The effects of Lead stress on flavonoid content and antioxidant activity from *Scrophularia striata* Boiss.

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Introduction

Global industrialization and anthropogenic sources increase the release of heavy metals into the environment. lead (Pb) is known as the second most toxic metal among heavy metals [1–3]. Pb absorption through plants is one of the most well-known paths, which enters into the food chain of various organisms such as humans, and finally threatens their health [4,5].

Ilam province, located in the west of Iran, is an area exposed to various metal mines and refineries, and the human health risk caused by heavy metals is increasing [6]. Ilam is a city rich in medicinal plants, and native inhabitants have traditionally used these plants to treat various diseases for many years. Heavy metal pollution, especially Pb, in the region and its absorption by plants can greatly hazard biosystems and human society. Among the plants of this region, which are consumed abundantly, we can mention *Scrophularia striata* Boiss. species from the Scrophulariaceae family with the local name of Teshnehdari. *S. striata* has been prepared in different forms, including infusion and compress, to treat several diseases such as pain and digestive disorders, colds, and infectious wounds. For preparing tea, aerial parts of Teshnedari plants have often been infused in boiling water and used for therapeutic purposes [7–9]. Because of its beneficial properties, Teshnehdari is sent to other areas of Iran. The *S. striata* plant was reported that has a remarkable bio-accumulation of Pb [10,11]. This herb is also known for its phenolic compounds, especially phenylethanoid glycosides, and its medicinal and therapeutic properties refer to these compounds [12,13].

In addition to Pb having destructive effects on human health, it can also have undesirable effects on plants, including the effect on the growth and metabolism, and force the plants to respond to this pollution [1,14,15]. In response to Pb toxicity, plants change metabolite biosynthesis pathways, and this causes a loss of plant energy. The studies demonstrated that the biosynthetic pathway of phenylpropanoid compounds is an important pathway that changes in different stresses such as lead pollution [16]. This change can move towards the production of other medicinal compounds. However, this plant grows in habitats exposed to lead pollution, but there is not adequate information about its metabolic pathways under Pb pollution and stress on this plant. Considering the mentioned cases, we decided to investigate the biosynthetic pathway of phenolic compounds in response to Pb stress in the *S. striata* plant.

Materials and Methods

Growth condition

The Teshnehdari seeds were collected from northern Ilam Province, Iran. the mature seeds were rinsed with water for 48h, then disinfected with 2% sodium hypochlorite, and then immersed in gibberellin (500 ppm, 24h). The seeds were washed and moved in perlite. After 35 days, the plants were moved on hydroponic and grown for 35 days. The growth chamber conditions were 16h light photoperiod (220 µmol photons m^{-2} s^{-1} light irradiance), 25ºC temperature, 70% air humidity. The plants were prepared to treat with adding 250 ppm Pb $(NO_3)_2$ in a fresh half-strength Hoagland's nutrient solution (PH 5.8) for three collecting times (24, 48 and 72 hours, respectively). The shoots were gathered and prepared for future analysis.

Enzyme activities assay

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assayed at 560 nm by Giannopolitis and Ries (1977) method, based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). The enzymatic activity of guaiacol peroxidase (POD; EC 1.11.1.7) was measured at 470 nm according to the method of Pandolfini et al. (1992). The enzymatic activity of catalase (CAT; EC 1.11.1.6) was analyzed at 240 nm using the method of Cakmak and Marschner (1992), based on the initial rate of disappearance of H_2O_2. The protein concentration was measured at 240 nm following Bradford's (1976) method.

Metabolite extraction and quantitative analysis of flavonoids

To analysis the Genistein, 0.1 g of shoots dried samples was extracted by using 1.5 ml of 40% methanol solution containing 0.5% acetic acid. All extracts were incubated overnight at room temperature at 50 rpm shaking. After that, the blend was centrifuged (13000 rpm, 12 min), and then analyzed by HPLC (Agilent Technologies 1260 Infinity, USA). the stationary phase (Perfectsil Target ODS-3 (5 µm), 250×4.6 mm; MZ Analysentechnik, Mainz, Germany) was located at 25 ℃. Genistein was identified at 254 nm using DAD detector (Agilent Technologies 1260 infinity, USA). HPLC grade water consisting of 0.5% phosphoric acid (A solvent) and HPLC grade acetonitrile (solvent B) were used as mobile phase with a flow rate of 0.8 $mL.min^{-1}$. The gradient elution was as follows: A/B (82/18 %), 0-30 min; (33/67 %), 30-60 min; (82/18 %), 60-65 min; (82/18 %), 65-70 min. The flavonoid of Genistein were identified by comparing chromatographs of samples with valid standards by using external and internal standards purchased from Sigma-Aldrich.

statistical analysis

Data, performed in a completely randomized design with three replications, were analyzed by using SPSS-16 software. A one-way ANOVA test was obtained to represent significant differences between groups ($P < 0.05$), then measured using the Duncan test. The correlation coefficient was accomplished using R software v.3.6.1 (http://www.r-project.org).

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Results and Discussion

The antioxidant capacity was illustrated by assessing of antioxidant enzymes using shoot extractions. Scavenger enzymes included SOD, CAT, and POD. POD significantly increased after 24 h treatment and compared to control samples. In this treatment, POD was the predominant antioxidant enzyme. However, SOD and CAT meaningfully increased at 72 h after stress and regulated the oxidative status. Flavonoid profiles were identified by HPLC analysis, and chromatograms are presented in figure 1. Genistein significantly increased at 24 and 72 h after treatment against control samples.

It has been reported that Pb stress increases the content of intracellular ROS. The ROS can also destroy cellular redox homeostasis, so the plant's antioxidant system is activated in response to Pb exposure, and the increased activities of this system go towards inducing them to quench and eliminate them. The antioxidative activity of flavonoid compounds is primarily operated by phenolic hydroxyl groups in their structure. In this study, we evaluated the effects of Pb treatment on the flavonoid contents (table 1) as non-enzymatic antioxidant and enzymatic antioxidant activities of *S. striata*. The contents of flavonoid and antioxidant capacities enhanced during Pb stress. In a previous study, Beshamgan et al. (2019) also stated that flavonoids and antioxidant enzymes increased in Teshnehdari at all times (12, 24, 48, and 72 h) after Cadmium (Cd) stress against control samples. SOD enzyme is often considered the first line of plant defense; through its activity, the O_2^- is converted to O_2 and H_2O_2. In the continuation of that, CAT and POD decompose H_2O_2 into H_2O. Zafari et al. (2016) results demonstrated that the production of POD increased slower than CAT; this indicates that CAT may be more responsible for H_2O_2 removal in the early hours of Pb stress than POD. In contrast, our findings showed that POD significantly increased after 24 h treatment, and POD increased faster than CAT. In the following, CAT enzymatic activity increased more than POD. These results were comparable to previous studies under various stress resulting in secondary oxidative stress.

The correlation heatmap of antioxidant capacities is shown in figure 2. Statistical analysis presented a negative relationship between POD with CAT and SOD. The correlation coefficient analysis of flavonoids also showed a significant correlation between flavonoids and antioxidant activity (CAT and SOD). Jakovljevic et al. 2021 also showed a negative relationship between POD with CAT and SOD by analyzing Pearson's correlation in Basil under temperature stress. In summary, it can be stated that secondary oxidative stress is occurred in plants under Pb stress, activating different shoots ' mechanisms in *S. striata* to cope with it.

Conclusions

Overall, our studies demonstrate that Theshnehdari can cope with Pb stress using diverse antioxidant defense responses. This species can also be a good source of different antioxidants with the medicinal application.

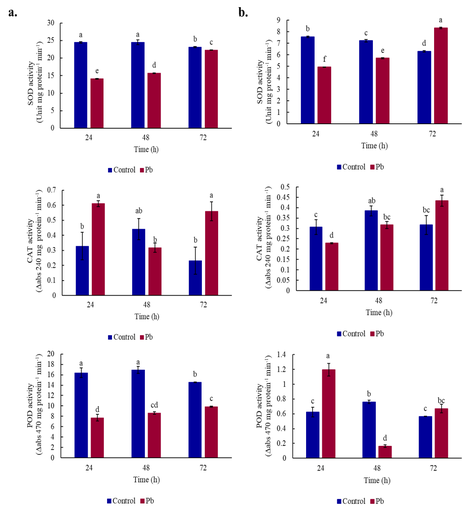

Fig. 1. Effects of Pb (250 mg L^{-1}) on enzymatic antioxidant activities of SOD, CAT and POD in A) roots and B) shoots of *S. striata* species grown in hydroponic conditions for 24, 48 and 72 hours after treatment. Data are expressed as Mean ± SD. Mean values sharing a common letter are not significantly different ($P \leq 0.01$).

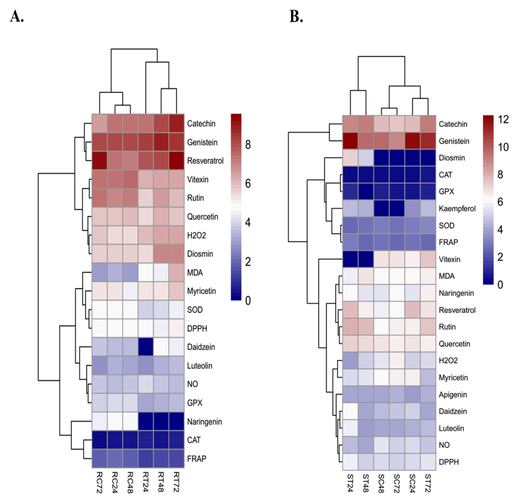

Fig. 2. The heatmap analysis of oxidative status and flavonoids contents in A) roots and B) shoots of *S. Striata* plants under Pb treatment: from low in dark blue to high in dark red

Table 1. The effect of Pb on flavonoid contents of *S. striata*.

Sample organ	Flavonoids (µg g^{-1} DW)	Control with harvesting time (Hour)			Pb treatment (250 mg L^{-1}) with harvesting time (Hour)		
		24	48	72	24	48	72
Root	Catechin	161.31±10.33^{c}	168.52±1.78^{c}	98.92±16.41^{c}	158.12±34.51^{c}	280.99±68.94^{b}	466.38±31.87^{a}
	Vitexin	159.85±10.70^{a}	190.40±27.49^{a}	167.20±4.56^{a}	76.36±14.78^{b}	87.22±11.33^{b}	84.33±14.57^{b}
	Rutin	124.14±4.74^{ab}	144.56±5.33^{a}	153.27±9.83^{a}	51.06±8.54^{c}	101.31±22.34^{b}	59.69±14.96^{c}
	Quercetin	55.64±8.96^{ab}	65.47±7.33^{ab}	55.16±6.74^{ab}	44.21±8.21^{b}	69.24±8.88^{a}	59.16±11.11^{ab}
	Genistein	268.50±11.75^{b}	267.71±37.68^{b}	267.48±42.64^{b}	331.99±97.82^{ab}	475.77±45.45^{a}	352.45±85.46^{ab}
	Diosmin	45.98±10.29^{b}	46.52±15.63^{b}	48.81±8.75^{b}	42.93±24.58^{b}	124.47±5.06^{a}	120.81±10.74^{a}
	Myricetin	35.69±3.81^{b}	21.97±3.83^{c}	35.60±0.37^{b}	35.28±0.61^{b}	37.32±3.11^{b}	53.56±2.06^{a}
	Resveratrol	160.89±6.38^{d}	148.02±33.41^{d}	582.55±1.22^{b}	231.31±14.73^{cb}	278.67±5.71^{c}	745.22±92.72^{a}
	Daidzein	11.19±1.86^{b}	10.24±0.45^{b}	12.11±0.73^{b}	-	24.83±3.79^{a}	20.83±2.01^{a}
	Luteolin	8.62±1.22^{ab}	7.47±1.43^{ab}	5.55±0.67^{b}	6.25±1.45^{b}	8.68±2.15^{ab}	11.27±1.64^{a}
	Naringenin	24.53±2.89^{a}	25.79±2.03^{a}	21.13±4.16^{a}	-	-	-
	Apigenin	-	-	-	-	-	-
	Kaempferol	-	-	-	-	-	-
Shoot	Catechin	225.69±56.18^{b}	204.18±52.56^{b}	164.63±21.62^{b}	488.73±11.58^{a}	576.93±31.65^{a}	570.07±99.36^{a}
	Vitexin	94.76±5.12^{b}	102.08±7.98^{b}	104.63±13.77^{b}	-	-	183.72±13.90^{a}
	Rutin	84.97±6.00^{c}	82.28±11.19^{c}	84.55±4.17^{c}	235.56±14.23^{a}	217.74±11.98^{a}	186.74±8.79^{b}
	Quercetin	105.44±5.80^{bc}	106.99±11.75^{bc}	97.54±13.70^{c}	143.71±3.73^{a}	127.74±7.73^{ab}	96.81±4.54^{c}
	Genistein	3709.69±334.12^{b}	772.36±132.47^{d}	494.61±74.87^{d}	4934.90±35.25^{a}	787.90±29.32^{d}	2224.79±195.87^{c}
	Diosmin	-	-	-	138.18±9.96^{a}	34.46±9.09^{b}	-
	Myricetin	89.71±10 86^{a}	78.91±10.08^{a}	84.38±7.60^{a}	37.72±2.15^{bc}	44.43±3.70^{b}	21.56±8.23^{c}
	Resveratrol	197.16±0.17^{a}	49.05±3.99^{c}	59.75±10.16^{c}	196.64±16.28^{a}	93.05±4.67^{b}	114.44±9.93^{b}
	Daidzein	30.58±5.38^{b}	24.08±3.94^{b}	26.10±3.86^{b}	73.88±15.75^{a}	14.97±1.70^{b}	14.83±4.76^{b}
	Luteolin	18.82±1.60^{b}	15.60±2.64^{b}	16.92±5.92^{b}	52.87±0.21^{a}	12.71±1.71^{b}	20.99±5.58^{b}
	Naringenin	44.62±2.61^{d}	44.37±2.94^{d}	60.80±8.60^{c}	77.12±6.04^{b}	45.32±2.11^{d}	94.37±5.44^{a}
	Apigenin	13.28±0.95^{c}	14.35±0.54^{bc}	19.43±1.42^{a}	14.88±0.48^{bc}	17.02±1.81^{ab}	17.52±1.61^{ab}
	Kaempferol	11.24±0.05^{b}	-	-	20.52±2.36^{a}	18.44±2.88^{a}	20.86±1.40^{a}