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Effect of Heavy Metal Phytoremediation on Phytochemical Fingerprint and Bioactivity of *Pistia stratiotes*: A Quest for Re-routing Disposal to Commercial Application

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ABSTRACT

Phytoremediation is one of the non-energy consuming processes of remediating polluted water. However, the disposal of post-remediated plants poses a threat of the re-introduction of pollutants back into the ecosystem. Re-routing remediated pollutants for commercial application could be one way to reduce the re-introduction of pollutants in an ecosystem. Heavy metal pollution in water bodies is one issue, which can be mitigated to an extent with phytoremediation. In the current study, the effect of heavy metal phytoremediation on the phytochemical fingerprint and bioactivity of *Pistia stratiotes* L. was investigated. *Pistia stratiotes* L. was subjected to different concentrations of iron (Fe) and lead (Pb), in the range of 5-20 ppm. Different parameters such as heavy metal estimation (in plants and water post-treatment), thin layer chromatography (TLC), antioxidant activity, and antiurolithic activity were measured. Post remediation, heavy metal concentration was found to be comparatively higher in roots (16.515 ± 0.008 $mg.g^{-1}$ and 5.25 ± 0.086 $mg.g^{-1}$ when treated with 15 ppm iron and lead respectively). TLC revealed differences between the fingerprints of treated and untreated plants. Some bands increased in intensity as the concentration of heavy metal increased, while some bands which were present in untreated, were absent in treated plant samples. Antioxidant activity of treated plants shows lesser IC_{50} values, compared to untreated, in that, treated leaves show better activity (IC_{50} = 1.8 ± 0.5220 $mg.mL^{-1}$ of leaf treated with 2 ppm iron as opposed to IC_{50} > 5 $mg.mL^{-1}$ of untreated leaf extract). The treated plants revealed good antiurolithic activity compared to untreated, in that, the percentage inhibition showed by Iron treated leaves and roots was better (96.87% and 98.95% exhibited by iron-10 ppm treated leaves and roots respectively), while the untreated showed a maximum of only 68.75% inhibition. The results suggest that the bioactivity of the plant extracts increases post-remediation. Potential applications of these extracts can be explored such as nanoparticle synthesis, drug discovery, etc.

INTRODUCTION

The introduction of heavy metals like lead, iron, mercury, and arsenic to water bodies due to human and natural sources has numerous ill effects because of their inability to degrade and remain until treatment. Lead (Pb) and iron (Fe) are one of the major heavy metal polluters. Many researchers have proposed various chemical methods for the extraction of heavy metals from the ecosystem. Some of these methods are reverse osmosis, electrodialysis, ion exchange, chemical precipitation, and ultrafiltration (Singh et al. 2012). These methods proved to be effective but cost-bearing and produced many non-biodegradable by-products. Green remediation technology uses plants to extract pollutants or transform them into non-toxic forms. *Eichhornia crassipes* L. (Malik et al. 2020), *Lemna minor* L. (Materac & Sobiecka 2017), *Phragmites australis* (Milke et al. 2020), and *Pistia stratiotes* L. (Tripathi et al. 2010) have been majorly used in phytoremediation of wastewater. The heavy metal is effectively extracted from the wastewater and concentrated in the plant; however, the main concern is the disposal of the plants (Farraji et al. 2016). When these plants are disposed of, the extracted heavy metal potentially re-enters the earth.

To break this cycle, we propose the utilization of the whole plant for potential commercial use. Plants under biotic and abiotic stress elicit secondary metabolites for combating the same. We hypothesized that because heavy metals elicit the production of secondary metabolites (Lajayer et al. 2017), the treated *Pistia stratiotes* L. plants can be expected to show a change in their phytochemical fingerprint and bioactive properties. *Pistia stratiotes* L. has already been studied for its wound healing, antifungal, anti-dermatophytic, and antimicrobial properties (Tripathi et al. 2010), so any

enhancement in the production of secondary metabolites or the aforementioned properties can be considered for a commercial application. Although the ill effects of heavy metals on the human body are not fully ruled out, the way they can be safely introduced can be investigated.

Lead and iron have been used in the current study, as heavy metal contaminants. As lead is not known to have any physiological functions in the human body, its concentration in the blood should be very low. The natural sources of lead include volcanic eruptions and forest fires and the artificial sources are batteries, toys, cosmetics, vehicles using leaded petrol or gasoline, lead smelters, burning of coal, and ammunition used in hunting (Iqbal 2012, Zhang et al. 2015). Pratush et al. (2018) mentioned that the lead added to gasoline in vehicles forms chloride, bromide, and oxide salts which exit through the exhaust pipe. The larger particles settle down and enter the soil or groundwater reserves whereas the smaller particles remain suspended in the air. As lead cannot be degraded by natural methods or microbial activity, it remains in the ecosystem for a long time (Zhang et al. 2015). Lead poisoning can cause cardiovascular diseases (Iqbal 2012), dysfunction of the endocrine and reproductive systems, infertility in men, and miscarriages in women (Ara et al. 2015). It can also affect neurological well-being, renal and gastrointestinal health, developmental and immunological issues (Zhang et al. 2015).

On the other hand, iron has many physiological functions in the human body like oxygen transport, DNA synthesis, transport of electrons, etc. Hence, iron is effectively absorbed in the small intestine and is stored in the form of ferritin and hemosiderin in the bone marrow, spleen, and liver (Abbaspour et al. 2014). The presence of iron contamination in water bodies can result from several natural sources including the action of microorganisms and the breaking down of minerals with high iron content. Iron is additionally introduced to water bodies by sewage discharge, corroded water pipes, and other human activities related to the metal industry (Sarkar et al. 2018). Excessive amounts of iron can harm human health by increasing the risk of conditions including diabetes, hepatic cancer, heart ailments, and other illnesses (Kumar et al. 2017). At higher concentrations, iron facilitates the synthesis of reactive oxygen species (ROS) which can damage cellular constituents (Wessling-Resnick 2017).

The presence of heavy metals in water bodies affects marine life adversely. A study conducted in the Mithi River in Mumbai (Kakde & Nagarsekar 2014) discusses the presence of elevated levels of many heavy metals, including lead and iron, and their adverse effects. Lead accumulates in the tissues of fish and causes degenerative diseases and changes in the circulatory and nervous systems (Afshan et al. 2014). In addition, lead pellets used for hunting swans, ducks, and waterfowl are conveyed through the food chain to the higher tropic levels and put bigger carnivores at risk as well (Zhang et al. 2015).

The current paper discusses the effect of increasing concentrations of lead and iron on the phytochemical makeup, antioxidant activity, and anti-urolithic activity of the plants for any potential change. Based on the results, further applications could be proposed for the whole plant utilization and diverting the re-introduction of heavy metals into the earth.

MATERIALS AND METHODS

Phytoremediation Studies

Sample collection, authentication and pre-treatment: *Pistia stratiotes* L., was collected from a local nursery and authenticated at the Blatter Herbarium in St. Xavier's College. The plants were then divided into equal numbers (10 plants each) and treated with increasing concentrations of lead and iron separately (2 ppm, 5 ppm, 10 ppm, 15 ppm and 20 ppm) in simulated wastewater for 10 days. One batch of plants was not treated with the heavy metals and is referred to as "untreated" in this paper. The leaves and roots of the plants were separated and air-dried for a few days before drying them in the oven at 50°C.

Lead (Pb) and iron (Fe) estimation in *Pistia stratiotes* L. and water by Atomic Absorption Spectroscopy (AAS): An amount of 0.1 g of the dried plant parts was taken in a 250 mL conical flask and to these flasks, 10 mL of concentrated nitric acid (69-72%) and 5 mL of perchloric acid (70%) was added. The flasks were then kept on a hot plate until complete acid digestion. The liquid obtained was filtered using Whatman filter paper and diluted to 100 mL with distilled water. The digested plant samples were analyzed for heavy metals by direct air acetylene flame method by using the lead and iron lamps in Atomic absorption spectroscopy (Thermo Scientific). The standards, water, and plant samples (treated & untreated) were measured at wavelengths 217 nm for lead and 246.3 nm for iron.

The bioconcentration factor is the ratio of a chemical's concentration in a living substance to the concentration of that chemical in the surrounding environment, in this case, water (Manahan 2009). It was calculated as follows:

BCF = Metal concentration in roots/leaves ÷ 2

BCF >1 indicates the concentration of metal in the plant is greater than its surroundings. The translocation factor (TF), also known as the shoot-root quotient, describes a plant's

ability to transport metal from its roots to its shoots and leaves, which is principally responsible for phyto-extraction (Nirola et al. 2015).

The translocation factor was also calculated using the formula:

TF = Heavy metal concentration in leaf ÷ Heavy metal concentration in roots

Phytochemical and Bioactivity Studies

Preparation of plant extracts for TLC and bioactivity studies: The dried plant material was ground and macerated to form a coarse powder. Methanol and ethanol were added to the dried powder in a ratio of 7:3. After 24 hours, the extract was filtered using Whatman filter paper. The extracts were stored in microfuge tubes in the fridge at 4°C.

Phytochemical fingerprinting by thin layer chromatography: Using the CAMAG Linomat 5 semi-automatic applicator, 5 microlitres of the plant extracts (Untreated & Treated with 2, 5, 10, 15, 20 ppm Pb and Fe respectively) were applied to a TLC plate of size 10 × 10 cm. The plate was dried and developed in a 10 × 10 twin trough chamber which was saturated with the 20ml mobile phase (toluene: chloroform: ethanol in the proportion 4:4:1) for 10 minutes. The mobile phase was run until 70 mm, after which the plate was dried using a dryer. The plates were first visualized using a TLC visualizer under 256 nm and 366 nm. It was then scanned with the CAMAG TLC scanner at 254 nm, 366 nm, and 540 nm. Further, the plates were derivatized with anisaldehyde reagent and visualized, and scanned again at 366 nm and 540 nm for better visualization. The software used for the entire process was VisionCATS.

Determination of antioxidant activity by DPPH assay: The antioxidant activity of treated and untreated leaf and root extracts of *Pistia stratiotes* L. was assessed using the microtiter plate method with 1,1-diphenyl-2-picrylhydrazyl (DPPH). A 0.1 mM DPPH reagent was prepared by dissolving 3.94 mg of DPPH powder in 100 mL of methanol. 100 µL plant methanolic extracts were added to the ELISA plate, followed by 100 µL of DPPH reagent in each well. Ascorbic acid was used as the standard. After an incubation period of 30 minutes, the absorbance was measured using an ELISA plate reader (Erba Mannheim). The percentage inhibition of each sample was calculated by subtracting the absorbance of the sample from the absorbance of the blank and then dividing it by the absorbance of the blank into 100.

Assessment of antiurolithic activity by methanolic extracts of *Pistia stratiotes* L.: The antiurolithic activity of the plant *Pisita stratiotes* was investigated using a synthetic urine assay on plants treated with varying doses of lead and iron, respectively. According to Atmani et al. (2000), a synthetic urine assay was used to evaluate the percentage inhibition and development of calcium oxalate monohydrate crystals at varied doses of plant extract.

In the laboratory, artificial urine was created by combining two solutions of the following composition: $Na_2C_2O_4$ (2 mmol.L^{-1}) and $CaCl_2.2H_2O$ (10 mmol.L^{-1}) (Beghalia et al. 2008). With the addition of NaCl, two solutions were created. To count the number of crystals, a drop of the solution was placed on a hemocytometer slide. To determine the percentage of inhibition, an equal volume of the above-mentioned solution and plant extract were mixed and incubated for 30 minutes. Following incubation, the number of crystals was counted using a hemocytometer slide under a 10X objective lens of a light microscope.

Percentage inhibition = (TSI-TAI) ÷ TSI × 100

TSI = number of Ca-oxalate monohydrate crystals before inhibitor (plant extract)

TAI = number of Ca-oxalate monohydrate crystals after adding inhibitor

RESULTS AND DISCUSSION

Heavy Metal Estimation in *Pistia stratiotes* L. and Water by Atomic Absorption Spectroscopy

From Table 1, it was observed that the treated roots showed a significant increase in iron concentration as compared to the leaves. The lowest concentration of iron in roots was seen at 10 ppm, at which the concentration significantly increased in leaves, which might suggest that at 10 ppm, iron might be translocated efficiently to the leaves from the roots. The concentration of iron in the water samples also significantly decreased with the increase in concentration, thereby indicating that the metal has been taken up by the plant. In this case, the Bioconcentration factor was found to be the highest at 2 ppm. The ratio decreased as the iron concentration increased, which may be because the plant was reaching a saturation level.

From the results obtained in Table 2, we can see that the roots showed a higher accumulation of lead as compared to the leaves. There was also a decrease in the concentration of lead from 15 ppm to 20 ppm in the roots, which could mean that 15 ppm is the saturation point for the uptake of the metal in the plant.

Considering the levels of lead in the water after treatment is also less, there is a possibility of biotransformation of lead within the roots, at 20 ppm. Furthermore, the concentration of lead in leaf

Table 1: Iron concentration in treated and untreated *Pistia stratiotes* L. and water samples. The values for treated samples reported are post-phytoremediation and their corresponding bioconcentration and translocation factors.

Samples	Roots ($mg.g^{-1}$)		Leaves ($mg.g^{-1}$)		Iron concentration of Water ($mg.g^{-1}$)*	Translocation factor (Iron treated)
	Iron concentration	Bioconcentration factor	Iron concentration	Bioconcentration factor		
Untreated	6.315 ± 0.087	–	0.09	–	0.000975	0.15
Treated 2 ppm	16.095 ± 0.017	8.0475	1.26	0.63	0.00048	0.078
Treated 5 ppm	15.315 ± 0.008	3.063	1.23 ± 0.008	0.246	0.00387	0.080
Treated 10 ppm	9.33	0.933	3.135 ± 0.008	0.3135	0.005625	0.336
Treated 15 ppm	16.515 ± 0.008	1.101	1.8 ± 0.008	0.12	0.00054	0.108
Treated 20 ppm	16.38 ± 0.008	0.819	2.085 ± 0.017	0.10425	0.00033	0.127

*Water analysis post-treatment

Table 2: Lead concentration in treated and untreated *Pistia stratiotes* L. and water samples. The values for treated samples reported are post-phytoremediation and their corresponding bioconcentration and translocation factors.

Samples	Roots ($mg.g^{-1}$)		Leaves ($mg.g^{-1}$)		Lead concentration of Water ($mg.g^{-1}$)*	Translocation factor (Lead treated)
	Lead concentration	Bioconcentration factor	Lead concentration	Bioconcentration factor		
Untreated	0	—	0.15 ± 0.086	—	0.00003	0.15
Treated 2 ppm	0.9 ± 0.086	0.45	0.15 ± 0.086	0.075	0.00015	0.166
Treated 5 ppm	1.8 ± 0.086	0.9	0.3	0.15	0.002745	0.166
Treated 10 ppm	3.6 ± 0.086	1.8	0.3 ± 0.086	0.15	0.000555	0.083
Treated 15 ppm	5.25 ± 0.086	2.625	0.6	0.3	0.00432	0.114
Treated 20 ppm	1.2	0.6	1.05 ± 0.086	0.525	0.000015	0.875

*Water analysis post-treatment

20 ppm is significantly higher as compared to the other leaves, which also indicates that at 15 ppm lead treatment, the metal could be translocated from roots to leaves.

As can be observed in Tables 1 and 2, the Translocation factor of iron-treated plants increases from 5 ppm to 10 ppm and then decreases from 10 ppm to 15 ppm. The metal is assumed to be translocated efficiently at 10 ppm treatment, following which the plant could be saturated at higher concentrations. However, in the case of lead-treated plants, there is an increase in the translocation factor as the concentration of lead increases in the plant. This is an indication that the metal is translocated from roots to leaves at higher lead concentrations, efficiently. Therefore, *Pistia stratiotes* L.could be an efficient accumulator of lead at higher concentrations.

Heavy metal analysis of iron in *P. stratiotes* L. has been previously studied and it shows a great increase in metal concentration in the treated parts of the plants and the highest concentration was always contained in the roots. The initial concentration was seen as 8.682 $mg.g^{-1}$ and the final was seen as 12.226 $mg.g^{-1}$ in 2 $mg.L^{-1}$ concentration of the Iron (Mishra & Tripathi 2008)**.** Another study reports that the concentration of iron and lead was found to be the highest in roots as compared to the leaves and the translocation factor did not exceed one, indicating that this plant exhibits rhizofiltration (Galal et al. 2018). Studies have also shown *P. stratiotes* L. as a hyperaccumulator of lead and iron and thus can be applied for the remediation of surface waters (Lu et al. 2011). With a clearance percentage of 99.31% at 1 $mg.L^{-1}$, *P. stratiotes* L. had the highest tolerance and removal efficiency for lead. The ability of *P. stratiotes* L. to remove heavy metals, particularly lead, suggested that they could be useful in the treatment of metal-polluted water (Zahari et al. 2021). The result obtained in our study is in alignment with the literature reported.

Phytochemical Fingerprinting By Thin Layer Chromatography

Heavy metals at a high concentration in the environment act as abiotic stress agents that lead to oxidative damage in plants. Plants growing in such environments respond to the threat with various defense mechanisms. A common defense

mechanism against heavy metals is excessive production of secondary metabolites. These secondary metabolites can precipitate metal ions, act as chelating agents, and help scavenge reactive oxygen species (ROS) (Anjitha et al. 2021). Hence, this suggests that to stimulate the production of excess secondary metabolites, one could treat the plants with heavy metals.

The leaves of *P. stratiotes* L. are said to have many secondary metabolites including alkaloids, phytosterols, triterpenes, flavonoids, and terpenoids (Desai & Aparadh 2014). They also have tannins, saponins, steroids, quinones, and anthraquinones, while the roots of *P. stratiotes* L. harbor flavonoids, quinones, and anthraquinones (Tyagi 2017). When used for phytoremediation of heavy metals, these plants are under abiotic stress which is then expected to produce an enhanced rate of production of some secondary metabolites as seen in the experiments performed by Rao et al. (2021), Zhu et al. (2020), Farrokhzad & Rezaei (2020), Drzewiecka et al. (2018), Kisa et al. (2016), Abnosi et al. (2015), and Kai et al. (2012).

To compare the production of these secondary metabolites in the untreated plants and the plants treated in simulated wastewater containing heavy metal (here, iron or lead), thin-layer chromatography was used. The aim was to compare the phytochemical fingerprints of the untreated plants to the treated ones by chromatography. Visually, many changes in the phytochemical fingerprint of the plant extracts could be observed. Some bands showed an increase in intensity with the increasing concentration of heavy metal in the simulated wastewater. However, some other bands disappeared altogether in the treated samples.

Previous studies such as the one where treatment of plant cell cultures with heavy metals have proven that exposure to heavy metal as an effective way to elicit secondary

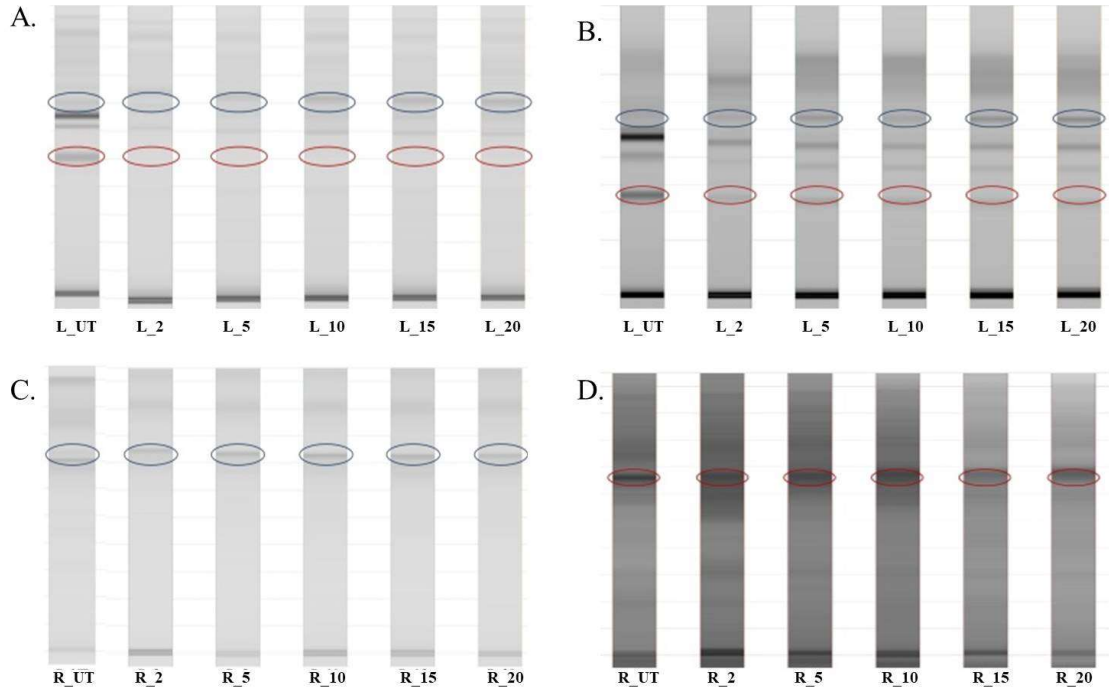

Fig. 1: Densitogram of the developed TLC plates scanned; (A) Methanolic extracts of leaves of *P. stratiotes* L. treated with iron scanned at 366 nm before derivatization with anisaldehyde reagent. The blue circles indicate the increase in the intensity of a band in the samples treated with iron as compared to the untreated. The red circles show a band disappearing after treatment with iron. (B) Leaf methanolic extracts of *P. stratiotes* L. treated with lead scanned at 366 nm before derivatization with anisaldehyde reagent. The bands encircled in blue show an absence of the compound in the untreated sample and an increase in density in the samples treated with lead. The bands encircled in red show a band present in the untreated sample which disappears in the treated samples. (C) Methanolic extracts of roots of *P. stratiotes* L. treated with iron scanned at 254 nm before derivatization with anisaldehyde reagent. The blue circles represent the band that is absent in the untreated sample and is present almost uniformly in the samples treated with iron. (D) Root methanolic extracts of *P. stratiotes* L. treated with lead scanned at 366 nm after derivatization with anisaldehyde reagent The bands encircled in red show the decreasing density of a band in the samples treated with lead as compared to the untreated sample. Key: L_UT – untreated leaves, L_2 – leaves treated at 2 ppm, L_5 – leaves treated at 5 ppm, L_10 – leaves treated at 10 ppm, L_15 – leaves treated at 15 ppm and L_20 – leaves treated at 20 ppm.; R_UT – untreated roots, R_2 – roots treated at 2 ppm, R_5 – roots treated at 5 ppm, R_10 – roots treated at 10 ppm, R_15 – roots treated at 15 ppm and R_20 – roots treated at 20 ppm.

metabolite production. Bota and Deliu noted that $CuSO_4$ can be used to stimulate the production of flavonoids in *Digitalis lanata* suspension cultures (Bota & Deliu 2011). Cultures of *Camellia sinensis* expressed an increase in the production of cinnamic acid in response to the addition of Co ions (Sutini et al. 2019). Resveratrol production can be stimulated in *Vitis vinifera* cell cultures by the presence of Co^{2+}, Ag^{+}, and Cd^{2+} (Cai et al. 2013). In Fig. 1, we can see a stark difference in the phytochemical fingerprint of untreated and treated plants of *P. stratiotes* L.. This suggests that exposure of plants to heavy metals during phytoremediation has led to a change in the production of secondary metabolites.

The Rf values obtained from the chromatogram of the untreated sample were compared statistically to that of the treated samples at a particular wavelength i.e. 254 nm, 366 nm, and 540 nm using the Kruskal-Wallis test. The null hypothesis of the test (Ho) was considered as "no significant differences between the number and pattern of compounds between untreated and each treated sample", and the alternate hypothesis as "there is a significant difference between the number and pattern of compounds between untreated and each treated sample".

It was seen from Tables 3 and 4, that at all wavelengths, the estimated p-value was greater than 0.05, which means

Table 3: Biostatistical analysis of Rf values obtained from the phytochemical fingerprint of lead-treated *Pistia stratiotes* L. methanolic extracts.

Sr. No.	Wavelength	Rf between samples	H statistic	P value	Significant difference (P<0.05)
1.	254 nm	UT_L, T_Pb_2_L, T_Pb_5_L, T_Pb_10_L, T_Pb_15_L & T_Pb_20_L	0.2738	0.9981	No
		UT_R, T_Pb_2_R, T_Pb_5_R, T_Pb_10_R, T_Pb _15_R & T_Pb_20_R	1.4270	0.9213	No
2.	366 nm	UT_L, T_Pb_2_L, T_Pb_5_L, T_Pb_10_L, T_Pb_15_L & T_Pb_20_L	0.2921	0.9978	No
		UT_R, T_Pb_2_R, T_Pb_5_R, T_Pb_10_R, T_Pb_15_R & T_Pb_20_R	2.6840	0.7485	No
3.	540 nm	UT_L, T_Pb_2_L, T_Pb_5_L, T_Pb_10_L, T_Pb_15_L & T_Pb_20_L	0.8141	0.9761	No
		UT_R, T_Pb_2_R, T_Pb_5_R, T_Pb_10_R, T_Pb_15_R & T_Pb_20_R	-	-	-
4.	366 nm Derivatized	UT_L, T_Pb_2_L, T_Pb_5_L, T_Pb_10_L, T_Pb_15_L & T_Pb_20_L	2.5032	0.7760	No
		UT_R, T_Pb_2_R, T_Pb_5_R, T_Pb_10_R, T_Pb_15_R & T_Pb_20_R	1.7225	0.8860	No
5.	540 nm Derivatized	UT_L, T_Pb_2_L, T_Pb_5_L, T_Pb_10_L, T_Pb_15_L & T_Pb_20_L	1.6709	0.8925	No
		UT_R, T_Pb_2_R, T_Pb_5_R, T_Pb_10_R, T_Pb_15_R & T_Pb_20_R	0.5769	0.9890	No

Key for leaf samples: UT_L: untreated leaves; T_Pb_2_L: leaves treated at 2 ppm Pb; T_Pb_5_L: leaves treated at 5 ppm Pb; T_Pb_10_L:leaves treated at 10 ppm Pb; T_Pb_15_L:leaves treated at 15 ppm Pb & T_Pb_20_L: leaves treated at 20 ppm Pb.

Key for root samples: UT_R: untreated roots; T_Pb_2_R: roots treated at 2 ppm Pb; T_Pb_5_R: roots treated at 5 ppm Pb; T_Pb_10_R:roots treated at 10 ppm Pb; T_Pb_15_R:roots treated at 15 ppm Pb & T_Pb_20_R: roots treated at 20 ppm Pb.

Table 4: Biostatistical analysis of Rf values obtained from the phytochemical fingerprint of iron-treated *Pistia stratiotes* L. methanolic extracts.

Sr. No.	Wavelength	Rf between samples	H statistic	P value	Significant difference (P<0.05)
1.	254 nm	UT_L, T_Fe_2_L, T_Fe_5_L, T_Fe_10_L, T_Fe_15_L & T_Fe_20_L	0.9464	0.96677	No
		UT_R, T_Fe_2_R, T_Fe_5_R, T_Fe_10_R, T_Fe_15_R & T_Fe_20_R	3.7474	0.58632	No
2.	366 nm	UT_L, T_Fe_2_L, T_Fe_5_L, T_Fe_10_L, T_Fe_15_L & T_Fe_20_L	1.6557	0.89443	No
		UT_R, T_Fe_2_R, T_Fe_5_R, T_Fe_10_R, T_Fe_15_R & T_Fe_20_R	0.7780	0.97842	No
3.	540 nm	UT_L, T_Fe_2_L, T_Fe_5_L, T_Fe_10_L, T_Fe_15_L & T_Fe_20_L	0.4867	0.99260	No
		UT_R, T_Fe_2_R, T_Fe_5_R, T_Fe_10_R, T_Fe_15_R & T_Fe_20_R	-	-	-
4.	366 nm Derivatized	UT_L, T_Fe_2_L, T_Fe_5_L, T_Fe_10_L, T_Fe_15_L & T_Fe_20_L	0.2877	0.99787	No
		UT_R, T_Fe_2_R, T_Fe_5_R, T_Fe_10_R, T_Fe_15_R & T_Fe_20_R	2.9761	0.7037	No
5.	540 nm Derivatized	UT_L, T_Fe_2_L, T_Fe_5_L, T_Fe_10_L, T_Fe_15_L & T_Fe_20_L	1.6826	0.89108	No
		UT_R, T_Fe_2_R, T_Fe_5_R, T_Fe_10_R, T_Fe_15_R & T_Fe_20_R	1.8428	0.87044	No

Key for leaf samples: UT_L: untreated leaves; T_Fe_2_L: leaves treated at 2 ppm Fe; T_Fe_5_L: leaves treated at 5 ppm Fe; T_Fe_10_L:leaves treated at 10 ppm Fe; T_Fe_15_L:leaves treated at 15 ppm Fe & T_Fe_20_L: leaves treated at 20 ppm Pb.

Key for root samples: UT_R: untreated roots; T_Fe_2_R: roots treated at 2 ppm Fe; T_Fe_5_R: roots treated at 5 ppm Fe; T_Fe_10_R:roots treated at 10 ppm Fe; T_Fe_15_R:roots treated at 15 ppm Fe & T_Fe_20_R: roots treated at 20 ppm Fe.

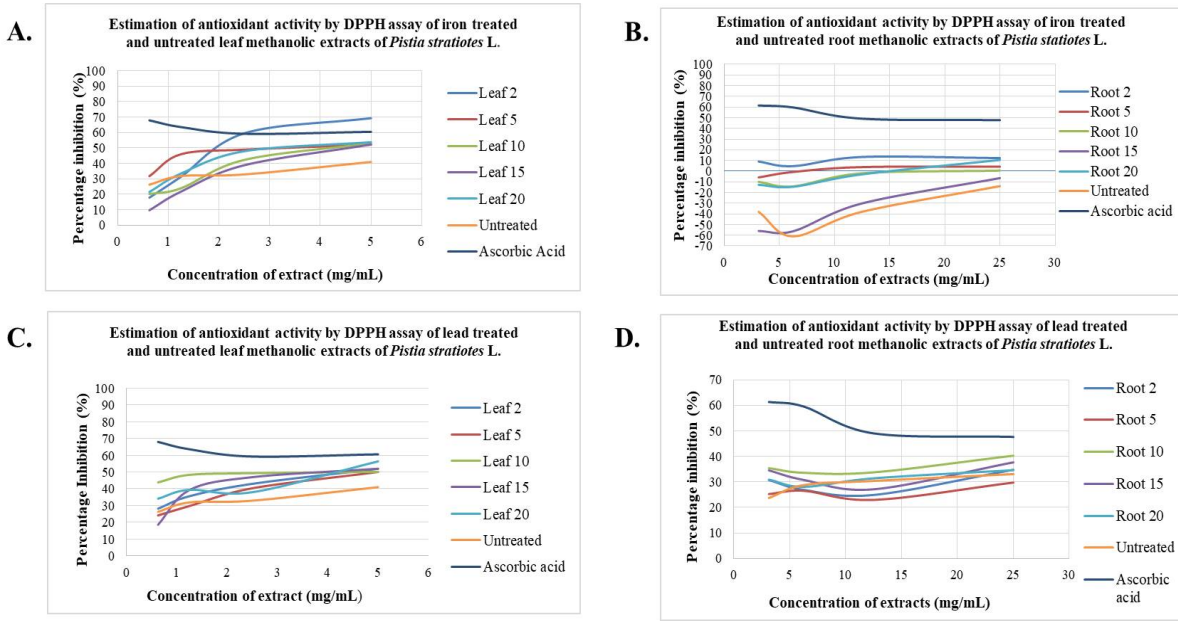

Fig. 2: Percentage inhibition Vs. Concentration of extract graph of: (A) Iron treated and untreated methanolic leaf extracts of *P. stratiotes* L. at different concentrations (0.625 to 5 mg.mL^{-1}); (B) Iron treated and untreated methanolic root extracts of *P.stratiotes* L. at different concentrations (3.125 to 25 mg.mL^{-1}); (C) Lead treated and untreated methanolic leaf extracts of *P. stratiotes* L. at different concentrations (0.625-5 mg.mL^{-1}); (D) Lead treated and untreated methanolic root extracts of *P. stratiotes* L. at different concentrations (3.125 to 25 mg.mL^{-1}). Key: Untreated– untreated leaves, leaf 2 – leaves treated at 2 ppm, leaf 5 – leaves treated at 5 ppm, leaf 10– leaves treated at 10 ppm, leaf 15– leaves treated at 15 ppm and leaf 20 – leaves treated at 20 ppm; Untreated– untreated roots, root 2 – roots treated at 2 ppm, root 5 – roots treated at 5 ppm, root 10– roots treated at 10 ppm, root 15 – roots treated at 15 ppm and root 20 – roots treated at 20 ppm.

that the difference in the band patterns of the treated and untreated samples was not statistically significant.

Determination of Antioxidant Activity of *Pistia stratiotes* L. Heavy Metal Treated and Untreated Samples by DPPH Assay

Antioxidant compounds such as phenolics, flavonoids, and certain enzymes play an important role in protection against cellular damage caused by Reactive Oxygen Species (ROS). Previous studies have shown the presence of antioxidant activity in methanolic extracts of leaves and roots of *Pistia stratiotes* L. (Tyagi & Parashar 2017). A comparative study was done to determine the difference in the antioxidant activity of the plant before and after treatment with heavy metals using the DPPH assay. The results were then plotted on a graph and the IC_{50} value was calculated. Leaf methanolic extracts in the range of 0.625-5 mg.mL^{-1} were used while root methanolic extracts in the range of 3.125 to 25 mg.mL^{-1} were used, since at lower concentrations, IC_{50} values could not be detected in roots.

Table 5: IC_{50} values of lead and iron treated and untreated leaf and root extracts antioxidant activity.

Samples (Treatment concentration)	IC_{50} values (mg.mL^{-1}) for Lead treated plants		IC_{50} values (mg.mL^{-1}) for Iron treated plants	
	Leaf methanolic extracts	Root methanolic extracts	Leaf methanolic extracts	Root methanolic extracts
Untreated	> 5	> 25	>5	>25
2 ppm	4.4 ± 0.0283	>25	1.8 ± 0.5220	>25
5 ppm	4.95 ± 0.1768	>25	3.4 ± 0.3535	>25
10 ppm	4.55 ± 0.4243	>25	4.2 ± 0.0707	>25
15 ppm	3.8 ± 0.2121	>25	4.55 ± 0.1414	>25
20 ppm	4.15 ± 0.5657	>25	3.05 ± 0.03535	>25

Table 6: Study of anti-urolithic activity in Lead and Iron treated and untreated root and leaf samples.

Concentration of extract ($\mu g.mL^{-1}$)	Lead treated samples		Iron treated samples	
	Leaves (Percent inhibition)	Roots (Percent inhibition)	Leaves (Percent inhibition)	Roots (Percent inhibition)
Untreated	16.66	68.75	16.66	68.75
2 ppm	76.04	78.12	91.79	63.54
5 ppm	86.98	79.16	94.87	65.12
10 ppm	92.7	89.58	96.87	98.95
15 ppm	94.79	84.37	92.3	92.3
20 ppm	33.33	86.97	87.17	89.58

From Fig. 2, it could be observed that the treated leaf extracts showed increased antioxidant activity as compared to the untreated extract. At a concentration of 5 $mg.mL^{-1}$, the untreated leaf extract showed the lowest percentage inhibition (40.98%), whereas a higher percentage inhibition was seen in leaf extract of the plants treated with lead and iron (Fig. 2A and 2C). In iron-treated leaf extracts (Fig. 2A), the highest percentage of inhibition was seen in plants treated with 2 ppm at an extract concentration of 5 $mg.mL^{-1}$ (69.34%). In lead-treated leaf extracts (Fig. 2C), the highest percentage of inhibition was seen in plants treated with 20 ppm at an extract concentration of 5 $mg.mL^{-1}$ (56.33%). However, IC_{50} values of root extracts of the untreated and treated plants (both iron and lead), were not achieved. The same has been reported in Table 5.

The presence of heavy metals is known to induce oxidative stress in plants which may lead to the synthesis of several secondary metabolites (Anjitha et al. 2021). To defend themselves from free radicals, plants may increase the production of bioactive antioxidant compounds (Fryzova et al. 2018). Studies on *Macrotyloma uniflorum* and *Cicer arietinum* L. showed an increase in activity of antioxidative enzymes like superoxidase, dismutase, and catalase on exposure to lead stress (Reddy et al. 2005).

Assessment of Antiurolithic Activity by Methanolic Extracts of *Pistia stratiotes* L.

The Antiurolithic activity was checked using artificial urine and from the results obtained it was found that the Treated parts of the samples showed a higher percentage of inhibition as compared to the untreated samples as seen in Table 6.

The plants treated with lead comparatively showed a higher percentage of inhibition in treated leaves rather than roots. The plant showed a greater percentage inhibition at 15 ppm as compared to the other concentrations. The highest percentage inhibition difference was seen between 20 ppm roots and leaves. Here, the plant extract acted as an inhibitor. At 2 and 20 ppm, the roots showed a higher percentage of inhibition as compared to the leaves, whereas at 5, 10 and 15 ppm, the leaves showed a higher percentage of inhibition as compared to the roots.

Plants treated with iron showed percentage inhibition was higher in roots as compared to the leaves. It can also be observed that the percent inhibition was seen as the highest in treated samples as compared to the untreated ones.

Previously, antiurolithic activity has been studied in plants like *Tephrosia tinctoria* (Fabaceae) and *Boehmeria macrophylla* (Urticaceae), wherein the methanolic extracts were used to check their ability to prevent the formation of calcium oxalate monohydrate crystals using artificial urine synthesized in the laboratory. Both tropical species inhibited the formation of calcium oxalate crystals in the presence of artificial urine (Bavishi et al. 2019).

Similarly, here methanolic extracts of *Pistia stratiotes* L. showed an inhibition against calcium oxalate crystals in the presence of heavy metals. Further toxicity studies need to be done on the introduction of such metal into the human body.

CONCLUSION

The results from atomic absorption spectroscopy showed that the *P. stratiotes* exhibit rhizofiltration which is in line with the literature available (Veselý et al. 2011). The highest amount of the metal was found to be stored in roots as compared to the leaves. Hence, *P. stratiotes* could be used in wastewater and industrial waste to reduce the content of heavy metals.

The results of thin layer chromatography show a change in the phytochemical fingerprint of plants treated with heavy metals as compared to the untreated ones. The secondary metabolites that increase in concentration can further be characterized and purified for commercial use. Plant secondary metabolites such as vincristine, paclitaxel, and homoharringtonine are being used in cancer therapy (Seca & Pinto 2018). Nicotine, veratrin, anabasine, anthocyanin

pigments, and tannins have been studied for their insecticidal properties (Rattan 2010). Aristolochic acid, furanoquinoline alkaloids, colchicine, vinblastine, phenylpropanoids, and isothiocyanates are some secondary metabolites that display anti-parasitic properties (Wink 2012). During the green synthesis of nanoparticles from plant extracts, secondary metabolites, especially flavonoids, play an important role in their formation and stabilization (Marslin et al. 2018). Other applications of secondary metabolites include their use as dyes (e.g. indigo), flavoring agents (vanillin), fragrances (essential oils of lavender), painkillers (morphine), and stimulants (caffeine, nicotine) (Rattan 2010).

The antiurolithic activity of the heavy metal-treated plant extracts showed a positive result in inhibiting the formation of calcium oxalate crystals. This can be correlated with the antioxidant activities, where, lead treated leaves at 15 ppm, shows both the highest antioxidant activity (IC_{50} = 3.8 $mg.mL^{-1}$) and antiurolithic activity (Percentage inhibition = 94.79%). Thus, these heavy metal-treated plants can further be analyzed for their use against kidney stones and toxicity studies would also have to be conducted for the introduction of plant extracts containing heavy metals into the human body.

This study paves the way to consider the possibility of using plants subjected to remediation for commercial use since the results strongly emphasize the change in phytochemical and bioactive properties. Being a preliminary study, further investigation of its applications with actual wastewater needs to be reconfirmed.

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