DOI: https://doi.org/10.52756/ijerr.2023.v36.001 *Int. J. Exp. Res. Rev.*, Vol. 36: 01-19 (2023)

Original Article | *Peer Reviewed* | *Open Access*

International Journal of Experimental Research and Review (IJERR)

ISSN: 2455-4855 (Online)
www.iaph.in
ISSN 2455-4855

A comprehensive study on the assessment of chemically modified *Azolla pinnata* as a potential cadmium sequestering agent

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Article History:

Received: 30th Jul., 2023
Accepted: 25th Nov., 2023
Published: 30th Dec., 2023

Keywords:
Azolla pinnata, bioactive profile, bioremediation, cadmium chelation, chemical treatment, surface modifications

How to cite this Article:
Kaakarlu Shivakumar Vinanthi Rajalakshmi and Kuppusamy Alagesan Paari (2023). A comprehensive study on the assessment of chemically modified Azolla pinnata as a potential cadmium sequestering agent. *International Journal of Experimental Research and Review*, *36*, 1-19.
DOI:
https://doi.org/10.52756/ijerr.2023.v36.001

Abstract: The major environmental issue raised throughout the world is the egression of toxic pollutants in water bodies. Hence, employment of novel technological interventions such as bioremediation and phytoremediation for mitigating the toxic effects caused by the pollutants has gained attention. The aquatic macrophyte, *Azolla pinnata* is utilized as a biofiltering agent in the present study for the chelation of metal toxicants from the artificial wastewater system. The nutritive value of *A. pinnata* was determined to be 268.99Kcal/100g energy and the mineral profiling showed the highest amount of calcium (54.7ppm), iron (14.04ppm) and manganese (7.96 ppm). The quantitative screening of total phenolic and total flavonoid contents showed a maximum of 402.33±4.29 mg/g GAE and 105.25±3.81 mg/g QE respectively and the sample exhibited strong antioxidant activity in quenching the DPPH radicals with an IC_{50} value of 88.27µg/ml. Similarly, the highest bioactivity was observed in methanolic and chloroform extract of *A. pinnata* biomass showing the zone of growth inhibition against *E. coli* (17mm) and *S. aureus* (18mm). The results recorded from the SEM-EDX, GCMS, FTIR and XRD confirmed the adsorptive properties of biomass. The chemically modified and unmodified *Azolla* exposed to cadmium metal solution showed the maximum adsorption of about 0.47±0.001 and 0.48±0.003 ppm in 60mins using the unmodified biomass with dosage of 0.75 and 1.0g respectively. Moreover, the results recorded from the instrumental characterization for the adsorptive properties of *Azolla* biomass proved that cadmium chelation is due to the modifications caused in porosity, surface structure and the addition of functional groups in the treated biomass surface.

Introduction

The aquatic fern, *Azolla* is a tiny, free-floating, nitrogen-fixing pteridophyte that lives in the area with abundance of water both in the tropics and subtropics regions. Around forty extinct species and seven extant species are categorized under the two sub-genera *EuAzolla* (Raja et al., 2012). *Azolla* is often referred as 'Green Gold mine' due to its high protein and amino acid content (Gregory and Wagner, 1997). *Azolla* is commonly co-cultivated in rice paddies due to the prevalence of perfect habitat for propagation and in turn, nitrogen supplementation to rice crops is reciprocated through the symbiotic association of N_2-fixing cyanobacterium *Anabaena Azollae* (Shi and Hall, 1988; Qiu and Yu, 2003). The unique properties, such as high multiplication rate, growth rate, nutritive value, biomass production and atmospheric nitrogen-fixing ability, make it an attractive candidate to execute potential applications in solving environmental concerns (Raja et al., 2012). *Azolla* has historically been used in traditional systems around the world to treat numerous ailments, including inflammation, fever, and liver disorders (Elrasoul et al., 2020). Many researches have also confirmed *Azolla* is a 'one wonder plant with many powers' having the ability to sequestrate carbon, act as green fertilizer, biofuels, be involved in nitrogen fixation, and also aid in water purification (Lumpkin and Plucknett, 1980; Raja et al., 2012).

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Aquatic macrophytes are considered to be strong indicators of heavy metal pollution in aquatic ecosystems, acting as biological filters by absorbing and hyperaccumulating heavy metals from polluted habitat (Sarojini et al., 2023; Sharma et al., 2023). The ability of water ferns to chelate heavy metals from aquatic environments has been well utilised due to its eco-sustainability and cost-effectiveness compared to conventional treatments (Sachdeva and Sharma, 2012; Naghipour et al., 2018). Recently, interest in exploring methodology to optimize the growth and cultivation of *Azolla* and its applicability in bioremediation is receiving much attention (Bianchi et al., 2020; Rezooqi et al., 2021). While *Azolla pinnata* shows great promise for various bioremedial applications, a significant number of challenges also exist. One major challenge to be addressed is to optimise the growth and cultivation process in varied environmental conditions. Developing cost-effective methods for harvesting and processing *Azolla pinnata* remains a challenge.

Very few studies have reported the biochemical profiling of *A. pinnata*. Additionally, no other explicit study has stated the efficacies of *A. pinnata* plant as biological tool for metal remediation. Hence, this research aims to identify the phytochemical profiling of A. pinnata and assess the phyto-remediating ability of cultured aquatic fern in the purification of waters polluted by Cd (II). Our study also describes the properties responsible for Cd adsorption through instrumental characterization using SEM, XRD, and FTIR as powerful tools for analysing Cd biosorption from metal solutions by *A. pinnata*. Optimization has been the major demand of any technology for minimizing the labour, input costs, time, and applicability at pilot scale. The present study aims to explore the optimization factors for enhancing the potentiality of the cultured water fern, *Azolla pinnata* in the bioremediation and wastewater treatment which are under heavy stress of anthropogenic pressure. The current study also provides insights to assess Azolla's complete bioactive profile, highlighting the correlation between the phytochemical profile and bioremediation potential of Azolla biomass. The study also revealed that modifications on the biomass surface through chemical treatment enhanced the metal uptake. The exposure studies are limited to only one species of *Azolla*, *Azolla pinnata*, on an artificial water system with a single metal, Cadmium. Since metals are in a wide range, future explorations by using aquatic plants as a bioremediation tool are needed.

Materials and Methods

Culture, identification and growth studies

The fern was collected from Vasundara farm, 14.23^{0}N to76.4^0 E coordinates of Karnataka, India and was cultured in the greenhouse at CHRIST (Deemed to be University) throughout the research study. Culture was maintained for 30 days to assess the isolated fern's growth rate and doubling time in the commercial nutrient medium. During the experimental period, the parameters such as pH 7.0 and temperature between 25-27^{o}C with 16:8 light: dark photoperiod were strictly maintained. Fresh biomass was collected and weighed at intervals of 24h to analyse the growth properties of *Azolla*. Cultured fern was identified and authenticated as *Azolla pinnata* (Taxonomic serial number: 181820).

Relative Growth Rate (g/g/d) was calculated following the standard protocols (Badayos, 1989).

Relative growth rate (RGR) = [ln (Final biomass) – ln (Initial biomass)/ Time interval in days]

Doubling time (D_t) = [0.693 (Growth period)/ ln (Final biomass/ Initial biomass)]

Physiochemical properties

Total Ash

The cleaned silica crucible was heated to redness and cooled in a desiccator. A total of 10g of precisely weighed *A. pinnata* powder was taken in a tared silica crucible and burned in an electric muffle furnace at 600^{0}C for 2 hrs until it was carbon-free. Once the powdered plant material in the crucible was turned to carbon-free ash, it was allowed to cool in a desiccator and the weight of the recovered carbon-free ash was recorded.

Total ash (%) = [Ash weight/ weight of sample] x 100

The acquired carbon-free ash was subjected to further experiments to measure acid-insoluble ash and water-soluble ash by following the standard protocol (AOAC, 2019).

Acid-insoluble ash (%) = [Acid insoluble ash weight/ weight of sample] x 100

Water-soluble ash (%) = [Total ash weight – water-insoluble residue in total ash/ weight of sample] x 100

Proximate analysis of *Azolla pinnata*

The whole plant powder was assessed for moisture, ash, crude fibre, crude fat, and protein content following the standard procedures (AOAC, 2019). Meanwhile, the total carbohydrate and nutritive value of A. pinnata was estimated using the above obtained approximate values.

Total Carbohydrate = 100 - (% of Ash + % of Moisture + % of Crude Fibre + % of Crude Protein)

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Finally, the nutritive value of the *A. pinnata* is expressed in kilocalories per 100 grams of dry weight of plant and is calculated using the formula

Nutritive Value = (4 × % of Protein) + (9 × % of Crude Fat) + (4 × % of Total Carbohydrate)

Mineral profile of *Azolla pinnata*

Micro and macro elements of *Azolla pinnata* were analysed by appropriate digestion methods. The *Azolla* powder (4g) was digested with di-acid Conc. The HNO3: HCl (3:1) mixture was on a hot plate till a clear solution was obtained and was suitably diluted with distilled H_2O to make up to 100ml. The elemental content in the biomass was determined using atomic absorption spectrophotometer (AAS).

The mineral content of *Azolla* was determined using the following formula:

Element in *Azolla* (µg/g) = Concentration of mineral in the sample solution (mg/L) x volume prepared (ml) / the weight of the sample (grams, g).

Preparation of plant extract

20 g of dried *Azolla* powder and 200 ml of solvent (1:10 w/v) were placed in a round-bottom flask of soxhlet apparatus with the heating mantle underneath. The extraction was performed with 4 different solvents: acetone, aqueous, chloroform and methanol. The extraction process was conducted at the boiling point of respective solvents until the solvent in the siphon arm became clear. Finally, the extracts were evaporated to dryness in the vacuum evaporator, stored at -4oC, and further used for qualitative and quantitative assays (AOAC, 2005).

Phytochemical screening by qualitative methods

The qualitative phytochemical assays were analysed for Alkaloids (Dragendroff's test); Amino acid and protein (Ninhydrin test), Carbohydrate (Molisch's test), Saponin (Foam/Frothing test), Quinone (Hydrochloric acid test), Phlobatannins (1% HCl test), Phenol (FeCl3 test), Flavonoids (Ammonia test), Coumarins (Sodium hydroxide test), Glycoside (Keller-Killiani test), Anthocyanin (Sulphuric acid test), Tannin (Braymer's test), Terpenoids/ Steroid (Liebermann-Burchard test), Oxalate (Glacial acetic acid test), Anthraquinones (Borntrager's test), Diterpenes (copper acetate test), and Resins (turbidity test) by following standardized protocols (Farook et al., 2019; Suresh and Xavier, 2023).

Phytochemical screening by quantitative methods

Total Phenolic content

Each extract was evaluated using the Folin–Ciocalteau reagent to determine the phenolic content. The quantification was performed based on a calibration curve (y = 0.0011x + 0.0021, R^2 = 0.9977) established with gallic acid ranging from 0-500 µg/ml.

To 10µl (1mg/ml) of plant extract, 100µl of 1/10 dilution of Folin-Ciocalteau's reagent and 80µl of Na_2CO_3 solution (1M) was added. The solution mixture was vortexed and kept incubated in the dark for 15-20 minutes. The absorbance of all the test samples was measured using a 96-well plate in microplate reader at 700 nm. The tests were performed in triplicate and the average of the three measured values was calculated. The results were expressed in terms of micrograms of gallic acid equivalents (GAE) per gram of dry extract weight (Jafri et al., 2014).

Total Flavonoid content

Flavonoid contents in specific plant extracts were quantitatively determined using aluminium chloride ($AlCl_3$) colorimetric method. Quercetin served as the reference or standard compound. The results were obtained through a calibration curve (y = 0.0042x - 0.0014, R^2 = 0.9979) established with quercetin ranging from 0–100 µg/ml.

A volume of 100µl of extract (1mg/ml) was mixed with 100µl of $AlCl_3$ reagent (10%, w/v) in 400µl methanol. To this mixture, 100µl of sodium acetate (1M, adjusted to 5.2pH using glacial acetic acid) solution was added. The reaction mixture was left undisturbed for 150 minutes in dark at room temperature. Subsequently, the absorbance of the resulting mixture was measured at 440 nm in 96-well plate microplate reader. The entire experiment was performed in triplicate, and the total flavonoid content (TFC) of the extract, expressed as quercetin equivalents (QE) per gram of dry extract weight was recorded (Kosalec et al., 2004).

Phytochemical screening by GCMS analysis

Phytochemical analysis was performed using a single quadrupole high-performance gas chromatograph and a mass spectrophotometer (Model: GCMS- QP2010 SE SHIMADZU). The GC system was equipped with an MS. The carrier gas used was helium. The specific GC-MS conditions were as follows: the ion-source temperature was set at 200°C, the interface temperature was 290°C, and the pressure was maintained at 76.2 kPa. The injection was performed in split less mode with total flow of 4.3 ml/min (Colum flow: 1.25ml/min and purge flow: 3.0ml/min), and the injection temperature was set at 280°C. The column oven temperature initially started at 60°C for 3 minutes, then increased at a rate of 12°C/min until reaching 200 °C, and it was held up to 2 minutes. It was further raised to 280°C at a rate of 20°C/min and was held for 16 minutes. The average peak area of each component was determined by assessing the total peak

area. Prior to phytochemical analysis, crude extract was dissolved in chloroform at 5mg/ml. The identification of the components was accomplished by utilizing their retention indices and by interpreting the mass spectra. The obtained mass spectra of the unknown components from the *A. pinnata* asper fraction were compared and interpreted using the standard mass spectra of the National Institute of Standards and Technology 2017 (NIST 17) database library.

Screening of Bioactive properties

Antioxidant activity using DPPH method

The antioxidant capacity was assessed using DPPH (2,2-diphenyl-1-picryl-hydrazyl, HI media, India) method with slight modifications according to the standard protocol (Brand-Williams et al., 1995). The fresh stock of 0.1mM DPPH solution was prepared on the day of analysis by dissolving 4mg in 100 ml of methanol. The reference standard, L-Ascorbic acid, was prepared at a concentration of 1mg/ml and linear calibration curve ($y = 2.6938x + 0.196$, $R^2 = 0.998$) was obtained in the range between 0-12 µg /ml. *A. pinnata* crude extracts were diluted with methanol at 120, 100, 80, 60, 40, and 20 µg/ml. An aliquot of diluted extract (1ml) was mixed with 3ml of 0.04% (w/v) DPPH solution (A_e). For blank, 1 ml of methanol was mixed with 3 ml of the methanolic DPPH solution (A_0). The mixture was incubated in the dark for 30 minutes, and the absorbance (A) was measured at 520 nm.

DPPH Radical Scavenging Activity (%) = $[(A_0 – A_e)/A_0] \times 100$

Half maximal Inhibitory Concentration (IC_{50}) of the sample required to scavenge 50% of DPPH free radical was calculated using the following equation:

Half maximal Inhibitory Concentration (IC_{50}) = (50 – c)/m

Antimicrobial activity

Prior to antimicrobial analysis, crude extracts were re-dissolved in respective solvents at specific concentrations. All the pathogenic strains were procured from ATCC. The procured cells were inoculated in the test tube and incubated under suitable conditions depending on the test microorganism. Two bacterial strains, *Escherichia coli* (ATCC-10536), *Staphylococcus aureus* (ATCC 25923), and two fungal strains, *Candida tropicalis* (ATCC *10231)* and *Aspergillus flavus* (ATCC 9643) were used as test microbes to check the efficacy of *Azolla pinnata* crude extracts and the zone of growth inhibition was measured (Bora et al., 2018; Efstratiou et al., 2012).

Metal chelating potential of *A. pinnata*

The metal chelating assay used the analytical grade of Cd $(NO_3)_2.4H2O$. The standard metal stock solution with different initial concentrations of 15.75, 31.25, 62.5, 125, 250 and 500ppm were prepared by dissolving Cd $(NO_3)_2.4H_2O$ in distilled water. The adsorption experiments were conducted with optimization parameters such as temperature of 25±2ºC, pH 6.8, contact time of 15-180mins, and *Azolla* adsorbent dosage of 0.25, 0.50,0.75 and 1.0g/25ml of 500pm cadmium.

Unmodified and chemically modified *Azolla pinnata* biomass were tested for their ability to sequester cadmium accumulation. The 0.25-1.0g biosorbent dosage was exposed to 25 ml of 500ppm of cadmium solution. Optimization factors such as temperature 35^0C, 1hr exposure period and pH of 6.8 were maintained. After 1hr of exposure, the liquid (supernatant) and solid (pellet) were separated using centrifugation and were subjected to di-acid digestion to free up the metal ions. The digested samples were analysed using atomic adsorption Spectroscopy (AAS) and the accumulation of cadmium metal ions from metal solution by A. *pinnata* (modified or unmodified) biomass was determined.

Instrumental Characterization of *A. pinnata* for its adsorptive properties

The adsorptive properties of the *Azolla* biomass before and after cadmium metal adsorption have been characterized using SEM-EDX analysis, FT-IR spectra, and XRD patterns. The morphological changes on the surface of *Azolla pinnata* powder were studied by using Apreo S LoVac Scanning Electron Microscopy (SEM, ThermoFisher Scientific Corporation). Fourier transform infrared (FTIR) spectrophotometer with QATR-S single-reflection ATR accessory having extended range diamond crystal (Shimadzu Model IR Spirit) was used to characterise the functional groups present in the sample. The changes in the crystallinity of the *Azolla* adsorbent, and the binding energies of the elements before and after adsorption and chemical treatment were analysed using Rigaku Mini Flex Powder X-ray Diffractometer (XRD, Rigaku Corporation) with Cu Kα radiation. The generator voltage and current were set at 35 KV and 25 mA respectively. The *A. pinnata* samples were scanned in the 2θ ranges 20-80°C in continuous scan mode with a 10°/ min scan speed.

Statistical analysis

The statistical analysis of the present study was analysed using SPSS statistics software version 27. The results were interpreted by One-way Analysis of Variance (ANOVA) to verify the significance by using Duncan's Test by the probability less than $p<0.05$.

DOI: https://doi.org/10.52756/ijerr.2023.v36.001

Results and Discussion

Cultivation and growth properties

Azolla pinnata was cultivated in the greenhouse for 30 days in a water-soil medium supplemented with commercial nutrients. The growth properties were observed on the 1st, 5th, 10th, 20th and 30th day. The mean relative growth rate was found to be of 0.229 g/g/d and the doubling time required was about 4.651 days. The plant growth performance was evaluated following the protocol of Kösesakal et al. (2019). The relative growth rate and doubling time were determined to be 0.148 g/g/d and 5.6 days, respectively, which are lesser than those reported in our current study. In contrast, the recent research investigation on the growth conditions for *A. pinnata* reported a growth rate and doubling time of 0.321 g/g/d and 2.16 days, respectively that was achieved using the optimised conditions of 15% strength Hoagland solution, higher light intensity, nitrogen present in the medium (Da Silva, 2022). *Azolla* strains exhibit diverse responses to environmental conditions such as temperature, light intensity, and soil nutrients, leading to variations in their growth characteristics and chemical composition (Chatterjee et al., 2013).

Physiochemical, Proximate and nutritional analysis

The proximate composition and nutritive value of *Azolla pinnata are* detailed (Table 1). revealed that the plant is rich in nutrition and can be used in multiple potential applications. The high ash content (27.9%) determines that the biomass was relatively rich in mineral elements. The results of ash content are in close agreement with earlier reports (Kumar et al., 2018; Querubin et al., 1986) on A. pinnata of about 25.50% and 28.70%, respectively. Total ash value is the significant parameter for the detection of adulteration, impurities, authenticity and quality of the sample. The relatively lower percentage of water-soluble ash (9.52±0.69) and acid-insoluble ash (4.5±0.45) indicated the low inorganic compounds and silica in *A. pinnata*. The moisture content in fresh *Azolla* ranges from 94.97- 95.45 with an average value of 95.03±0.09. However, similar results with 93.5 % moisture were reported earlier (Balaji et al., 2009).

The results of proximate estimation of crude fat, crude fibre, crude protein and total carbohydrates were found to be 5.711%, 7.93%, 28.87% and 25.52% (Table 1). The similar findings of crude fat content ranging between 3.5-5% was reported (Anitha et al., 2016; Dutt Choudhary et al., 2020). The total crude fibre of 7.44% recorded by Mishra et al. (2013) shows slight similarity with the data reported in the present study. The higher difference in the fibre content of about 12.5% was also observed (Parashuramulu et al., 2013), which may be due to change in dry matter content and maturity status of the *Azolla*. Recent studies indicated that the crude protein content in *Azolla* might be around 24.65-28.96% (Bhatt et al., 2020), which was concurrent with the result of 28.87±0.02 mentioned in our research. However, the nutritional value of 268.99 Kcal/100g energy was determined using the obtained proximate values of *Azolla pinnata*.

Mineral Profiling

The elemental analysis performed using the di-acid digested sample for Fe, Mn, Zn and Cu was obtained in the order of 351.02>199.03>38.99>21.62 µg/g in shade dried *Azolla pinnata* biomass. Despite, the microelement, calcium was found to be rich (1367.75 µg/g), and the presence of magnesium and potassium in trace amounts was observed (Table 2). Earlier findings with similar order of elemental analysis were documented in *A. pinnata,* indicating it to be a rich source of micronutrients (Anitha et al., 2016).

Soxhlet crude extraction yields

The extraction of *Azolla pinnata* with four different solvents, such as acetone, aqueous, chloroform and methanol using Soxhlet apparatus yielded higher crude extractable components. The yield percentage was 6.2, 9.37, 7.27 in acetone, aqueous and chloroform respectively. Whereas, for methanol, a highest yield of 12.32% crude components was obtained.

Table 1. Results on Physicochemical and Proximate analysis of *A. pinnata.*

Parameters value (W/W %)								
Total ash	Acid insoluble ash	Water soluble ash	Total Moisture		Total crude fat	Total crude fibre	Total crude protein	Total Carbohydrate
			Fresh	Dry				
27.9±0.13	4.5±0.45	9.52±0.69	95.03±0.09	9.63±0.16	5.714±0.07	7.93±0.05	28.87±0.02	25.52±0.13

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Table 2. Results on Mineral profile of *Azolla pinnata.*

Types of elements	Concentration (ppm)	Elemental content in plant (µg/g)
Micro elements		
Cu	0.8649	21.62
Fe	14.0407	351.02
Mn	7.9612	199.03
Zn	1.5599	38.99
Macro elements		
Ca	54.7100	1367.75
Mg	1.4206	35.515
K	-3.3318	Below detectable limits

Phytochemical screening

The preliminary phytochemical analysis of different solvents extract was tested for 17 phytochemicals (Table 3). The screening of *A. pinnata* for the presence of secondary metabolites such as phenols, flavonoids and saponins in various solvent extracts of *Azolla pinnata* was reported (Mithraja et al., 2011). Alkaloids, quinones, glycosides, oxalates, terpenoids/ steroids and diterpenes are found to be present in all three extracts except in aqueous extract. Screening of *A. microphylla* and *A. caroliniana* showed the presence of alkaloids, flavonoids, phenols and tannins, which are known to possess antimicrobial properties (Yadav et al., 2016) and could serve as good sources of medicine in pharmacological sectors (Mishra et al., 2016; Park and song, 2017). The findings of Thagela et al. (2017) in *A. microphylla* witnessed the significance of saponins and steroids as the main source for the normal functioning of central nervous system using proteome analysis. In contrast, amino acids and resins were detected only in the aqueous extract of *A. pinnata*. Carbohydrates and coumarins are detected only in acetone and aqueous extracts, which correlate with the results obtained by Farook et al. (2019). Likewise, tannin, which was highly screened in methanolic extract in the present research, is known to elicit profound medicinal properties such as astringent, anti-inflammatory and antimicrobial, which is in concordance with the previous study (Sreenath et al., 2016). Vannini et al. (2018) demonstrated the role of tannins as antimicrobial agents by inhibiting extracellular enzymes, leading to the depletion of essential substrates necessary for microbial growth and hindering oxidative phosphorylation in microbial metabolism (Sarkar et al., 2022; Sadhu et al., 2022). Similar to the current investigation, Sathammaipriya et al. (2018) reported the presence of almost all the phytochemical compounds and screened the maximum antimicrobial properties of *A. microphylla* leaves against *Bacillus* and *Staphylococcus* species. Phlobatannins are absent in all the extracts of the current study, whereas similar findings were observed earlier (Sathammaipriya et al., 2018; Farook et al., 2019). The natural phytocompounds of *A. jacquemontii* are reported to be involved in the bioremediation of pollutants Sudan et al. (2014).

The quantitative screening of phytochemicals from fern, *A. pinnata* are also illustrated (Table 4), and their potential applications in pharmaceutical and medical aspects, especially in health promotion such as antioxidant, antibacterial, anti-cancer, cardioprotective effects, immune system promotion and anti-inflammatory effects are highlighted (Table 5).

Phenolic compounds, which are essential components found in plants, possess redox properties that contribute to their antioxidant activity (Scalbert et al., 2005). The presence of hydroxyl groups in plant extracts enables effective scavenging of free radicals. The quantification of total phenolic content in *A. pinnata* varied in different solvents extracted in the following order as aqueous<methanol<acetone<chloroform showing the mean ± SD in mg/g of GAE (Table 4). On the other hand, total flavonoid contents are in the order as aqueous<acetone<methanol<chloroform that exhibited in mg/g of QE (Table 4). Flavonoids, the largest group of naturally occurring phenolic compounds, can be used as metal hyperaccumulators. Recent studies by Tanui et al. (2021) have identified the novel approach of using specific phytochemical like flavonoids as a green solution to remediate heavy metals from a cocktail solution containing iron, nickel, lead, copper, cobalt, manganese, cadmium, and zinc in equal concentrations. Moreover, the present findings documented higher amounts of phenols and flavonoids in chloroform extracts compared to acetone, aqueous and methanolic extracts, indicating its capacity for remediation.

Table 3. Preliminary phytochemicals screening in different solvents of *A. pinnata.*

Sl. No	Phytochemical test	Acetone	Aqueous	Chloroform	Methanol
1	Alkaloids (Dragendroff's test)	+	-	-	+
2	Amino acid and protein (Ninhydrin test)	-	+++	-	-
3	Carbohydrate (Molisch test)	+++	+	-	-
4	Saponin (Foam/Frothing test)	+++	++	+	+++
5	Quinone (Hydrochloric acid test)	++	-	+	++
6	Phlobatanins (1% HCl test)	-	-	-	-
7	Phenol (FeCl3 test)	++	+	+++	+
8	Flavonoids (Ammonia test)	++	+	+++	++
9	Coumarins (Sodium hydroxide test)	++	+	-	-
10	Glycoside (keller-killiani test)	+++	-	++	++
11	Anthocyanin (Sulphuric acid test)	++	+++	+	-
12	Tannin (Braymer's test)	+	+	-	+++
13	Terpenoids/ Steroid (Liebermann-Burchard test)	+++	-	++	+++
14	Oxalate (Glacial acetic acid test)	+	-	+	++
15	Anthraquinones (Borntrager's test)	-	-	-	-
16	Diterpenes (copper acetate test)	++	-	+	++
17	Resins (turbidity test)	-	++	-	-
NB: - Absent, +++Highly Present, ++ Moderate and + less present.					

Table 4. Results on Total phenolic content, Total flavonoid content of *Azolla pinnata* extracts.

Type of Extract	Total phenolic content (mg/g of GAE)	Total flavonoid content (mg/g of QE)
Acetone extract	198.09±3.27^{b}	38.59±1.19^{c}
Aqueous extract	130.82±2.72^{d}	20.25±0.90^{d}
Chloroform extract	402.33±4.29^{a}	105.25±3.81^{a}
Methanol extract	157.18±0.91^{c}	42.71±1.26^{b}

Values are expressed as mean ± SD for triplicates, SD: Standard deviation, GAE: Gallic acid equivalent, QE: Quercetin equivalent. *p value significant ($p \leq 0.05$) in accordance to *Duncan's multiple range test* in different solvent extracts are expressed in increasing order as a-d.

GCMS analysis

GCMS analysis

The GC-MS chromatogram of the phytochemicals present in 4 solvents extract of A. pinnata are displayed (Figure 1). The analysis resulted in the identification of 32 different compounds in total in all the solvent extracts. The area percentage, retention time, molecular formula, and molecular weight of phytocompound are listed in Table 5. The phytochemicals detected in the chromatogram analysis majorly contain long-chain hydrocarbons such as alkanes and alkenes. The analysis showed the presence of neophytadiene, heneicosane, eicosane, 1-pentadecene, squalene, nonadecane, hexadecane, hexacosane and trifluoroacetoxy hexadecane that are reported to possess antimicrobial activity.

RT-Retention time, MF-Molecular formula, MW-Molecular weight, ACE-Acetone extract, AQE-Aqueous extract, CFE-Chloroform extract, MEE-Methanol extract

In vitro DPPH free radical scavenging activity

The present investigation of phytochemical analysis indicated a considerable quantity of secondary metabolites that are convincingly expected to elicit good antioxidant property. Hence, an *in vitro* DPPH radical scavenging assay was employed to cover the most significant mechanisms by which different antioxidants react to inhibit the generation of free radicals. In this study, the adsorption of DPPH solution in the presence of various concentrations of *Azolla pinnata* extracts was measured at 520nm. The results of DPPH antioxidant indicate that the chloroform extract of *A. pinnata* exhibited strong scavenging activity (IC_{50}) on the DPPH free radical of 88.27 µg/ml, which is consequently increasing with increase in the concentrations of plant extract. In addition, the methanolic extract exhibited with IC_{50} value of 104.06 µg/ml, followed by acetone and aqueous extract with similar inhibition values of 137.56 µg/ml and 140.02 µg/ml, respectively (Table 6). The scavenging effect of L-ascorbic acid standard indicated a higher activity than that of the extracts that agreed with the earlier reports (Sreenath et al., 2016; Noor et al.,

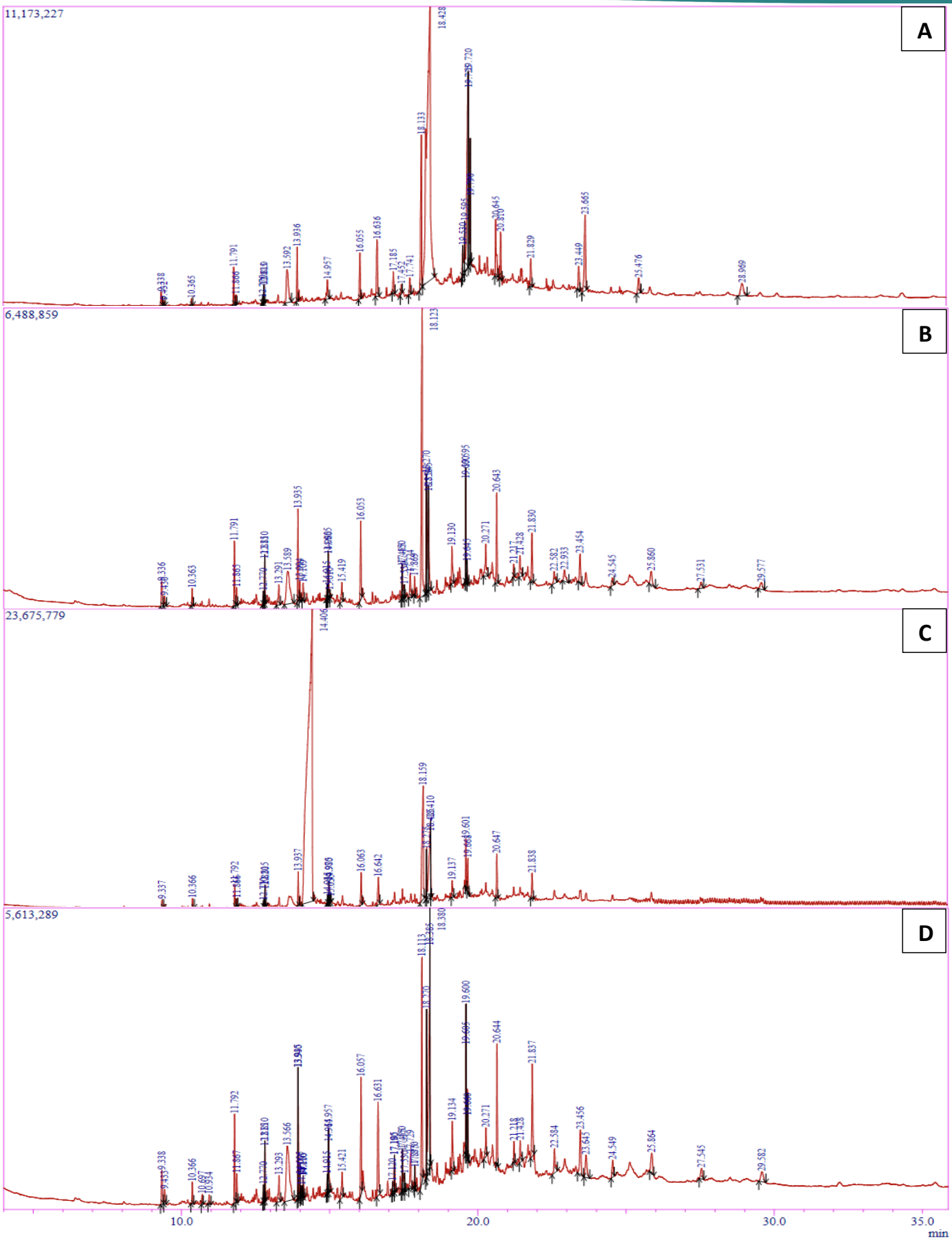

Figure 1. GCMS Chromatograms represented A-Acetone extract, B-Aqueous extract, C-Chloroform extract, D-Methanolic extract of *Azolla pinnata*.

Table 5. Results on GC-MS analysis of plant compounds in different solvent extracts of *A. pinnata.*

Name of the compound	Category	Area (%)	RT	MF	MW	Extract	Pharmacological activities or uses
1-Dodecene	Alkene	0.58	9.338	C12H24	168	ACE, AQE, CFE, MEE	Not intended for diagnostic or therapeutic use
E-14-Hexadecenal	Aldehyde	1.71	16.055	C16H30O	238	ACE, AQE, CFE, MEE	Antibacterial
Neophytadiene	Alkene	2.63	16.636	C20H38	278	ACE, CFE, MEE	Antimicrobial, Anti-inflammatory, larvicidal and insecticidal activities.
3.7,11,15-Tetramethyl-2-hexadecen-1-ol	Alcohol	0.77	17.185	C20H40O	296	ACE, CFE, MEE	Antimicrobial, Anti-inflammatory, Anticancer, Antidiuretic, and also precursor for the synthesis of vitamin E and Vitamin K1.
Heneicosane	Alkane	0.30	17.452	C21H44	296	ACE, AQE, CFE, MEE	Antimicrobial, Plant metabolite
Phenol, 3.5-bis(1,1-dimethylethyl)-	Alcohol	3.28	13.592	C14H22O	206	ACE, AQE, MEE	Anticancer agents and protagonists in many drugs discovery
n-Hexadecanoic acid	Fatty acid	44.61	18.428	C16H32O2	256	ACE	Antioxidant, Hypocholesterolemic Nematicide, Pesticide, Lubricant, Antiandrogenic and Flavor
Dibutyl phthalate	Fatty acid	6.21	18.133	C16H22O4	278	ACE, AQE, CFE, MEE	Antimicrobial activity, Inhibitory effects on phytopathogenic bacteria and cancer cell line
Eicosane	Alkane	2.56	20.271	C20H42	282	ACE, AQE, CFE, MEE	Antimicrobial, anti-inflammatory, antitumor, analgesic and antipyretic
Heneicosyl heptafluorobutyrate	Fatty acid	3.04	21.830	C25H43F7O2	508	AQE	No activity reported

1-Pentadecene	Alkene	1.58	13.935	C15H30	210	ACE, CFE	Cytotoxic activity, antimicrobial, antibacterial activity
Tridecane	Alkane	0.27	11.866	C13H28	184	ACE, AQE, CFE, MEE	No activity reported
Heptadecyl heptafluorobutyrate	Fatty acid	2.42	20.645	C21H35F7O2	452	ACE, AQE, CFE, MEE	No activity reported
Octadecanamide	Amide	1.60	20.810	C18H37NO	283	ACE	No activity reported
1-Dodecanol, 2-octyl-	Alcohol	1.20	21.829	C20H42O	298	ACE, AQE, CFE, MEE	No activity reported
Squalene	Phenolic compound	4.54	24.665	C30H50	410	ACE	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive, lipoxygenase-inhibitor, pesticide
Hexadecanoic acid, dodecyl ester	Fatty acid	1.06	25.476	C28H56O2	424	ACE	Antistatic agent, emollients, plant and algal metabolite
Dodecane, 4,6-dimethyl-	Alkane	0.20	10.365	C14H30	198	ACE, AQE, CFE, MEE	No activity reported
1-Tetradecene	Alkene	1.07	11.791	C14H28	196	ACE, AQE, CFE, MEE	No activity reported
Cetene	Alkene	1.52	13.936	C16H32	224	ACE, AQE, CFE, MEE	No activity reported
Nonadecane	Alkane	1.11	14.957	C19H40	268	ACE, AQE, MEE	Anti-HIV, Antioxidant, Antibacterial, Antimicrobial, Cytotoxic effect, Antimicrobial. Antimalarial, Unini uses like weakness of the principal organs like heart, Brain, liver, General weakness, Haemoptysis, Palpitation, Conjunctivitis, Earache, Stomatitis

Hexadecane	Alkane	1.40	13.291	C16H34	226	ACE, AQE, CFE, MEE	Cytotoxicity, Antimicrobial, Antioxidant, Antipyretic, Anthelmintic, Tumour, Bronchitis, Asthma, Tuberculosis, Dyspepsia, Constipation, Anaemia, Throat diseases, Elephantiasis, Antidiabetic, Anti-inflammatory, Antidiarrheal
Diethyl phthalate	Fatty acid	0.99	14.109	C12H14O4	222	AQE, CFE, MEE	Anticancer (MDA-MB-231 cell lines), Antibacterial and Insecticidal activity.
Tetra tetracontane	Alkane	1.35	22.582	C44H90	618	AQE, MEE	Antioxidant and cytoprotective activities. Acts as human metabolite
1-Decanol, 2-octyl-	Alcohol	2.17	23.454	C18H38O	270	ACE, AQE, CFE, MEE	No activity reported
Hexacosane	Alkane	0.76	27.531	C26H54	366	AQE, MEE	Antimicrobial
Octadecanoic acid	Fatty acid	1.23	19.668	C18H36O2	284	CFE	No activity reported
1-Heptacosanol	Alcohol	3.93	21.837	C27H56O	396	AQE, MEE	No activity reported
9-Octadecenamide, (Z)-	Amide	1.86	23.645	C18H35NO	281	MEE	No activity reported
Hexadecane, 1-iodo-	Alkane	1.21	17.185	C16H33I	352	AQE, MEE	No activity reported
Trifluoroacetoxy hexadecane	Alkane	1.24	19.601	C18H33F3O2	338	AQE, CFE, MEE	Antifungal

DOI: https://doi.org/10.52756/ijerr.2023.v36.001

Table 6. DPPH radical scavenging assay for *Azolla pinnata* extracts.

Concentration of extract (µg/ml)	% Scavenging activity of *Azolla pinnata* extracts			
	Acetone extract	Aqueous extract	Chloroform extract	Methanol extract
20	5.562±0.017	7.497±0.029	9.0.17±0.023	13.301±0.028
40	13.180±0.005	13.341±0.005	25.030±0.03	19.589±0.017
60	20.798±0.015	21.241±0.006	37.64±0.004	27.449±0.003
80	27.449±0.010	29.021±0.011	44.683±0.012	32.164±0.012
100	30.472±0.004	34.905±0.003	56.409±0.031	48.972±0.027
120	48.730±0.010	43.370±0.014	66.397±0.024	60.822±0.019
IC_{50} value (µg/ml)	**137.537**	**140.167**	**88.275**	**104.063**

Table 7. Antimicrobial activity of *Azolla pinnata* with zone of growth inhibition against test microbial strains.

Type of extract	Concentration of extract (µg/ml)	Zone of growth inhibition against test microbial strains (mm)			
		Bacterial strains		Fungal strains	
		Escherichia coli	*Staphylococcus aureus*	*Aspergillus flavus*	*Candida tropicalis*
Acetone extract	25	11±0.6	-	-	-
	50	12±0.8	-	10±0.7	11±0.6
	75	13±0.3	12±0.4	11±0.2	12±0.2
	100	14±0.4	13±0.2	13±0.4	13±0.3
	Control	17±0.2	16±0.2	23±0.3	25±0.2
Aqueous extract	25	-	-	-	-
	50	-	-	-	-
	75	-	-	-	-
	100	-	-	-	-
	Control	18±0.1	18±0.3	23±0.4	23±0.2
Chloroform extract	25	14±0.3	15±0.4	-	-
	50	15±0.2	16±0.4	10±0.3	-
	75	16±0.4	17±0.2	10±0.6	-
	100	17±0.3	18±0.3	11±0.8	12±0.4
	Control	19±0.5	20±0.4	24±0.3	23±0.2
Methanol extract	25	14±0.3	15±0.3	-	-
	50	15±0.2	16±0.2	11±0.4	-
	75	16±0.4	17±0.1	13±0.2	13±0.3
	100	17±0.2	18±0.1	14±0.4	15±0.2
	Control	19±0.3	20±0.2	23±0.1	25±0.2

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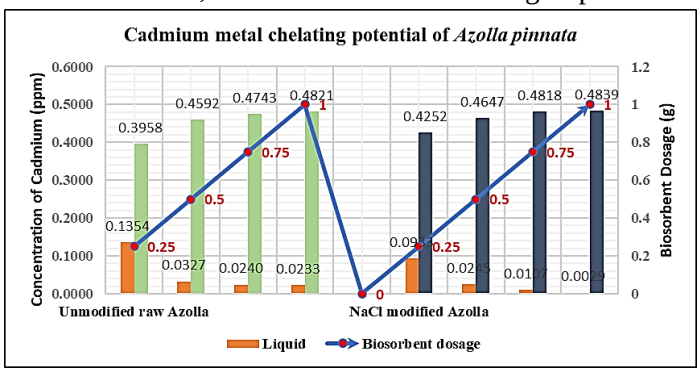
2014). However, a substantial hydrogen donating capacity in all the tested extracts of *Azolla pinnata* was observed, which could serve as primary antioxidants for better scavenging of free radicals (Table 6). These results observed the positive correlation between extracts' antioxidant potential and phenols and flavonoids' contents.

The crude extracts of *Azolla pinnata* biomass obtained using Soxhlet extraction method are used in this study. The present investigation is the foremost report on the bioactivity of acetone, aqueous, chloroform and methanolic extracts of *Azolla pinnata* (25-100 µg/ml) against clinically significant bacterial and fungal strains. The highest bioactivity was observed in the methanolic and chloroform extracts of *A. pinnata* against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* bacteria, whereas the standard, chloramphenicol reached 19 and 20mm respectively. *S. aureus* growth was hindered by the organic extract of *A. filiculoides* (Lumpkin and Plucknett, 1980), validating the present data for their use in traditional medicine. The zone of growth inhibition increased with an increase in the concentration (100 µg/ml) of methanolic extract and showed up to 17mm and 18mm against *E. Coli* ATCC 10536 and *S. aureus* ATCC 25923 respectively. Similar inhibition was found in chloroform extract of *A. pinnata* against both bacterial strains (Table 7). Acetone extract of *Azolla* (25-100µg/ml) showed the maximum of 11-14mm zone of inhibition against *E. coli*. The aqueous extract showed no bioactivity against growth of *E. coli* and *S. aureus*, which is in close agreement with the previous study (Pereira et al., 2015).

The antifungal activity of *A. pinnata* extracts was assessed against *Aspergillus flavus* ATCC *10231* and *Candida tropicalis* ATCC 9643 and fluconazole showed growth inhibition ranging 23±0.3 and 25±0.2 for *A. flavus* ATCC *10231* and *C. tropicalis* ATCC 9643 was used as a standard antifungal agent. The aqueous extract did not show bioactivity against any tested fungal strains. Whereas acetone, chloroform and methanolic extracts of *A. pinnata* did not exhibit inhibition against both *A. flavus* and *C. tropicalis* at the concentration of 25 µg/ml. However, acetone and methanolic extracts (100 µg/ml) showed minimum bioactivity between 13 and 15mm against both the fungal strains (Table 7). As per the review of the literatures, there are no reports witnessing the antifungal activity for *A. pinnata* extracts. Despite demonstrating ethnopharmacologically relevant bioactivities, the present research did not purify or identify the *Azolla*'s metabolites responsible for inhibiting Gram-positive bacteria and fungi. So, further exploration is needed to analyse *Azolla* metabolites.

Phytoremediation potential of *Azolla pinnata*

The present research assesses the potential of aquatic macrophytes, *Azolla pinnata*, in the remediation of water contaminated by heavy metals like cadmium ($1/100^{th}$ dilution of 500ppm). The adsorption study results showed that cadmium accumulation on biomass's surface increases with the increase in biosorbent dosage from 0.25 to 1.0g (Graph 1). The unmodified raw *Azolla* showed the maximum adsorption of about 0.47±0.001 and 0.48±0.003 ppm in 60mins using the biomass dosage of 0.75 and 1.0g respectively. The exposure study using the chemically (NaCl) modified *Azolla* exhibited the maximum adsorption of 0.42±0.001 with 0.25g of biosorbent, which gradually increases as the dosage increased. The adsorption of cadmium onto the surface of modified *Azolla* was observed to be 98-100% with 0.46±002<0.48±003<0.48±005 ppm using biomass dosage of 0.50<0.75<1.0g, respectively. The results showed an increase in metal accumulation onto the biomass, which might be due to the increase in porosity, surface structure, and addition of functional groups.



Graph 1. Cadmium metal chelating potential of raw (plotted in green) and NaCl modified (plotted in dark blue) *Azolla pinnata* biomass in 60 mins of exposure period.

Instrumental characterization for adsorptive properties

SEM-EDX analysis

The SEM images for *Azolla pinnata* biomass showed the changes in the apparent surface of the adsorbent before and after cadmium ion adsorption. Modifications in porosity, pore size, morphological structural change and enhancement of surface structure were noted. The SEM micrograph of *A. pinnata* before cadmium adsorption (Figure 2). The characterization showed the changes in the diverse structure with wide surface porosity, enhancing heavy metal adsorption. In addition, the rough surface area with wide pores was found to be distributed on the surface, offering potential binding of heavy metals. The average porosity size observed on the

DOI: https://doi.org/10.52756/ijerr.2023.v36.001

raw biomass surface was up to 263.39 nm, whereas the chemically modified biomass surface showed a two-fold increase in the porous size of about 675.71nm (magnified under 20kv, x10000). The micrographs of both modified and unmodified *Azolla* are represented). Metal adsorbed unmodified *Azolla* biomass (AM) was magnified under 5kv with 5000x to study the surface and porous structure after cadmium accumulation. The transition of heterogeneous structures to smooth surfaces with no visible pores on the particle was observed after the exposure of biomass to cadmium solution which can be due to accumulation of cadmium (Figure 2). This analysis also showed the major alterations on the biomass surface after the NaCl treatment by enhancing the adsorptive properties such as pore size and surface structure, facilitating higher amounts of cadmium metal uptake.

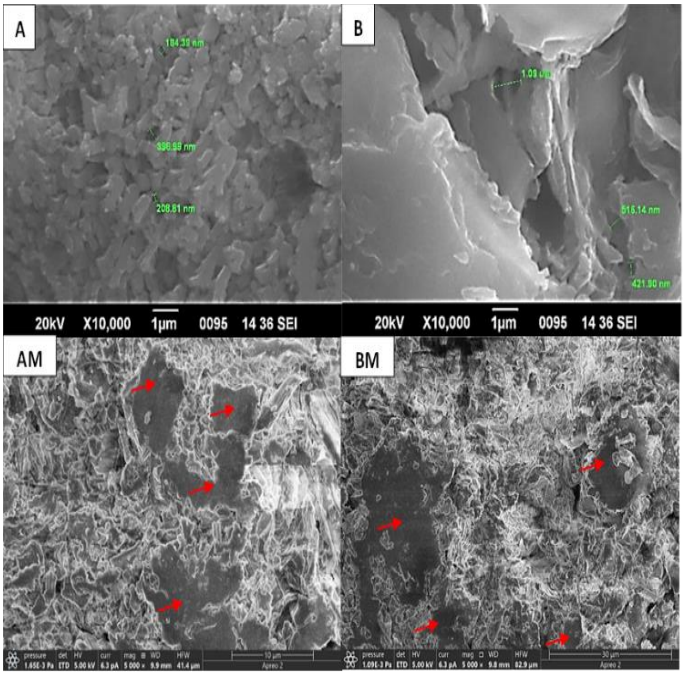


Figure 2. SEM images of raw (A) and NaCl modified (B) *Azolla pinnata* biomass before (marked porous size in green numericals). Metal exposed raw (AM) and NaCl modified (BM) *Azolla pinnata* after (showing non-porous structure in red arrow) cadmium biosorption.

Energy Dispersive X-ray Spectroscopy (EDX) is an analytical capability coupled with SEM that provides elemental composition of individual points (on areas as small as nanometres in diameter). Lateral distribution of elements from the imaged area before and after cadmium adsorption was assessed. The data for raw biomass showed the presence of C- 46.31, O-31.94, Na-2.24, Mg-0.91, Al-1.58, Si-3.89, Cl-4.54, P-0.94, S-0.73, K-1.78, Ca-1.83, Fe-2.54 and Cu-0.77 percentage of weight which agrees with XRF analysis of *A. filiculoids* (Balarak et al., 2020).

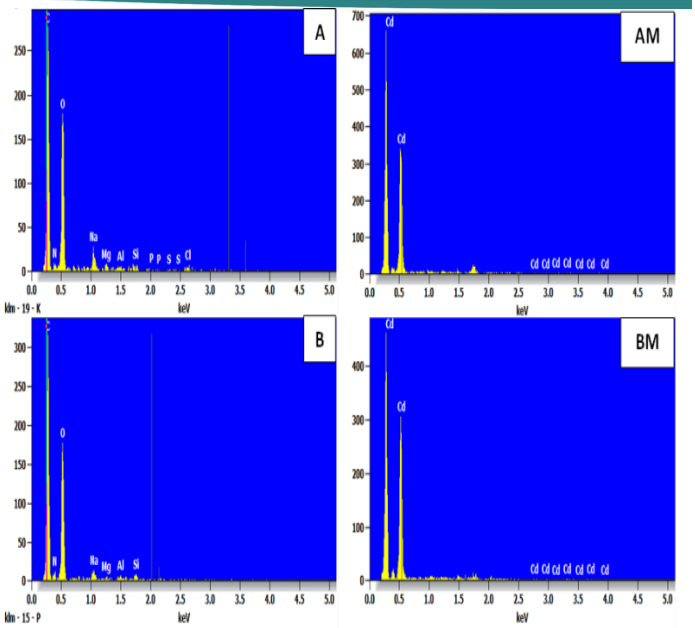


Figure 3. EDAX analysis showing raw unmodified (A), NaCl modified (B), metal adsorbed raw unmodified (AM) and metal adsorbed NaCl modified (BM) *Azolla pinnata* biomass.

respectively. These results may be responsible for the increase of C=O and other functional groups that facilitate the binding of cadmium onto the bio adsorbent surface. The presence of cadmium was observed in both modified and unmodified Azolla biomass after exposure to cadmium solution (Figure 3).

FTIR analysis

In FTIR, the shift in the bands and the changes in signal intensity in the adsorbent allowed the identification of the functional groups involved in adsorption. The FTIR spectrum of the untreated *Azolla pinnata*, the presence of various functional groups at wavelengths 3293 cm-1 (O–H stretch), 2918 cm-1, and 2855 cm-1 (C–H stretch), 1627 cm-1 (C=O bending) and 1029 cm-1 (out-of-plane bending of carbonate) was noted. The spectrum of metal-treated *Azolla pinnata* displayed bands at 3280 cm-1 (O–H stretch), 2918 cm-1 and 2858 cm-1 (C–H stretch), 1630 cm-1 (C=O bending) and 1031 cm-1 (out-of-plane bending of carbonate), where the shift of the bands indicate that these functional groups could interact with the cadmium metal ions. The three bands that are present only on treated *A. pinnata* are 1527 cm-1 (–COO- anti-symmetric stretching), 1417 cm-1 (–COO- symmetric stretching), 1226 cm-1 (C–N stretching), and 443 cm-1(C–H bending) confirmed the loading of cadmium metal ions onto the adsorbent (Figure 4). This data also suggested that functional groups of –COO- may be responsible for the interaction between the adsorbent and the metal.

DOI: https://doi.org/10.52756/ijerr.2023.v36.001

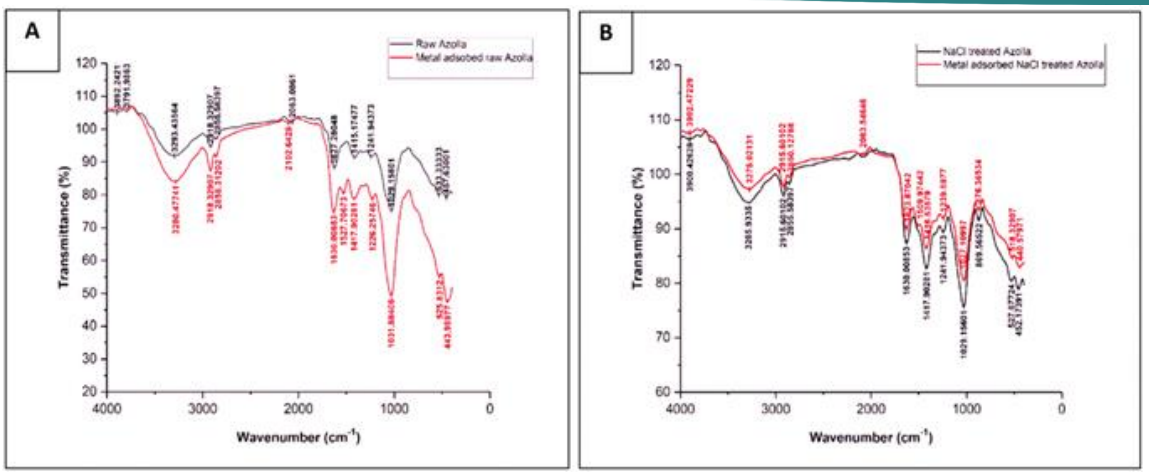


Figure 4. The FTIR spectra showing raw (A) and NaCl modified (B) Azolla pinnata biomass before (black graph) and after (red graph) cadmium biosorption.

The changes in the band intensity due to chemical treatment were observed in NaCl modified *Azolla* biomass. The presence of bands at 3285 cm-1, 2915 cm-1, 452 cm-1 marked the band shift pattern compared to unmodified raw biomass. The band at 1417 cm-1 was found to be more intense after chemical modifications, and a new band at 869 cm-1 (C-H bending) was also recorded, confirming the addition of functional groups on the sorbent surface. The spectrum of cadmium-exposed modified biomass showed the shift of all the major bands of NaCl modified *Azolla*, including 3275 cm-1 (O-H stretching), 1623 cm-1(C=C stretching), 876 cm-1 (C-H bending) and 440 cm-1 (out of plane bending) which can enhance the effectiveness of cadmium adsorption (Figure 4). The minor changes in the FTIR spectra before and after cadmium adsorption are due to the binding interactions between the metal ions and the sorbent material (Balarak et al., 2020).

XRD analysis

XRD studies show the changes in the crystallinity of the *Azolla pinnata* adsorbent. Well-defined peaks show the crystalline nature and the hallow peak shows the non-crystalline amorphous nature of the plant. The XRD pattern of raw *Azolla* biomass (Figure 5.) revealed the presence of sharp peaks majorly at 2θ=29.6º, 2θ=31.7º, 2θ=44.4º, 2θ=45.5º, 2θ=56.5º, 2θ=75.2º that can be related to the presence of complex minerals in the structure of the fern biomass. Noticeably the peak 2θ=29.6º in the *Azolla pinnata* after adsorption of metal ions attained a high intensity and all other peaks mentioned are not noticed in cadmium-exposed unmodified *Azolla*. The NaCl-modified biomass results showed a high high-intensity peak only at 2θ=29.5, while the remaining peaks were found unaltered even after cadmium adsorption (Figure 5). These findings can be attributed due to biosorption of cadmium ions onto active sites of *Azolla pinnata* biomass. The instrumental characterization of biomass proved the presence of heavy metal chelating properties in *Azolla pinnata*.

In summary, the overall findings indicated the use of chemical-treated *Azolla pinnata* biomass as an adsorbent for cadmium remediation from artificial wastewater which involves a trade-off between enhanced adsorption efficiency, long-term stability, and potential environmental and economic costs. Here, the cadmium adsorbed onto modified and unmodified *Azolla* biomass are subjected to di-acid digestion using HNO_3 and HCl (3:1) to free up the metal ions into the water which

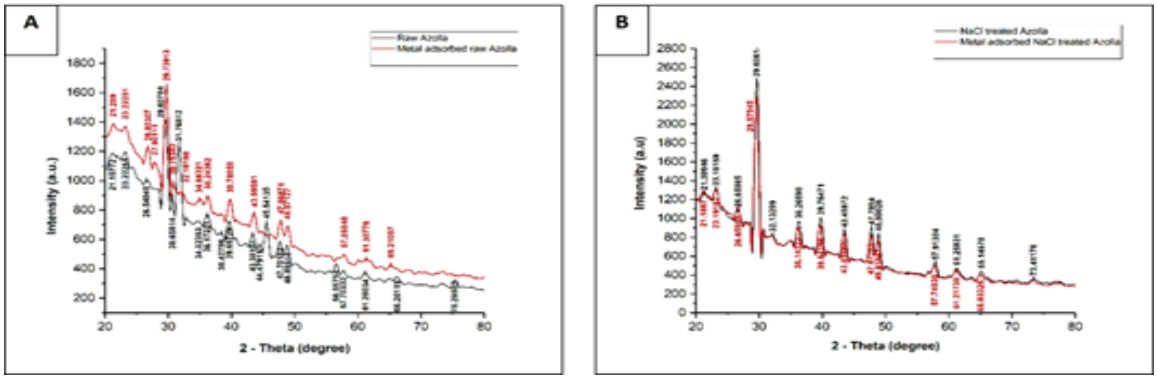


Figure 5. The XRD patterns of raw (A) and NaCl modified (B) Azolla pinnata biomass before (black graph) and after (red graph) cadmium biosorption.

facilitates to measure the metal ion concentration. Since, the present research limits with individual metal, cadmium, the NaCl modified-adsorbent can enhance the adsorption, they may not be equally effective for all contaminants. So, the present research continues to discover the more effective chemical methods to introduce physical modifications and metal desorption techniques to alleviate environmental and human risks. Therefore, careful consideration of the potential positive and negative impacts of specific treatment methods is essential in making informed decisions about the use of these technologies.

Conclusion

The application of novel technologies for the attainment of a sustainable environment gained attention in recent research trends. Phytoremediation approach in the removal of heavy metal from the waste water and adsorption of metal ions onto the active sites of biosorbent surface has become a promising approach for biomonitoring of ecosystem. The current challenging study findings however employed the assistance of chemical modifications to increase the efficiency of phytoremediation, which witnessed the cent percent removal of cadmium metal from polluted water in minimal biosorbent dosage and exposure time. The present study raised a challenge to fill the research gaps and to overcome problems of remediation such as more exposure time, suitability only for low polluted areas, entry of pollutants to adjacent media, dependency on seasonal conditions, etc. The study also provides a basement for future researchers in using ferns for metal remediation. Therefore, future studies using aquatic macrophytes on various heavy metals uptake and designing suitable chemical treatments for the enhancement of chelating properties must be focused.

Acknowledgement

The laboratory facilities and support for this study was provided by CHRIST (Deemed to be University), which the authors gratefully acknowledge and would like to extend their sincere gratitude to Centre for research for providing necessary facilities (MRP DSC - 1936).

Conflict of Interest

The authors declare no conflict of interest.

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How to cite this Article:

Kaakarlu Shivakumar Vinanthi Rajalakshmi and Kuppusamy Alagesan Paari (2023). A comprehensive study on the assessment of chemically modified Azolla pinnata as a potential cadmium sequestering agent. *International Journal of Experimental Research and Review*, *36*, 01-19.

DOI : https://doi.org/10.52756/ijerr.2023.v36.001