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Original Article

THE EFFECT OF LEAD TOXICITY ON GROWTH AND ANTIOXIDANT ENZYME EXPRESSION OF *ABUTILON INDICUM* L

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ABSTRACT

Objective: The focus of the present study was to analyse the oxidative effects of lead nitrate on biosynthesis of antioxidant enzyme activities (superoxide dismutase (SOD) and glutathione peroxidase (GPX)) in *Abutilon indicum.*

Methods: Seedlings of *A. indicum* were grown with supplementation of different concentrations (0, 25, 50, 75 and 100 µM) of lead nitrate for 15 and 30 days. The various growth parameters like shoot length, size and total number of leaves per plant were recorded. Extraction, assay and expression of super oxide dismutase (SOD) and glutathione peroxidase (GPX) were carried out with control (without metal salt) and lead nitrate treated plants.

Results: The growth parameters exhibited a declining trend in the metal treated plants in a dose dependant manner. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of leaf and root protein extracts exhibited the appearance of some new proteins due to metal stress. At lower concentrations of the metal, antioxidant enzyme activity was enhanced with a gradual declination of higher concentration (75 and 100 µM). The enzyme activity reflects the deleterious impact of lead on the plants. Isoforms of SOD and GPX were separated using non-denaturing polyacrylamide gel electrophoresis and new isoforms were noticed in the stressed plants rather than the control during the investigation.

Conclusion: Isoforms of the antioxidant enzymes synthesized due to Pb stress may be used as biomarkers for analysing the impact of heavy metals on other medicinal plants under metal stress condition.

Keywords: Antioxidant, Enzyme, GPX, Medicinal plant, SOD.

INTRODUCTION

Heavy metal toxicity due to environmental pollution are the increasing concern as they are creating hazardous health problems and other societal issues. Though metals are necessary for the normal metabolic functions of the plants [1-2] but heavy metals in excess amount posses serious threat towards human health. Lead is one of the heavy metal and simultaneously a toxic element which can be accumulated in plant organs and agricultural products. Subsequently the metal can enter to the human food chain [3]. Lead (Pb) is one of the ubiquitously distributed most abundant toxic element in the soil. It exerts adverse effect on morphology, growth and photosynthetic processes of plants. A high lead level in soil induces abnormal morphology in many plant species. For example, lead causes irregular radial thickening in pea roots, cell walls of the endodermis and lignification of cortical parenchyma [4]. The metal is known to inhibit seed germination of *Spartiana alterniflora* [5]. It also induces irregular proliferation on the repair process of vascular plants [6]. In *Allium* species lead also inhibits the elongation and expansion of above ground and underground plant parts [7]. In plants, all the metabolic steps are to some extent regulated by the association and diassociation of metals [8].

Abutilon indicum L. belongs to family Malvaceae is a medicinal plant and regionally known as *Atibala (Kanghi)*. Its medicinal potential and ethical importance place it among the essential plants traditionally used by mankind. It has wide uses in Ayurveda, Siddha and unani system of medicine. It is an annual, erect, woody, velvety shrub available mainly in the warmer climates i. e. Srilanka, Bangladesh, India, Pakistan etc. The plant is decorated with beautiful bright yellw flowers, with cordate leaves that are velvety and large sized [9]. Seeds of this plant are minute and black, arranged inside the fruit i. e. Scrobilate [10]. All parts of this plant are useful and have medicinal properties [11]. It is reported that the plant parts are useful for cure of ulcer, piles, gonorrhea, inflammation, dysentery, urinary disorders etc. Its hyper-hepatoprotective activity is of optimum level and it cures liver damage. Antibacterial, antiarthritic, antimycotin, antiasthmatic, antileprotic activities are also ensured [12]. Diuretic activities are also verified from the seed extracts. The plant has essential flavonoids that are known to rejuvinate the damaged pancreatic B-cells that stimulate the secretion of insulin. Wound healing capacity of this plant is well known among regional people [9]. Besides this other medicinal properties of this plant extracts are useful as demulcent, diuretic, laxative, cure for urinary disorders, chronic disorders, dysentery etc. One of the important property of *A indicum* includes larvicidal activity i. e antimalarial activity [13].

Plants produce significant amounts of antioxidants to prevent the oxidative stress caused by photons and oxygen. It has been established that oxidative stress is a major causative factor for chronic and degenerative diseases. Both the exogenous and endogenous antioxidants are found to be effective in prevention of free radical formation [14]. Now a days, search for antioxidant compounds that are pharmacologically important having low or, no side effects as a preventive medicine has been increased to many fold.

The present investigation was undertaken to study, protein profiling and to study the effect of lead (Pb) on the expression of antioxidant enzymes i.e. SOD and GPX in the leaf and root of the fifteen and thirty days treated plants.

MATERIALS AND METHODS

Plant materials and treatment conditions

Seeds of *Abutilon indicum* L. were collected from Botanical garden, Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India and were shown on the earthen pots containing a mixture of soil, sand and vermicompost at a ratio of (1:1:1). After germination, the seedlings were allowed to grow for a period of 15 days in the same condition. Healthy plants of *A. indicum* were selected from the group of small plants and transferred to the pots containing aforesaid soil, sand and compost mixture. After one month different concentrations (0, 25, 50, 75 and 100 µM) of lead nitrate was supplemented to the pots containing the healthy plants and allowed to grow for a period of 15 and 30 days.

Growth parameter

After 15 and 30 days of treatment, plants were carefully removed from the pot and washed twice with distilled water. The plants were blotted dry with tissue papers and then the various morphological parameters such as shoot length, leaf length and total numbers of leaves per plant were recorded.

Extraction and estimation of total soluble protein

For estimation of total soluble protein, plant tissues were extracted by homogenizing in pre-chilled mortar and pestle with 50 mM potassium phosphate buffer (pH 7.8), 50 mM ethylenediamine-tetra-acetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 % (w/v) insoluble polyvinylpyrrolidone (PVP). The homogenate were centrifuged at 14000 rpm for 20 min at 4 ºC and the supernatants were processed for estimation of protein and antioxidant enzymes. Quantitative estimation of protein was done according to the standard procedure [15] by measuring absorbance at 750 nm, using bovine serum albumin as a standard and the quantity of estimated protein was expressed as mg/g $^{-1}$ fresh weight (g. f. w.).

Analysis of protein profile by SDS-PAGE

The supernatant obtained earlier from the leaf and root samples were used for protein profiling by SDS-PAGE. Equal amounts (50 µg) of proteins were separated on 5 % stacking and 10 % resolving polyacrylamide slab gels [16] at a constant current of 35 mA for 4 h. Separated polypeptides on the gel were visualized by Coomassie Brilliant Blue (CBB) staining method [17]. The gels were then scanned and photographed by gel documentation system and analyzed with quantity one software from Bio-Rad (Bio-Rad, Italy). In order to achieve the precise sizing of the separated polypeptides, the protein molecular weight marker [phosphorylase b (97.4 kDa); bovine serum albumin (66.0 kDa); ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa); soyabean trypsin inhibitor (20.1) and lysozyme (14.3 kDa)] (Merck Specialities Pvt. Ltd.) were used as standard.

Assay and in-gel activities of antioxidant enzyme

Assay and activity staining of SOD

The activity of SOD was assayed according to the procedure of [18]. The reaction mixture was prepared by mixing 1.110 ml of 50 mM phosphate buffer (pH 7.4), 0.075 ml of 20 mM L-methionine, 0.040 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM hydroxylamine hydrochloride and 0.1 ml of 50 µM EDTA. To this mixture 100 µl of enzyme extract (50 µg protein) and 80 µl of riboflavin (50 µM) were added. The cocktail was mixed and illuminated for 10 min in an aluminium foil coated wooden box containing two 20 W-Philips fluorescent lamps fitted parallel to each other. Equal amount of buffer was added to the control tube instead of sample. The sample and its respective control were run together. After 10 min of exposure, 1 mL of Greiss reagent (prepared freshly by mixing equal volume of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-1-napthyl ethylene diamine) was added into each tube and immediately the absorbance was measured at 543 nm. The activity was calculated as nKat/mg $^{-1}$ of protein.

The specific activity staining of SOD was performed as per the classical method of [19]. The gel was completely submerged in freshly prepared staining buffer containing 50 mM phosphate buffer, 0.1 ml EDTA, 28 mM TEMED, 0.003 mM riboflavin and 0.25 mM nitroblue tetrazolium for 30 min in dark condition. Thereafter, the gel was placed on an illuminated glass plate until the bands become visible.

Assay and activity staining of GPX

Peroxidase activity was measured in a reaction mixture (3 ml) containing 100 mM potassium phosphate buffer pH 7.0 (2.8 ml), 10 mM H_2O_2 (50 µl), 0.018M guaiacol (50 µl) and 100µl enzyme extract, according to the method of [20]. The increase in absorbance due to formation of tetra guaiacol was recorded at 436 nm. GPX activity was calculated by using the extinction coefficient of 26.6 M^{-1} cm^{-1} for H_2O_2 at 436 nm and was expressed as nKat/mg $^{-1}$ of protein. The 10 % gel was stained by the procedure of [21]. Gel was immersed in 0.018 M guaiacol for 30 min at room temperature rinsed twice in double distilled water and immersed in 0.015 % of H_2O_2 in 1 % glacial acetic acid till the development of dark brown bands.

RESULTS

Growth parameter

The effect of lead toxicity on various morphological parameters was clearly detected even in low concentration (25 µM) of Pb. After 15 and 30 days of aqueous exposure of Pb, considerable reduction in shoot length, leaf size and number of leaves per plant were observed. As the concentration of Pb increases, inhibition seems to be prominent and the maximum destruction was found in 100 µM in both 15 and 30 days of treatment (Table 1).

Table 1: Growth parameter of treated and control plants under Pb stress

Treatment (µM)	Shoot length (cm) mean±SE	Leaf length (cm) mean±SE	No. of leaves/plant mean±SE
Growth parameter after 15 days of treatment			
0	17.21±0.27	1.26±0.43	10.00±0.96
25	16.11±0.93	1.19±0.82	10.00±0.03
50	15.12±0.11	1.06±0.33	9.00±0.34
75	14.01±0.39	0.92±0.22	8.00±0.54
100	12.61±0.02	0.79±0.15	7.00±0.69
Growth parameter after 30 days of treatment			
0	20.01±0.92	1.59±0.11	12.00±0.31
25	18.92±0.33	1.37±0.02	12.00±0.11
50	17.09±0.44	1.18±0.55	11.00±0.26
75	16.05±0.23	1.02±0.13	10.00±0.85
100	14.66±0.25	0.86±0.07	8.00±0.24

The data represent of replicates (n=5). Significant at P ≤0.05

Protein content and SDS-PAGE analysis

The total soluble protein content was found to be comparatively higher in leaf and root samples of 15 days treated plants than 30 days (Fig. 1) and (Fig. 2).

When analyzed in SDS-PAGE, the total number of protein bands were found to be (7 and 6) in leaf and (5 and 4) respectively in root samples of 15 and 30 days treated plants. It was also evidenced that about 2-fold increment in protein content was observed in the leaf than the root with 50 and 75 µM metal treated plants for 15 days. At 100 µM the protein content of both 15 and 30 days root was found to be almost similar (Fig. 3, Fig. 4).

SOD activity

The SOD activity of *A. indicum* was higher in 15days treated plants in comparision to day 30 plants. However SOD activity was significantly increased in leaf and root samples supplemented with 50 µM of metal both in 15 and 30 days of treatment respectively

(Fig. 7, fig. 9). The leaf extracts of 15 days treated plants exhibited declined value in SOD activities at 75 µM of Pb concentration. In both the treated plants (15 and 30 days) SOD activity was found to be minimum than other concentrations at 100 µM metal supplementation. Distinctly resolved bands of SOD were clearly observed in the native gel of both types of treated leaf tissues in all the concentrations (Fig. 8).

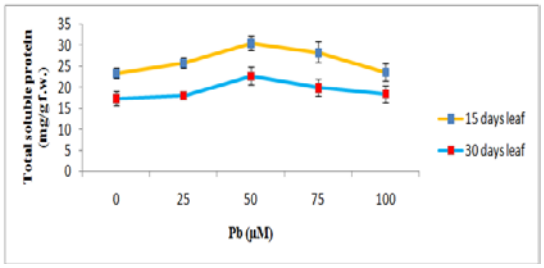

Fig. 1: Total soluble protein estimation under different Pb treatment (0, 25, 50, 75 and 100 µM) in leaf samples of *A. indicum* L. The data plotted represent mean±SE of replicates (n=5)

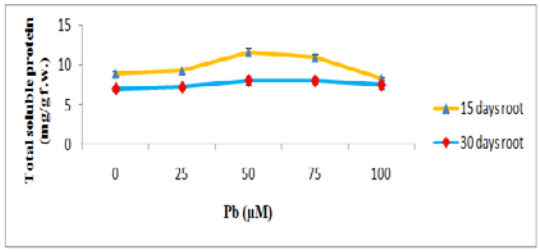

Fig. 2: Total soluble protein estimation under different Pb treatment (0, 25, 50, 75 and 100 µM) in root samples of *A. indicum* L. The data plotted represent mean±SE of replicates (n=5)

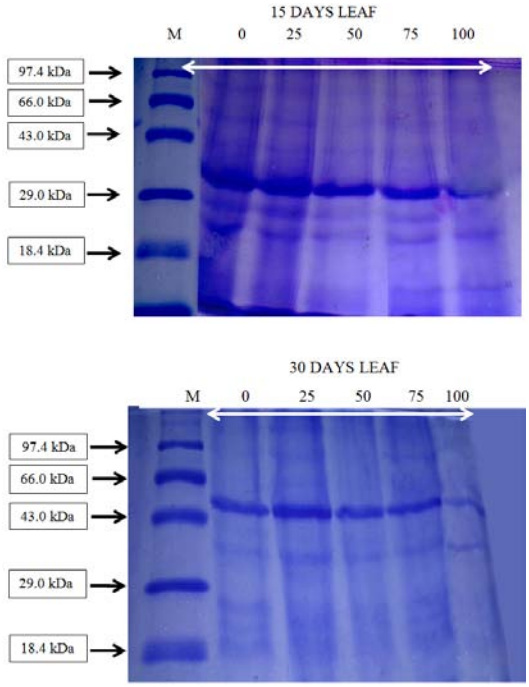

Fig. 3-4: Protein profile from leaf samples of *Abutilon indicum* L. treated with different concentration of Pb (0, 25, 50, 75 and 100 ppm) after 15 days and 30 days

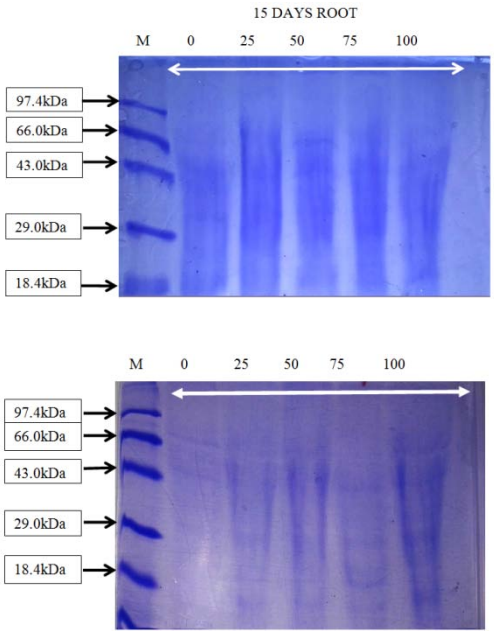

Fig.5-6: Protein profile from root samples of *Abutilon indicum* L. with different concentration of Pb (0, 25, 50, 75 and 100 ppm) after 15 days and 30 days

In 15 and 30 days of treatment two isoforms (SOD-1 and SOD-2) were observed in the leaf samples but the intensity was different and more intense bands were observed at 50 µM lead supplementation which is quite similar with the observations obtained in the activity study of SOD. Prominent bands of SOD isoforms were also viewed in the root tissues of all the metal stressed sample of *A. indicum*. Three isoforms (SOD-1, SOD-2 and SOD-3) were found in 30 days of treatment where as only two isoforms (SOD-1 and SOD-2) were observed in the root samples of 15days treated plants (Fig. 10).

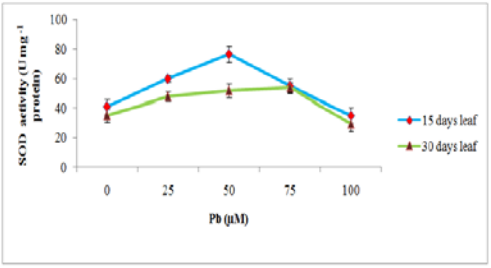

Fig. 7: SOD activity with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the leaf extracts of *A. indicum* L. after 15 and 30 days of treatment

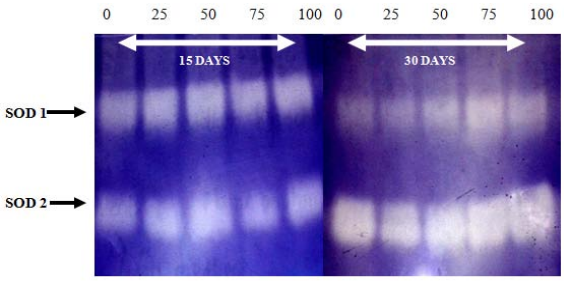

Fig. 8: SOD isoenzyme pattern with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the leaf extracts of *A. indicum* L. after 15 and 30 days of treatment

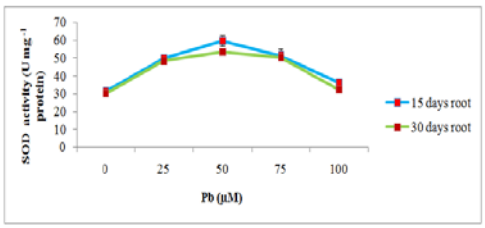

Fig. 9: SOD activity pattern with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the root extracts of *A. indicum* L. after 15 and 30 days of treatment

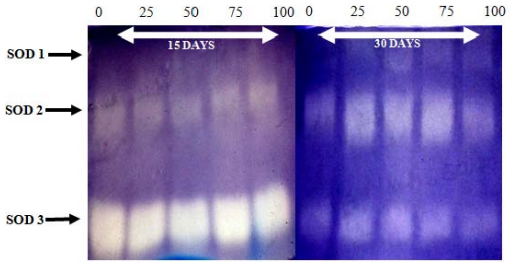

Fig. 10: SOD isoenzyme detected with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the root extracts of *A. indicum* L. after 15 and 30 days of treatment

GPX activity

Changes in the activity of GPX in *Abuliton. indicum* during the exposure to varying concentrations of the metal was clearly observed (Fig. 11, Fig. 13). After 15 and 30 days of treatment, the leaf synthesized more GPX with (50 µM) metal stress than the root. As observed earlier in leaf samples, the SOD activity was gradually declined with 100 µM treated plants. The native gel profile of GPX isoenzyme exhibited two isoformic bands (GPX-1 and GPX-2) in the 30 days treated leaf however only one band is found in the day 15 treated plants (Fig. 12). The isozymic pattern of GPX in the root sample revealed two bands. However more intense bands were observed at (50 µM) supplemented plants of *Abutilon. Indicum* L., both in 15 and 30 days of treatment (Fig. 14).

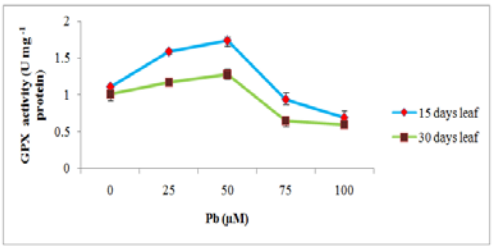

Fig. 11: GPX activity with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the leaf extracts of *A. indicum* L. after 15 and 30 days of treatment

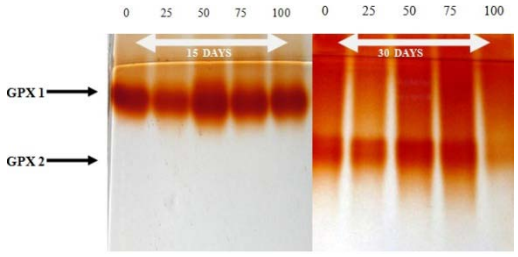

Fig. 12: GPX isoenzyme pattern with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the leaf extracts of *A. indicum* L. after 15 and 30 days of treatment

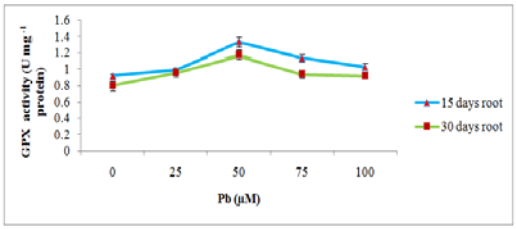

Fig. 13: GPX activity with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the root extracts of *A. indicum* L. after 15 and 30 days of treatment

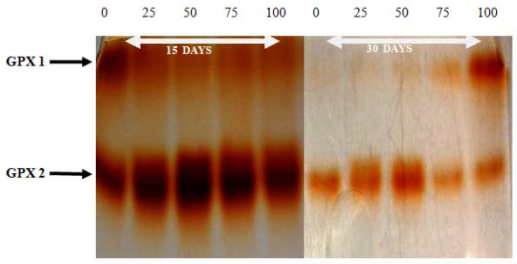

Fig. 14: GPX isoenzyme pattern with different concentration of Pb (0, 25, 50, 75 and 100 µM) in the root extracts of *A. indicum* L. after 15 and 30 days of treatment

DISCUSSION

In *Abutilon indicum* L. declination in growth with all the morphological parameters were observed by supplementation of different concentrations of $Pb(NO_3)_2$. This might be due to the toxic effect of Pb at higher concentrations (Table.1). Hence lead pollution is deleterious to the plant at higher concentrations [22]. The reduction of morphological characters may be due to the apoplastic or symplastic transportation of the metal from roots to the other organs. However protein content in treated leaves increased significantly both in 15 (30.54±1.7 mg/g) and 30 (22.71±2.1 mg/g) days plants when supplemented with 50 µM metal salt as compared to the control (Fig.1). Similar type of result was also observed with 15 days (11.67±0.51 mg/g) and 30 days (8.09±0.59 mg/g) treated root samples in comparison with the control root of *A. indicum* L. signalling in biosynthesis of soluble protein in plants was due to a wide variety of stress caused by natural and xenobiotic compounds [23]. In this study, the content of soluble protein was enhanced by the metal stress up to a certain limit and then declined. The present investigation corroborates with the earlier findings [24] where high levels of Pb enhanced the protein content in leaves, of *Jatropha curcas* L. The mechanism by which Pb affects protein biosynthesis is quite complex and needs a further study. Different isoforms of antioxidative enzymes were obtained in control and Pb treated plants of *Abutilon indicum* L. [in leaf (Fig.3 and Fig.4); in root (Fig.5 and Fig.6)] but their intensity decreases with increase in stress metal with disappearance of some bands or appearance of other proteins. Similar observations were also reported under copper stress in *Withania somnifera* L. plants [25].

In *A. indicum* L. supplementation of 25 and 50 µM Pb in day 15 plants induced a progressive increase in SOD activity. However two-fold increment of SOD was observed with 50 µM treatment. But the activities were relatively lower at 75 and 100 µM salt stress. Earlier report indicates that Pb induced alteration of the SOD activity were observed in *Oryza sativa* [26], horsegram [27], *Sesbania drummondii* [28], *Sedum alfredii* [29] and *Cassia angustifolia* Vahl, *Jatropha curcus* [30, 24]. Enhancement in SOD activity might be either due to increased production of ROS or be a protective measure adopted by *A. indicum* plant against oxidative damage. Two isoforms of SOD (I and II) (Fig. 4) were detected in the leaf whereas three bands of SOD isoenzymes (I – III) (Fig. 10) were detected by native gel electrophoresis in 30 days treated root sample. However two isoforms were observed in 15 days treated leaf and root samples of

A. indicum L. But the intensity of isoenzymes were variable with different metal stress (Fig. 10). The diverse responses of SOD isoenzymes suggested that each supplementation produces its own unique effects, and further study of these should help to clarify the overall importance of oxidative stress phenomena in plants.

A continuous increment in the GPX activity of *Abutilon. indicum* was observed till the concentration of the metal was up to 50 μM in leaf and root in day 15 and day 30 treated plants. Declined activities were also observed at higher concentration (Fig. 11 and Fig. 13). One isoform (GPX-1) was observed in 15 days treated plant but one additional expression of the isoform (GPX-2) was with 30 days treated plant (Fig. 12). With increase in the metal concentration the number of isoenzymes were enhanced, but at higher concentration, the intensity of the bands decreased with both 15 and 30 days treated plants (Fig. 14). The enhanced activity of GPX suggests that this enzyme serves as an intrinsic defensive tool to resist Pb-induced oxidative damage in *Abutilon* plant. The GPX activity has been documented earlier with a variety of stressful conditions, such as Pb [26], Cu [31] and salinity stress [32]. But all of the studies agreed that high concentration of stress is always harmful for the plant defence system.

CONCLUSION

The study revealed that *A. indicum* can tolerate moderate concentrations of Pb but at higher concentration this metal will induces oxidative stress and the magnitude of the stress is proportional to the concentration of the stressor. The accumulation of Pb in this plant may result the formation of reactive oxygen species, which confirmed via the antioxidative enzymatic defense system by stimulating an increment in SOD and GPX activity. Study on effect of lead toxicity on individual tissues is still continuing to confirm the effect of lead accumulation in the bioactivity based application of *A. indicum* L.

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CONFLICT OF INTERESTS

Declared None

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