

A study on the role of secondary metabolites in combating lead-induced stress in *Pistia stratiotes* (Water lettuce)

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Abstract: Invasive aquatic organisms represent a substantial danger to marine ecosystems, causing disruptions in ecology and hazards to species. Our study represents the potential of *Pistia stratiotes* to absorb excess nutrients and heavy metals. This study involves the adverse effects of abiotic stress, especially lead nitrate, on aquatic environments. Lead is a toxic heavy metal that acts as an inhibitor to seed germination, hinders seedling growth, etc. This research examined the changes in secondary metabolites like polyphenols and flavonoids as which are known because of their competence for binding heavy metals and for their antioxidant properties. We have studied phenylalanine ammonia-lyase gene activity, lipid peroxidation, proline content, and gene expression that play a crucial role in reducing metal stress. This research will help in the development of sustainable strategies for alleviating heavy metal pollution.

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1 Introduction

Exotic species are organisms that are moved by humans from their natural habitat to a new place [1]. *Pistia stratiotes* originally comes from the Nile River in Africa [2].

It is free-floating and stoloniferous, blocking sunlight and oxygen exchange, harming native plants, fish and other aquatic life [3]. *P. stratiotes* act as a potential candidate for a plant that can be used in wastewater treatment, as its roots absorb extra nutrients and heavy metals [4]. Abiotic stress is referred to as the damage caused by non-living factors such as drought, floods, etc [5]. Lead disrupts plant respiration, nutrient uptake, etc. Lead stress also changes secondary metabolites, which help plants defend against stress. Lead stress acts as an inducer of the phenylpropanoid pathway and upregulates PAL gene activity [13]. *Pistia* absorbs lead nitrate, making it useful for wastewater treatment [6]. Plants handle stress through physiological and biochemical changes, mainly by making secondary metabolites. PAL forms polyphenols, flavonoids, anthocyanins, etc [7]. Lead-induced stress results in excessive generation of reactive oxygen species (ROS) in plant cells, which acts as a critical signaling molecule activating stress-responsive metabolic pathways. Elevated ROS levels trigger the phenylpropanoid pathway, primarily through the upregulation of phenylalanine ammonia-lyase (PAL), the key regulatory enzyme linking primary and secondary metabolism. Activation of PAL enhances the biosynthesis of phenolic compounds and flavonoids, which function as potent antioxidants and metal chelators. These secondary metabolites mitigate oxidative

damage by scavenging ROS and binding lead ions, thereby contributing to cellular protection and stress tolerance in *Pistia stratiotes*. This mechanistic relationship forms the basis of the present investigation.[1,2]

2 Materials and methods

The lethal dose (LD₅₀) of lead nitrate was determined experimentally by exposing *Pistia stratiotes* plants to a range of Pb(NO₃)₂ concentrations (10–80 μM) for a defined exposure period and monitoring visible toxicity symptoms such as leaf chlorosis, necrosis, growth inhibition, and plant mortality. Based on survival percentage and physiological damage, the LD₅₀ value was determined to be 60 μM. Therefore, all experimental treatments (10–50 μM) were maintained below the LD₅₀ threshold to study sub-lethal stress responses.

2.1 Estimation of stress

2.1.1 Preparation of plant extract

The plant extract was prepared following standard protocol [8].

2.2 Estimation of phenols

Polyphenol and flavonoid estimation were calculated using the appropriate methodology [8].

2.3 Analysis and measurement of different polyphenols

For the measurement of various types of polyphenols under the action of lead

stress, High Performance Liquid Chromatography (HPLC) analysis was performed [8].

2.4 Activity assay of PAL enzyme

PAL activity was determined using the method of Dos Santos et al. (2004) with minor modifications. Plant tissue (1 g) was homogenized in sodium borate buffer (pH 8.8) at 4 °C, centrifuged, and the supernatant was incubated with 100 mM phenylalanine for 60 min before stopping the reaction with 5 N HCl. [8].

2.5 Estimation of total antioxidant assay

It was done by performing the phosphomolybdenum assay [8-9].

2.6 Estimation of lipid peroxidation

Lipid peroxidation was done following standard protocols with slight adjustments [10].

2.7 Estimation of proline

It was performed using standard guidelines [11].

2.8 Gene expression analysis of PAL

Total RNA was isolated and used to produce cDNA (cDNA kit - Applied Biosystem, CA, USA). Then reverse transcription PCR was carried out by using a specific primer of PAL. [12]. The data analysis was done by ANOVA and t-test (KyPlot; Version 3.0).

3 Results

3.1 Total polyphenol content

The total polyphenol content of the control was found to be low in Pistia [3.523µg gallic acid equivalent (GAE) g⁻¹ FW], but the polyphenol content started increasing in the samples when treated with increasing concentrations of Pb(NO₃)₂. The sample treated with 10 µM Pb(NO₃)₂ was deciphered to have 6.287µg GAE g⁻¹ FW polyphenol content, while the sample treated with 20 µM Pb(NO₃)₂ exhibited the greatest polyphenol content, which was 7.84µg GAE g⁻¹ FW (Fig. 1).

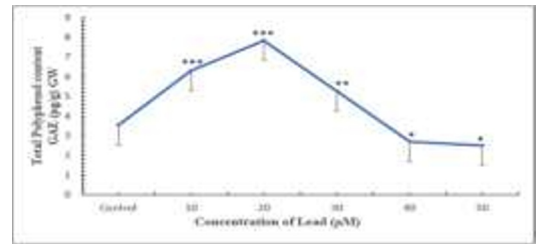


Fig. 1. The variation of polyphenol content in *P. stratiotes* under the treatment of lead.

3.2 Total flavonoid content

Flavonoid content was also seen to increase in the samples treated with lower concentrations of lead nitrate (10 and 20 µM). flavonoid content in the control was found to be low in Pistia [4.84 µg rutin equivalent RE g⁻¹ FW]. The accumulation of flavonoids was increased in the samples treated with 10µM lead nitrate, which was 7.08µg RE g⁻¹ FW, and the maximum flavonoid content was reported in the 20µM lead nitrate-treated samples (Fig. 2).

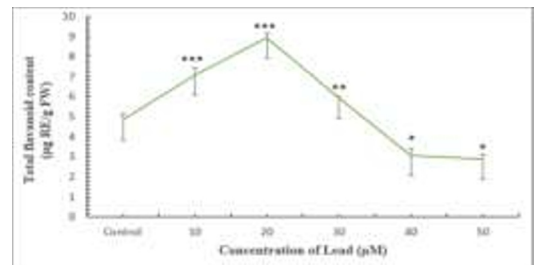


Fig. 2. The variation of flavonoid content in *P. stratiotes* under the treatment of lead.

3.3 Determination of flavonoid and phenolics by HPLC

Table 1. Various phenolics compounds present at different concentration of lead

Conc. of Lead	Polyphenol composition in $\mu\text{g g}^{-1}$ FW								
	Gallic acid Rt- 3.7 min	Chlorogenic acid Rt- 7.1 min	Caffeic acid Rt-9.8 min	Coumaric acid Rt- 12.8 min	Cinnamic acid Rt- 18.6 min	Rutin Rt- 11.8 min	Quercetin Rt- 16.5 min	Catechin Rt- 10.2 min	Kaemferol Rt- 23.8 min
Control	345.2±6.7	36.7±2.6	29.1±2.1	15.1±1.2	11.2±3.1	186.8±4.7	74.3±3.4	11.23±1.3	6.33±0.8
10 μM	447±5.1	56.4±3.2	30.1±2.6	18.4±1.9	23.1±1.2	297.6±10.7	161.9±3.5	17.45±1.9	8.56±1.2
20 μM	613±10.7	93.3±6.7	64.5±2.7	59.2±3.8	33.7±2.3	477.5±14.1	231.1±8.7	32.66±2.7	14.45±1.8
30 μM	334.9±5.8	32.4±2.7	21.3±0.8	9.7±0.7	8.1±0.2	132.2±3.1	43.3±1.8	13.45±1.1	7.65±1.2
40 μM	105.3±3.5	4.3±0.4	14.4±0.8	6.4±0.3	1.3±0.1	41.2±2.8	18.2±2.4	10.23±1.4	4.33±0.7
50 μM	85.3±3.5	3.3±0.4	10.4±0.8	4.7±0.3	1.1±0.1	31.2±2.8	11.2±2.4	7.23±1.4	3.33±0.7

min-1 g-1 FW. PAL activity decreases with increasing lead nitrate concentrations (30, 40, and 50 μM). (Figure 4).

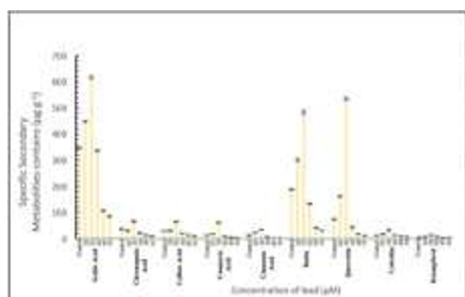


Fig. 3. Variation of different phenolics and flavonoids in *Pistia stratiotes* under the different doses of lead treatment.

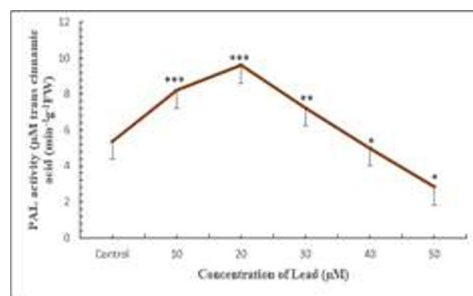


Fig. 4. The variation of *PAL* activity under the lead treatment.

3.4 Phenylalanine ammonia lyase (*PAL*) activity

The untreated *Pistia* sample (control) showed *PAL* activity of 5.36 $\mu\text{mol trans-cinnamic acid min}^{-1} \text{g}^{-1} \text{FW}$. The *PAL* activity rose considerably to 8.22 $\mu\text{mol trans-cinnamic acid min}^{-1} \text{g}^{-1} \text{FW}$ in 10 μM lead nitrate treated samples. The maximum *PAL* activity was reported in 20 μM lead nitrate treated samples, which was 9.62 $\mu\text{mol trans-cinnamic acid min}^{-1} \text{g}^{-1} \text{FW}$.

3.5 Total antioxidant assay

Total antioxidant content was found to be low (4.31 $\mu\text{g AAE g}^{-1} \text{FW}$) in the control or untreated samples of *Pistia*. This antioxidant activity increased in both 10 and 20 μM lead nitrate-treated samples, and it was observed to be the highest in the samples treated with 20 μM lead nitrate, which was 7.177 $\mu\text{g AAE g}^{-1} \text{FW}$ while the value was comparatively

lower (6.27 $\mu\text{g AAE g}^{-1}$ FW) in the samples treated with 10 μM . Then the antioxidant activity started declining in the samples with increasing concentrations of lead nitrate, and the least antioxidant activity was recorded in the sample treated with 50 μM lead nitrate, which was 3.29 AAE g^{-1} FW, while a slightly higher value of 3.33 AAE g^{-1} FW was observed in the sample treated with 40 μM lead nitrate (Fig 5).

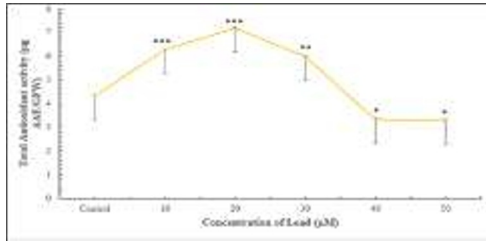


Fig. 5. The variation of antioxidant activity in *P. stratiotes* under the treatment of lead.

3.6 Expression of *PAL* gene and real-time PCR

Phenylalanine ammonia-lyase (*PAL*) was selected for gene expression analysis as it is the gateway enzyme of the phenylpropanoid pathway and plays a central role in regulating the synthesis of phenolics and flavonoids, thereby providing a comprehensive molecular insight into secondary metabolite regulation under lead stress [fig. 6].

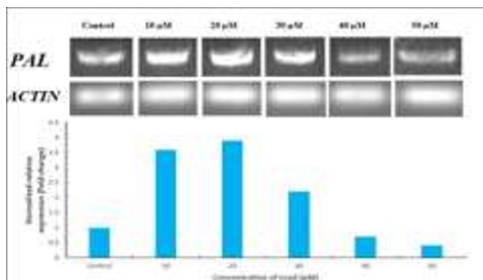


Fig. 6. Expression of the *PAL* gene under different concentrations of lead by PCR

3.7 Effect of lead on growth and tolerance of *Pistia stratiotes*

Growth response analysis revealed that *Pistia stratiotes* tolerated lower concentrations of

lead nitrate (10 and 20 μM) without significant visible damage. At these concentrations, plants maintained normal morphology and biomass. However, higher concentrations of lead nitrate (40 and 50 μM) resulted in noticeable growth inhibition, reduced fresh biomass, and leaf chlorosis, indicating physiological stress. These observations confirm that *Pistia stratiotes* exhibits tolerance to lead stress up to 20 μM , beyond which toxicity effects become prominent.

4 Discussion

According to WHO guidelines, the permissible limit of lead in water is 0.01 mg L^{-1} , whereas highly contaminated sites exhibit concentrations as high as 1.809 mg L^{-1} . The introduction of *Pistia stratiotes* significantly reduced lead levels in the water, bringing the concentration down to a range of 0.034–1.94 mg L^{-1} . In the present study, it states that *P. stratiotes* tolerate concentrations of lead nitrate which are low (up to 20 μM), which activates a strong antioxidant defence system. Increase in accumulation of proline, intact chlorophyll content, and reduced lipid peroxidation at Pb low levels indicates effective stress mitigation. Analysis of HPLC shows rutin and gallic acid as the major polyphenol and flavonoid, respectively. Elevation of *PAL* activity and upregulation of phenylpropanoid pathway genes, which contribute to increasing the synthesis of secondary metabolites. These findings state that *P. stratiotes* adopt biochemical and molecular strategies which specifically boost polyphenol and flavonoids to counteract the toxicity of lead. Overall, this study emphasizes the potential of plants for phytoremediation and also their ability to enhance the quality of wastewater under metal-induced stress.

Although low lead concentrations stimulated secondary metabolite synthesis, higher concentrations caused a marked decline in polyphenol and flavonoid content. This decline can be

attributed to severe oxidative stress, as evidenced by increased lipid peroxidation and proline accumulation at higher lead levels. Under excessive stress, cellular metabolic balance is disrupted, leading to reduced biosynthetic capacity and suppression of secondary metabolite production. Thus, the reduction in metabolite levels at higher Pb concentrations reflects toxicity-induced metabolic impairment rather than adaptive defence.

5 Conclusion

The study shows that *Pistia stratiotes* increases the production of secondary metabolites when exposed to lead stress, which helps the plant to reduce oxidative damage, and cellular stability is maintained. These metabolites act as natural defence compounds, which improve the tolerance of plants to the toxicity of heavy metals. Overall, the findings suggest that *P. stratiotes* inhibit strong biochemical mechanisms which help to combat the stress of lead-induced stress and can serve as a promising species for phytoremediation of contaminated water bodies.

Acknowledgement: The Authors acknowledge the University of Engineering and Management Kolkata, for this work.

Funding: Grant provided by the university was utilised for this work.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contribution statement: PT conceptualized and supervised and cross-checked the manuscript. All the authors contributed to the experiment, analysis and write-up of this work.

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