italy. *International Journal of Agronomy* **2010**, 1-5, https://doi.org/10.1155/2010/152158.
7. Mosha, S. A Review on Significance of *Azolla* Meal as a Protein Plant Source in Finfish Culture. *Journal of Aquaculture Research and Development* **2018**, *9*, https://doi.org/10.4172/2155-9546.1000544.
8. Shukla, M; Bhattacharyya, A; Shukla, P. K.; Roy, D.; Yadav, B.; Sirohi, R. Effect of *Azolla* feeding on the growth, feed conversion ratio, blood biochemical attributes and immune competence traits of growing turkeys. *Veterinary World* **2018**, 11, 459-463, https://doi.org/10.14202/vetworld.2018.459-463.
9. Arora, M., Kaur, A. *Azolla* pinnata, Aspergillus terreus and Eisenia fetida for enhancing agronomic value of paddy straw. *Scientific Reports* **2019**, 9, 1341, https://doi.org/10.1038/s41598-018-37880-1.
10. Sathammaipriya, N.; Thamilmaraiselvi, B.; Steffi, P.F.; Sangeetha, K. Investigation of phytochemical constituents in *Azolla microphylla* for antibacterial activity. *National Journal of Physiology, Pharmacy and Pharmacology Online* **2018**, *8*, 1500-1504, https://doi.org/10.5455/njppp.2018.8.0310430072018.
11. Ravi, R.; Zulkrnin, N. S. H.; Rozhan, N. N.; Nik, Yusoff N. R.; Mat, Rasat M. S.' Ahmad, M. I.; Intan, H. I.; Mohamad, F. M. Amin. Chemical composition and larvicidal activities of *Azolla pinnata* extracts against *Aedes* (Diptera: Culicidae). *PLoS ONE* **2018**, 13, e0206982, https://doi.org/10.1371/journal.pone.0206982
12. Lu, X., Lu, P. Response of microbial communities to pesticide residues in soil restored with *Azolla imbricata*. *Applied Microbiology and Biotechnology* **2018**, *102*, 475–484, https://doi.org/10.1007/s00253-017-8596-7
13. Liu, J.; Xu, H.; Jiang, Y.; Zhang, K.; Hu, Y.; Zeng, Z. Methane Emissions and Microbial Communities as Influenced by Dual Cropping of *Azolla* along with Early Rice. *Scientific Reports* **2017**, *7*, https://doi.org/10.1038/srep40635.
14. Mensor, L.L.; Menezes, F.S.; Leitão, G.G.; Reis, A.S.; Santos, T.C.d.; Coube, C.S.; Leitão, S.G. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. *Phytotherapy Research* **2001**, *15*, 127-130, https://doi.org/10.1002/ptr.687.
15. Talebi, M.; Tabatabaei, B.E.S.; Akbarzadeh, H. Hyperaccumulation of Cu, Zn, Ni, and Cd in *Azolla* species inducing expression of methallothionein and phytochelatin synthase genes. *Chemosphere* **2019**, 230: 488-497, https://doi.org/10.1016/j.chemosphere.2019.05.098.
16. Banach, A.M; Kuźniar, A; Grządziel, J; Wolińska, A. *Azolla filiculoides* L. as a source of metal-tolerant microorganisms. *PLoS One* **2020**, 15, e0232699, https://doi.org/10.1371/journal.pone.0232699.
17. Haldar, S.; Ghosh, A. Microbial and plant-assisted heavy metal remediation in aquatic ecosystems: a comprehensive review. *3 Biotech* **2020**, 10, 205, htpps://doi.org/10.1007/s13205-020-02195-4.
18. Sood, A.; Uniyal, P.L.; Prasanna, R.; Ahluwalia, A.S. Phytoremediation potential of aquatic macrophyte, *Azolla*. *Ambio* **2012**, *41*, 122–137, https://doi.org/10.1007/s13280-011-0159-z.