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Effect of heavy metals on germination, biochemical, antioxidant and withanolide content in *Withania somnifera* (L.) Dunal

Akshatha Banadka & Praveen Nagella*

Department of Life Sciences, CHRIST (Deemed to be University), Bengaluru 560 029, India

***Email:** praveen.n@christuniversity.in

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Abstract

Withania somnifera (L.) Dunal., commonly referred to as 'Ashwagandha', is a medicinal plant from the solanaceae family with a wide range of pharmacological properties. *W. somnifera* is a rich source of withanolides, such as withanolide A, withanolide B, withanolide D, withaferin A and many others which are attributed for a large number of pharmacological activities. In the present study, the impact of heavy metals such as cadmium (Cd), mercury (Hg) and lead (Pb) has been assessed on the growth, biochemical parameters, antioxidant activity and withanolide A and withaferin A content of *W. somnifera*. The seeds of *W. somnifera* were germinated in cocopeat and treated with different concentrations of Cd (20-200 ppm), Hg (10-100 ppm) and Pb (200-2000 ppm) for 21 days. There have been substantial differences between the heavy metal-treated plants and the control plants with the lowest germination of 20% observed in the plants treated with 2000 ppm Pb. The selected metals inhibited vegetative growth with lowest length of 3.07cm and lowest biomass of 0.74g in 180 ppm Cd and 200 ppm Cd treated plants respectively. With the addition of heavy metals, biochemical parameters like protein, carbohydrate, chlorophyll, total phenol, flavonoid and proline content varied significantly and showed metal tolerance by exhibiting antioxidant activity at lower concentrations. The metal accumulation occurred in a dose-dependent manner with highest Cd accumulation of 14.30mg kg^{-1}, Hg accumulation of 42.45mg kg^{-1}, and Pb accumulation of 217.46mg kg^{-1} of dry biomass of the plants. The withanolide content increased up to a specific metal concentration and decreased with a further increase in heavy metal concentration. The seeds treated with 1200 ppm of Pb showed the highest withanolide A content of 1.7mg g^{-1} dry weight (DW), and the seeds treated with 80 ppm of Cd showed the highest withaferin A content of 3.2mg g^{-1} DW.

Keywords

Withania somnifera, LD_{50}, withanolide A, withaferin A, antioxidant activity, heavy metal stress

Introduction

Withania somnifera (L.) Dunal, commonly referred to as ashwagandha ("the smell of a horse") and winter cherry, belongs to the family solanaceae. This ancient therapeutic herb grows in hot and dry tropical regions of Afghanistan, Balochistan, Congo, Djibouti, Egypt, India, Jordan, Liberia, Mali, Morocco, Nigeria, Sind, South Africa, Sri Lanka, Swaziland and Tanzania(1). In India, it is widely grown in Gujarat, Madhya Pradesh, Punjab, Rajasthan and Uttar Pradesh(2). This extensively prescribed botanical dates back to

thousands of years in the Indian Ayurvedic system(3).It has a wide range of pharmacological properties, which include ameliorative effects, antidiabetic, antihypertensive, anti-inflammatory, antioxidant, anti-stress and immunomodulatory properties (4). It also plays a major role in the proper functioning of various organs, exhibits hepatoprotective and cardioprotective properties and maintains reproductive health (5). These medicinal properties can be attributed to the withanolides, the major phytochemicals present in different parts of the plant.

Withanolides are a group of C28-steroidal lactones that include withanolide A, B and D, withaferin A, withanoside IV and V, withanone, withasomniferin A, withasomniferin B, 12-deoxy withastramonolide and many others (6). The withanolides were expected to dominate the global ashwagandha market by 5% in 2022 as per the report by Data bridge market research. Further, the research suggests that global market demand for ashwagandha is expected to reach 102.72 million USD by 2029 (7). The annual production of drugs from *Withania* in India is 5905 tonnes annum^{-1} and the expected production is approximately 12120 tonnes annum^{-1}, which exceeds its annual production. Thus, there is high market demand for ashwagandha (8).

With climate change and urbanization, encroaching on natural fertile lands, plants are vulnerable to various abiotic stressors such as drought, extreme temperature, heavy metal toxicity, light intensity, salinity, UV radiation (9). Medicinal plants are susceptible to heavy metal accumulation like arsenic (As), cadmium (Cd), chromium (Cr), mercury (Hg) and lead (Pb) in the soil due to heavy metal contamination from industrial effluents, pesticides and fertilizers (10). The Cd metal, which is toxic at lower concentrations, reduces nutrients and water uptake and causes oxidative damage, inhibiting plant growth, morphology and physiology (11). Hg causes growth retardation, displaces essential elements and causes protein disruption (12). Pb is toxic at higher concentration. It inhibits seed germination and overall plant growth, suppresses chlorophyll synthesis, ATP production and transpiration and reduces protein and water content (13).

The heavy metals in the soil gains entry to the plant roots through apoplastic and/or symplastic pathways. It then enters into the xylem stream via root symplasm and translocate to shoot via the transpiration stream in the xylem and transpiring shoot parts (14). Moreover, the entry and bioaccumulation of heavy metals into humans and animals through the intake of medicinal herbs grown in heavy metal-contaminated sites is a major concern for traditional and herbal medicine(15). Mobile heavy metals can affect the production of secondary metabolites in medicinal plants by either inhibiting or stimulating the biosynthetic pathways. In some plant species, these metals can deactivate the genes responsible for producing the enzymes involved in biosynthesis, leading to a suppression of secondary metabolite production. In contrast, in other plant species, heavy metals can activate gene expression for enzymes involved in biosynthesis, leading to an increase in secondary metabolite production (10).

W. somnifera, one of the most adaptogenic and commercially important medicinal plants, is widely cultivated in Africa, Asia, Australia and Europe. The plant is prone to heavy metal contamination when grown in heavy metal contaminated soil. There is scanty research on the effect of Pb and Cd with a limited range of metal concentrations (16) and no studies on the effect of other heavy metals such as Hg reported in *W. somnifera.* There are no substantial reports on metal accumulation and its effect on the biosynthesis of important metabolites in *W. somnifera*. With this research gap, the present study investigates the effects of Cd, Hg and Pb on germination, vegetative growth, biochemical variations, antioxidant activity and withanolide content in *W. somnifera*.

Materials and Methods

Impact of heavy metals on seed germination of W. somnifera

The authenticated seeds of *W. somnifera* (L.) Dunal (Arka ashwagandha) were procured from ICAR-Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru, India (13°08'04.7"N 77°29'27.2"E). The seeds were thoroughly washed with soap solution (1:10 soap: water) for 2-3 minutes and surface sterilized with 0.5%(w/v) sodium hypochlorite (4–5min). The seeds were transferred to seed trays filled with cocopeat pretreated with 10 different concentrations of Cd (20-200 ppm), Hg (10-100 ppm) and Pb (200-2000 ppm) solutions and allowed to germinate in the greenhouse and the germination was monitored at regular intervals of 7 days. The plants were harvested 21 days after sowing (DAS) and the germination % was calculated using the following formula (17).

Germination (%) =(No. of seeds germinated/Total no. of seeds sown) x 100

Measurement of Vegetative Growth Characteristics

The length and biomass of 21 days old germinated seedlings were measured to assess their growth and development. The seedlings were collected after harvest, washed under running tap water to remove any soil or debris from the roots and then the individual seedlings were separated from each other. To measure plant growth in terms of length and weight, only germinated seeds were considered and ungerminated seeds were excluded. The average plant length and the biomass of the germinated seeds were calculated for both the control and test concentrations. The length of each seedling was measured from the base of the root to the tip of the longest leaf using a ruler. The weight of the seedling was recorded using a calibrated weighing balance (18).

Evaluation of biochemical parameters

The biochemical parameters such as protein, carbohydrate, chlorophyll and proline content were evaluated in the 21-day-old ashwagandha plant treated with various heavy metals and untreated control plants. The protein content was estimated by Folin phenol–Lowry's method (19), the total carbohydrate content was estimated by phenol sulphuric acid method (20) with slight

modifications where 1g of homogenized sample was incubated in 5ml of 2.5N HCl, boiled for 3hrs and the cooled crude homogenate was centrifuged at 10000rpm for 10min, then 1ml of the resulting supernatant was mixed with 1ml of 2% (v/v) phenol and 5ml of 96% (v/v) sulfuric acid, followed by measuring the absorbance at 490nm using the UV–VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). The total chlorophyll content was estimated by Arnon's method (21) with slight modifications where 0.1 g of leaf sample was homogenized with 5ml of 80% (v/v) acetone, incubated overnight at room temperature, centrifuged at 5000rpm for 5min and then the optical densities of the supernatant were measured at 645 and 663nm wavelengths using the UV–VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan) and proline content by Bate's acid ninhydrin method (22).

Methanolic plant extract preparation

Dried, powdered plant samples (control and treated, each) of 0.2g were immersed in 5ml of methanol for 24h with constant stirring. The mixture was then filtered, following which the filtrate was evaporated to extract the residue. The residue was weighed and redissolved in a fixed volume of methanol to obtain 10mg ml^{-1} concentration. The extract was stored at 4°C until its next use and was further diluted based on the experimental setup.

Total phenol content (TPC) estimation by Folin–Ciocalteu method

The total phenolic content of the treated and untreated samples was performed as described by standard procedure(23). To 0.25ml of methanolic plant extract (5mg ml^{-1}), 0.25ml Folin–Ciocalteu reagent (1:1) and 0.5ml of 7.5% (w/v) sodium bicarbonate solution was added and incubated in dark conditions for 1hr at room temperature. The absorbance was recorded at 765nm after incubation using the microplate reader (BIO-RAD, iMARKTM, Japan). Using the standard calibration curve of gallic acid (0 to 50µg ml^{-1}), the phenol content of the samples was calculated.

Total Flavonoid Content (TFC) by the aluminum chloride method

The total flavonoid content of the treated and untreated samples was performed by the method described by Sembring et al. (23). 0.5ml of methanolic plant extract (10mg ml^{-1}) was taken, to which 0.1ml of 10% (w/v) aluminum chloride and 0.1ml of 1M sodium acetate were added and incubated at room temperature for 45min. The incubated samples' absorbance was recorded at 415nm using the microplate reader (BIO-RAD, iMARKTM, Japan). Using the standard calibration curve of quercetin (0 to 100µg ml^{-1}), the flavonoid content of the sample was calculated.

Total Malondialdehyde (MDA) content estimation by Thiobarbituric acid reactive substance (TBARS) assay

The total MDA was measured using the TBARS assay as described by Chandana (24). 0.1g of fresh plant sample was homogenized using 10ml of 0.1% (w/v) TCA and was centrifuged at 10000rpm for 5min To 1ml of the supernatant, 4ml of 20% (w/v) TCA containing 0.5% (w/v) TBA was added and heated at 95°C for 30min. It was then cooled, and the absorbance was recorded at 532nm using UV–VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan).

Antioxidant Activity

Non-enzymatic antioxidant assays:

Radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH):

The DPPH radical scavenging activity was assessed by the method described by Blois (25). 30µl (10mg mL^{-1}) of the plant extract was made up to 3ml with methanol, to which 1 ml of DPPH solution (0.1mM) was added and incubated in dark condition for 30min. The absorbance was recorded at 517nm using the UV–VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). Methanol (3ml) served as the blank. The DPPH radical scavenging activity was calculated using the formula:

DPPH radical scavenging activity (%) = $(A_{control} - A_{sample} / A_{control}) \times 100$

where $A_{control}$ and A_{sample} are the absorbance of control without plant extract and sample with plant extract, respectively.

Metal chelating activity and reducing power assay:

The metal chelating activity was assessed by the method described by Chew et al. (26) and the reducing power was measured by the FRAP method as described by Chung et al. (27) in 500µg ml^{-1} extract. The absorbance was measured by using a UV–VIS spectrophotometer (Shimadzu, UV-1900, Kyoto, Japan). The metal chelating activity was calculated using the formula:

Metal chelating activity (%) = $(A_{control} - A_{sample} / A_{control}) \times 100$

where $A_{control}$ and A_{sample} are the absorbance of control without plant extract and sample with plant extract respectively.

Enzymatic antioxidant assays:

Ascorbate peroxidase (APX) activity:

The APX activity was measured following the method of Uarrota et al. (28) by monitoring the change in absorbance in the assay mixture containing 1.2ml potassium phosphate buffer (50mM; pH 7.0), 0.2ml EDTA (0.1mM), 0.2ml ascorbate, 0.2ml sample and 0.2ml of H_2O_2 (0.1mM) at 290 nm. The APX activity was expressed in µmoles min^{-1} mg^{-1} protein. (Extinction coefficient for ascorbate at 290 nm is 2.8 mM^{-1} cm^{-1}).

APX activity = (Change in absorbance min^{-1} X total reaction volume in mL X amount of protein in g in 1 ml of sample) / (Extinction coefficient for ascorbate) X volume of sample taken in ml)

Catalase (CAT) activity:

The catalase activity was measured following the method of Uarrota et al. (28) by monitoring the change in absorbance in the assay mixture containing 1.95ml potassium phosphate buffer (50 mM; pH 7.0). 0.05 ml

sample and 1.0ml of 0.059M H_2O_2 solution at 240nm. The catalase activity was expressed in µmoles min^{-1} mg^{-1} protein (the extinction coefficient for H_2O_2 at 240 nm is 43.6 M^{-1} cm^{-1}).

CAT activity = (Change in absorbance min^{-1} X total reaction volume in ml X amount of protein in g in 1 ml of sample) / (Extinction coefficient for H_2O_2) X volume of sample taken in ml)

Superoxide dismutase (SOD) activity:

The SOD activity was measured following the standard method (29). For the estimation of superoxide dismutase activity, 3ml of assay mixture containing 0.8ml of phosphate buffer (50mM; pH 7.0), 0.5ml methionine (13 mM), 0.5ml Nitro blue tetrazolium (NBT; 75mM), 0.5ml Ethylenediaminetetraacetic acid (EDTA; 0.1mM), 0.5ml riboflavin (2mM) and 0.2ml sample were incubated for 10min at room temperature under illumination from fluorescent lamp. The change in absorbance due to formazan formation was read at 560nm. The 50% inhibition was taken as equivalent to one unit of SOD activity.

X % inhibition of NBT reduction by SOD = ($A_{control}$– A_{sample} / $A_{control}$) X 100

where $A_{control}$ and A_{sample} are the absorbance of control without plant extract and sample with plant extract respectively.

50% inhibition is equal to 1 unit of the enzyme/ ml

Y unit/ ml = 1/X

Analysis of heavy metal accumulation in 21 days old W. somnifera

The heavy metals were analyzed by the wet acid digestion method as described by Turek et al. (30). The oven-dried (35°C±5°C) ashwagandha plant samples were powdered, and 0.5g of this sample was digested using an aqua regia mixture (HNO_3 and HCl in a 3:1 ratio) and was heated to 45°±5°C on a hot plate maintained in a fume hood until the volume was reduced to 10ml. It was then made up to 25ml with deionized water and filtered using Whatman No.42 filter paper. The heavy metals were analyzed using atomic absorption spectrophotometer (Shimadzu, AA-6880, Japan).

Quantification of withanolides using High performance liquid chromatography (HPLC)

The withanolide content in the heavy metal treated and untreated 21-day-old plants were carried out by method followed by Praveen and Murthy (31). 1.0g of dried plant material was dissolved in 5ml methanol and subjected to sonication for 20min, followed by centrifugation for 5min at 3000rpm. The supernatant was made up to 5ml with methanol. The samples were filtered through a 0.45µM nylon membrane filter and then subjected to the HPLC analysis. RP-HPLC system (Shimadzu scientific instrument LCMS-8040, Kyoto, Japan) with Sharpsil-U C-18, 250 (L) ×4.6mm and equipped with SPD40 UV-Vis detector and analytical software (Lab Solutions). The mobile phase consisted of a mixture of water and acetonitrile (30:70,v/v) at a flow rate of 1ml min^{-1} and the column temperature was maintained at 40°C. Absorbance was set at 212nm with isocratic elution and a run time of 20min. Withanolide A and withaferin A standard ranging from 0.4- 2mg ml^{-1} was employed to quantify the withanolides present in the sample. The HPLC grade Withanolide A and Withaferin A (purity- 99.3%) were procured from Natural Remedies Pvt. Ltd., Bengaluru, India.

Statistical Analysis

All the experiments were performed in triplicates and were statistically analysed using IBM SPSS Statistics software version 22.0. The validity and variability of results were confirmed using One-way ANOVA. The significant differences among means of control and metal-treated groups were analyzed by post hoc Duncan's multiple range test (DMRT) at $P \leq 0.05$. The data obtained was presented as means ± SE followed by the letter obtained from post hoc test (DMRT).

Result and Discussion

Impact of heavy metals on seed germination of W. somnifera

Seed germination is an important stage in the plant life cycle. It is one of the major reproduction methods in nature and the most extensively used propagation method in agriculture due to its high efficiency (32). The seeds sown in the soil is prone to various stress, and the metals present in the soil hamper the seed germination process (33). In the present study, as the concentration of metals in the growing media increased, there was a decrease in seed germination. The germination in the seeds of untreated and heavy metal treated *W. somnifera* is presented in Fig. 1. The lowest germination of 20% was observed in seeds treated with 2000 ppm lead compared to untreated control plants which showed 100% germination. The heavy metal concentration of 70 ppm of Hg, 140 ppm Cd and 400 ppm Pb showed 50% inhibition of seed germination (LD_{50}) (Fig. 2). In *Solanum nigrum*, the rate of seed germination decreased in seedlings treated with 200–300mol l^{-1} cadmium(34). The heavy metals cause inhibition of radical formation and mobilization of food storage, disturb cellular osmoregulation and suppress proteolytic activities, in turn causing inhibition of seed germination and seedling development (35).

Vegetative growth of W. somnifera under Cd, Hg and Pb stress

The vegetative stage indicates a period of growth between the germination and flowering stages of plant growth and is prone to exposure to various stress parameters. Heavy metals are such stress inducers that cause harmful effects such as growth retardation, low biomass accumulation, retardation of growth, disruption of nutrient assimilation, and senescence in the vegetative stage of the plant, and the response to heavy metal stress varies in different plant species (36). The heavy metal treated *W. somnifera* showed variation in the length and biomass in comparison to the control. The highest plant length of 10.37cm and the

lowest of 3.07cm were observed in plants treated with

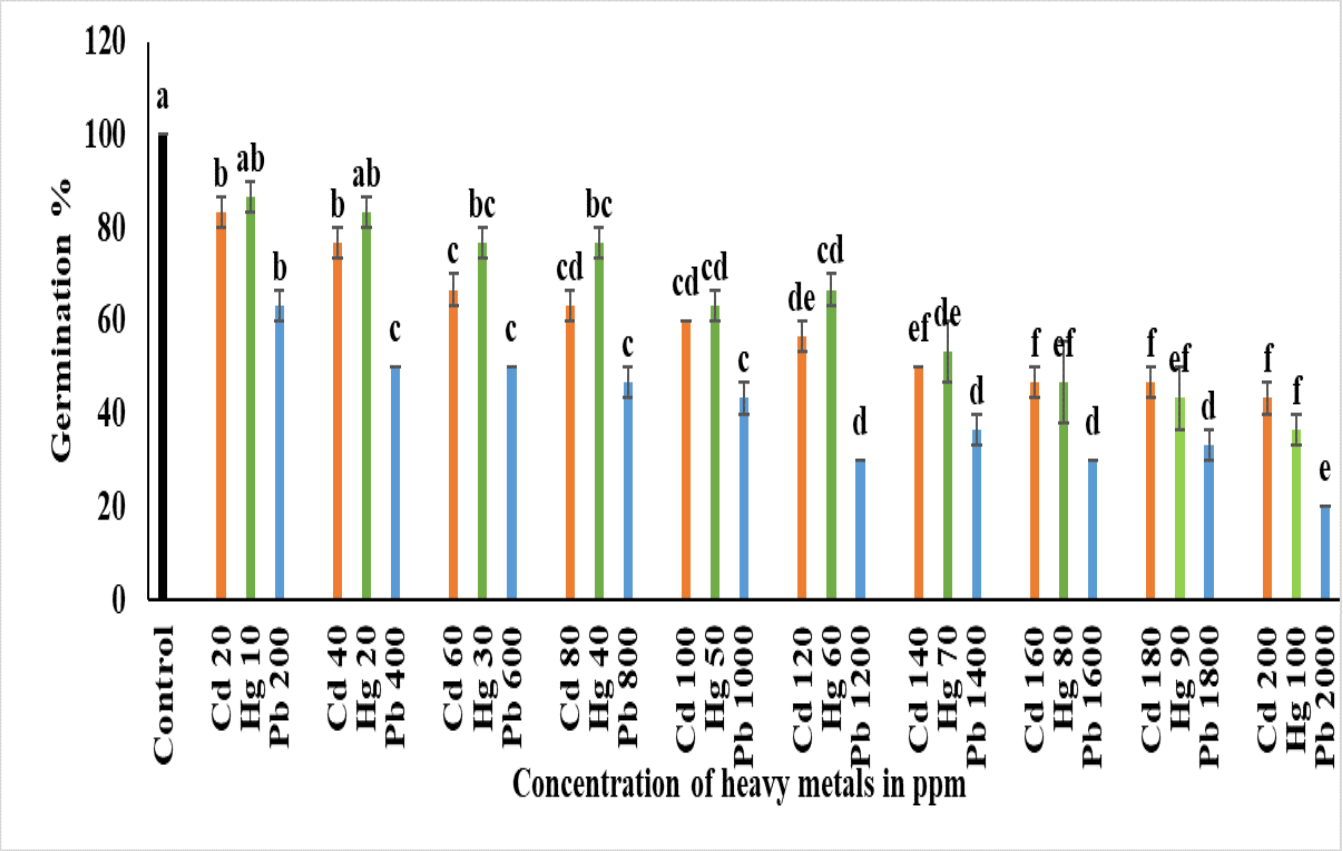

Fig.1. Effect of heavy metals on seed germination of *Withania somnifera* (L.) Dunal. Data represent mean values ± SE of 3 replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). Cd: Cadmium, Hg: Mercury, Pb: Lead.

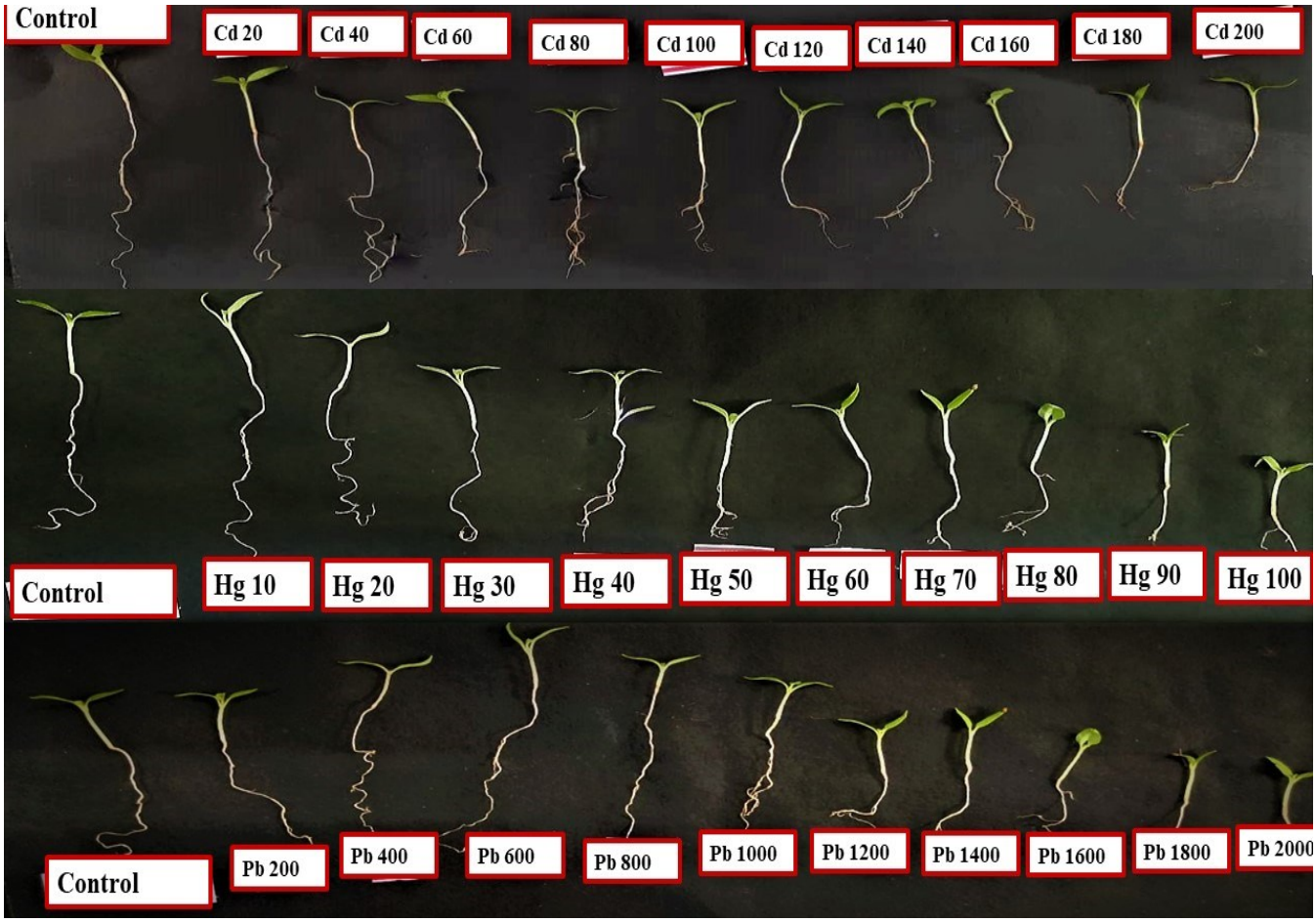

Fig.2. Germination of *Withania somnifera* (L.) Dunal treated with different concentrations of cadmium and untreated control.

lowest of 0.74g in 200 ppm cadmium (Table 1). The heavy metals inhibit root growth which alters water and nutrient uptake leading to suppression in shoot growth and thus affecting the overall plant growth (36).

Impact of different concentrations of Cd, Hg and Pb on biochemical parameters

Plants produce reactive oxygen species (ROS) as a primary response to heavy metal stress, which in turn alters the protein synthesis and its activity, enhances proline content, causes lipid peroxidation of membrane lipids, disruption of the chlorophyll molecule and metabolic pathways (37). Protein, an important component of the cell, has various biological activities and is easily prone to damage under heavy metal stress(38). Chlorophyll, an essential photosynthetic pigment, has been found to be decreased under metal stress, in turn influencing carbohydrate content. Further, the amount of multifunctional amino acid proline enhances in response to heavy metal stress in order to scavenge ROS, reconstruct chlorophyll, stabilize protein synthesis and other macromolecules and act as a metal chelator, thereby alleviating the stress caused by heavy metals (37). The presence of malondialdehyde (MDA), a product of lipid peroxidation, is an indication of tissue damage by ROS generated due to metal stress(39). The effect of different heavy metals on various biochemical parameters is represented in Table 2.

In the present study, the highest protein content of 2.63mg g^{-1} FW and lowest protein content of 1.17mg g^{-1} FW has been observed in untreated control and 2000 ppm lead-treated plants, respectively (Table 2). The decrease in protein content could be due to protein denaturation, enhanced protease activity, or the replacement of thiol residues in metalloproteins with heavy metals (40). The proline content increased with increasing concentration and the highest proline content of 17.22µ mol g^{-1} FW and the lowest proline content of 1.01µ mol g^{-1} FW were achieved in 200 ppm Cd treated and untreated control plants respectively (Table 2). Enhanced proline accumulation with increasing metal concentration could be due to proline dehydrogenase activity and decreased usage of proline (41).

The chlorophyll content was affected and showed a decline with increasing metal concentration. The highest chlorophyll content of 1.5mg g^{-1} FW and the lowest of 0.45mg g^{-1} FW have been observed in control and 100 ppm Hg treated plants, respectively (Table 2). The reduction in chlorophyll content could be due to the replacement of Mg with heavy metals, a decrease in essential metals (Zn and Mg) and essential enzymes (δ-aminolevulinic acid) involved in chlorophyll synthesis or lipid peroxidation in chloroplast membrane (42). The carbohydrate content decreased with increasing metal concentration and the carbohydrate content of 23.4mg g^{-1} FW (highest) and 12.83±0.56mg g^{-1} FW (lowest) was obtained in control and 100 ppm Hg treated plants respectively (Table 2). The decrease in carbohydrate content is due to the disrupted photosynthetic machinery or loss of chlorophyll pigment (43). The MDA content also increased with the highest of 3.44µ moles g^{-1} FW and the lowest of 1.01µ moles g^{-1} FW observed in 200 ppm and control plants respectively (Table 2). The increasing heavy metal concentration increased the generation of free radical species, thereby increasing membrane lipid peroxidation, subsequently forming MDA (39).

Impact of different concentrations of Cd, Hg, and Pb on TPC and TFC

Phenolic compounds are an important group of secondary metabolites containing one or more hydroxyl groups. They are sub grouped as simple phenolics and polyphenols such

Table 1. The effect of heavy metals on plant growth

Cadmium conc. (ppm)	Biomass (g)	Length (cm)	Mercury conc. (ppm)	Biomass (g)	Length (cm)	Lead conc. (ppm)	Biomass (g)	Length (cm)
Control	1.85±0.1^{a}	6.03±0.61^{fg}	Control	1.85±0.1^{a}	6.03±0.61^{bc}	Control	1.85±0.1^{g}	6.03±0.61^{fg}
20	1.91±0.09^{a}	7.2±0.69^{cde}	10	1.85±0.06^{a}	8.17±0.03^{a}	200	3.2±0.13^{bc}	7.77±0.33^{cde}
40	1.79±0.1^{ab}	7.03±0.38^{b}	20	1.6±0.08^{ab}	6.67±0.19^{b}	400	3.38±0.14^{bc}	9±0.3^{b}
60	1.69±0.09^{abc}	6.2±0.53^{a}	30	1.37±0.08^{bc}	6.17±0.5^{b}	600	3.87±0.11^{a}	10.37±0.13^{a}
80	1.53±0.1^{bcd}	5.83±0.09^{bc}	40	1.23±0.08^{cd}	5.73±0.47^{bcd}	800	3.12±0.1^{bc}	8.57±0.55^{bc}
100	1.42±0.08^{cde}	5.41±0.52^{bcd}	50	1.09±0.07^{de}	5.73±0.64^{bcd}	1000	2.96±0.06^{cd}	7.93±0.44^{bcd}
120	1.29±0.11^{de}	5.27±0.15^{de}	60	1.07±0.09^{de}	4.63±0.44^{de}	1200	2.72±0.09^{de}	7.37±0.39^{de}
140	1.21±0.09^{ef}	4.68±0.18^{ef}	70	1.01±0.08^{de}	4.7±0.32^{de}	1400	2.65±0.08^{e}	6.67±0.33^{ef}
160	1±0.1^{fg}	3.29±0.36^{fg}	80	0.88±0.07^{e}	4.77±0.27^{cde}	1600	2.16±0.07^{f}	5.6±0.35^{fg}
180	0.77±0.09^{g}	3.07±0.51^{gh}	90	0.62±0.1^{f}	3.47±0.3^{e}	1800	1.98±0.1^{fg}	5±0.31^{gh}
200	0.74±0.11^{g}	3.18±0.43^{h}	100	0.5±0.1^{f}	4.13±0.32^{e}	2000	1.41±0.1^{h}	4.07±0.07^{h}

Data represent mean values±SE of three replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P≤0.05 according to Duncan's multiple range test (DMRT)

Table 2. The effect of heavy metals on biochemical parameters in *Withania somnifera*

Mercury concentration in ppm	**Control**	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**
Protein (mg g^{-1}FW)	2.63±0.13^{a}	2.43±0.02^{b}	2.06±0.04^{c}	2.08±0.0^{c}	2.08±0.0^{c}	1.9±0.02^{d}	1.83±0.0^{d}	1.71±0.04^{ef}	1.64±0.02^{fg}	1.58±0.02^{fg}	1.52±0.02^{g}
Carbohydrate (mg g^{-1} FW)	23.44±0.52^{a}	21.47±0.39^{b}	20.58±0.71^{b}	17.78±0.53^{c}	17.25±0.25^{cd}	16.63±0.25^{cde}	16.33±0.19^{de}	15.99±0.1^{def}	15.38±0.42^{ef}	14.93±0.39^{f}	12.83±0.56^{g}
Proline (µ mol g^{-1} FW)	1.01±0.11^{h}	2.07±0.12^{g}	3.54±0.07^{f}	3.98±0.1^{e}	4.09±0.0^{e}	4.61±0.1^{d}	4.72±0.1^{c}	4.85±0.11^{bcd}	5±0.13^{abc}	5.16±0.14^{ab}	5.29±0.1^{a}
Chlorophyll (mg g^{-1} FW)	1.5±0.05^{a}	1.37±0.02^{ab}	1.36±0.02^{ab}	1.32±0.02^{ab}	1.25±0.02^{bc}	1.23±0.02^{bc}	1.23±0.02^{bc}	1.19±0.02^{bc}	0.59±0.02^{cd}	0.51±0.01^{d}	0.45±0.0c^{d}
MDA (µ moles g^{-1} FW)	1.01±0.01^{i}	1.1±0.02^{i}	1.24±0.06^{h}	1.36±0.02^{g}	1.49±0.03^{f}	1.61±0.02^{e}	1.65±0.01^{e}	1.8±0.03^{d}	1.98±0.01^{c}	2.11±0.02^{b}	2.39±0.0^{a}

Cadmium concentration in ppm	**Control**	**20**	**40**	**60**	**80**	**100**	**120**	**140**	**160**	**180**	**200**
Protein (mg g^{-1} FW)	2.63±0.13^{a}	2.47±0.05^{b}	2.39±0.01^{b}	2.17±0.0^{c}	2.02±0.0^{d}	1.98±0.0^{d}	1.91±0.03^{de}	1.89±0.01^{de}	1.88±0.02^{def}	1.83±0.01^{ef}	1.74±0.01^{f}
Carbohydrate	23.44±0.52^{c}	23.78±0.54^{c}	24.28±0.44^{bc}	25.13±0.14^{ab}	25.64±0.13^{a}	22.18±0.31^{d}	19.94±0.35^{e}	18.41±0.22^{f}	18.14±0.13^{f}	16.64±0.55^{g}	15.53±0.74^{g}
Proline (µ mol g^{-1} FW)	1.01±0.11^{k}	3.99±0.06^{j}	4.21±0.06^{i}	6.94±0.06^{h}	7.9±0.06^{g}	8.74±0.05^{f}	9.73±0.06^{e}	11.88±0.05^{d}	12.7±0.05^{c}	14.42±0.05^{b}	17.22±0.05^{a}
Chlorophyll (mg g^{-1} FW)	1.5±0.05^{ab}	1.54±0.01^{a}	1.54±0.01^{a}	1.48±0.02^{ab}	1.44±0.01^{ab}	1.18±0.01^{abc}	1.08±0.01^{abc}	0.95±0.01^{bc}	0.87±0^{c}	0.82±0.01^{c}	0.72±0^{c}
MDA (µ moles g^{-1} FW)	1.01±0.01^{de}	1.02±0.03^{e}	1.09±0.04^{de}	1.11±0.03^{de}	1.2±0.02^{d}	1.25±0.03^{cde}	1.29±0.03^{cd}	1.39±0.02^{cd}	1.79±0.06^{c}	2.99±0.26^{b}	3.44±0.13^{a}

Lead concentration in ppm	**Control**	**200**	**400**	**600**	**800**	**1000**	**1200**	**1400**	**1600**	**1800**	**2000**
Protein (mg g^{-1} FW)	2.63±0.13^{c}	3.02±0.05^{b}	3.25±0.03^{a}	2.16±0.01^{d}	2.09±0.01^{e}	1.91±0.01^{e}	1.83±0.02^{e}	1.78±0.02^{ef}	1.68±0.02^{fg}	1.57±0.02^{g}	1.17±0.04^{h}
Carbohydrate	23.44±0.52^{a}	22.59±0.25^{b}	21.73±0.29^{c}	21.33±0.22^{c}	18.86±0.39^{d}	18.07±0.16^{d}	17.26±0.14^{e}	16.52±0.11^{e}	15.69±0.24^{f}	15.34±0.13^{f}	13.98±0.23^{g}
Proline (µ mol g^{-1} FW)	1.01±0.11^{i}	1.36±0.09^{h}	1.65±0.07^{g}	2.88±0.07^{f}	2.86±0.04^{f}	4.82±0.04^{e}	4.91±0.08^{e}	5.12±0.06^{d}	6.65±0.04^{c}	7.6±0.08^{b}	8.79±0.06^{a}
Chlorophyll (mg g^{-1} FW)	1.5±0.05^{a}	1.48±0.12^{a}	1.35±0.01^{ab}	1.2±0.01^{a} ^{bc}	1.15±0.02^{abc}	1.08±0.02^{abc}	0.94±0.01^{bc}	0.8±0^{cd}	0.75±0^{cd}	0.7±0^{cd}	0.55±0^{d}
MDA (µ moles g^{-1} FW)	1.01±0.01^{g}	1.17±0.04^{fg}	1.2±0.04^{fg}	1.25±0.03^{efg}	1.3±0.03e^{fg}	1.37±0.02^{efg}	1.56±0.02^{de}	1.71±0.1^{c} ^{d}	1.92±0.04^{bc}	2.12±0.03^{bc}	2.84±0.33^{a}

Data represent mean values±SE of three replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P≤0.05 according to Duncan's multiple range test (DMRT)

as flavonoids and tannins. The hydroxyl and carboxyl groups of phenolic compounds suppress the ROS-producing Fenton reaction (44). In heavy metal-treated *W. somnifera*, the highest phenol content of 7.53mg g^{-1} DW and lowest phenol content of 2.20mg g^{-1} DW have been observed in 120 ppm Cd treated plant and untreated control plants respectively (Fig. 3). The highest flavonoid content of 5.55mg g^{-1} DW and the lowest of 1.36 mg g^{-1} DW in 100 ppm Cd treated plants and 100 ppm Hg treated plants respectively (Fig. 3). The increase in total phenol and flavonoid content at lower metal concentrations could be due to the enhanced activity of enzymes involved in de novo synthesis of phenolics and glycosidic conjugate hydrolysis, respectively (44). However, at higher doses of metal concentration, the phenol and flavonoid content decreased due to a decline in the activity of enzymes involved in the biosynthesis of phenolic compounds (45).

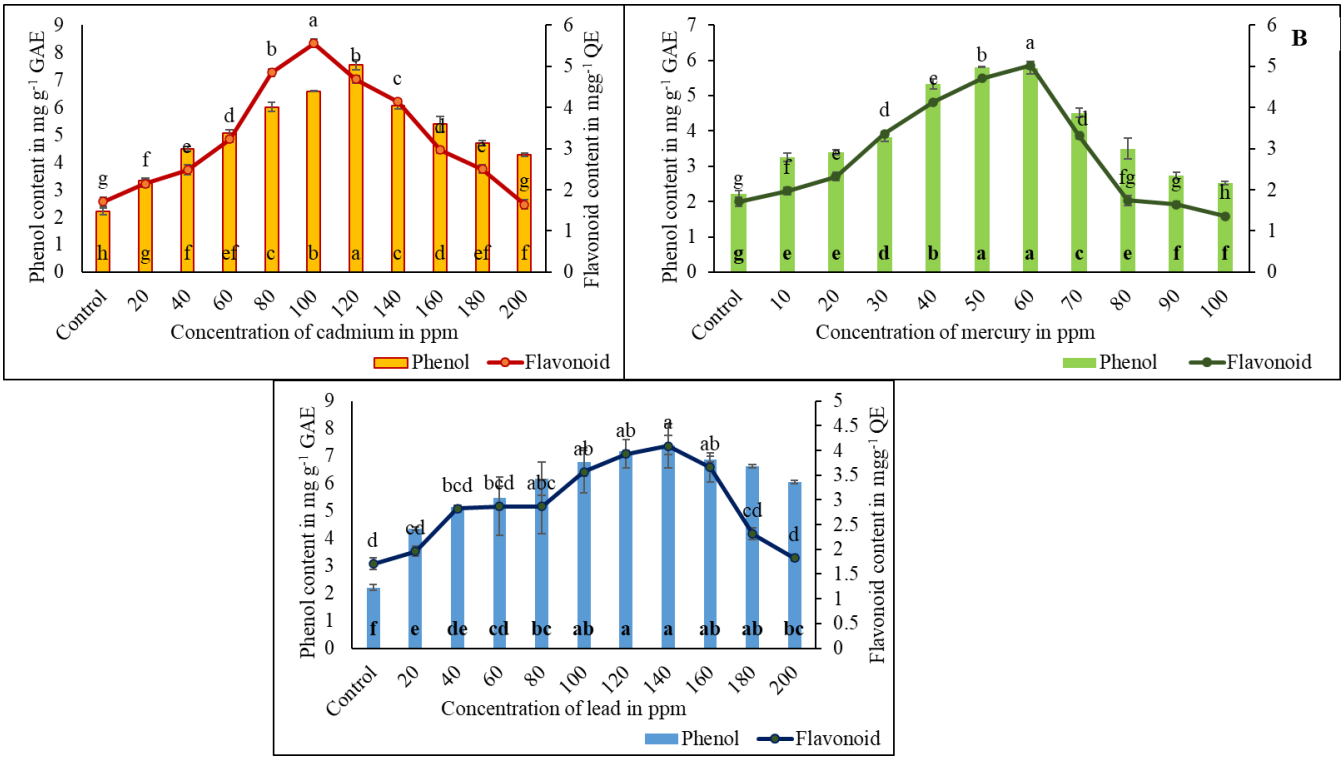

Fig. 3. Total phenol and flavonoid content in (A) Cadmium treated, (B) Mercury treated, (C) Lead treated *Withania somnifera*. Data represent mean values ± SE of 3 replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P ≤ 0.05 according to Duncan's multiple range test (DMRT).

Non-enzymatic and enzymatic antioxidant activity in heavy metal treated W. somnifera

Plants under heavy metal stress have developed enzymatic and non-enzymatic antioxidant systems to scavenge the reactive oxygen species. Proline, phenolic compounds and glutathione act as non-enzymatic antioxidants to protect against oxidative stress, whereas enzymes such as catalase, superoxide dismutase, and peroxidase function as enzymatic antioxidants, playing a crucial role in protecting organisms from oxidative stress (46, 47). DPPH radical scavenging assay, metal chelating activity and FRAP assay indicate the non-enzymatic antioxidant activity. In the present study, the non-enzymatic activity increased at lower metal concentrations and decreased with a further increase in metal concentration which has been represented in Fig. 4. The highest DPPH activity of 84.02% and highest metal chelating activity of 68.55% in 1400 ppm Pb treated plants,

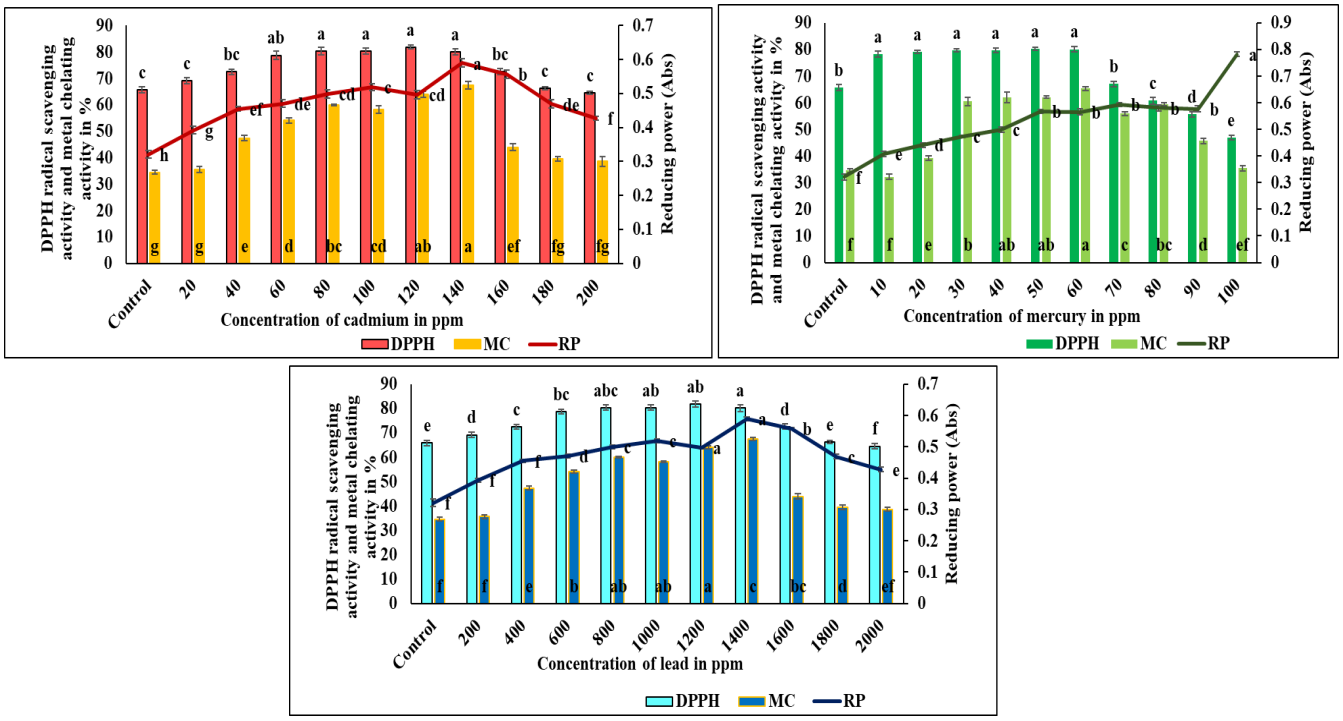

Fig. 4. Non enzymatic activity in (A) Cadmium treated, (B) Mercury treated, © Lead treated *Withania somnifera*. Data represent mean values ± SE of three replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P ≤ 0.05 according to Duncan's multiple range test (DMRT).

whereas the lowest DPPH activity of 46.92%, and the lowest metal chelating activity of 35.39% were observed in plants treated with 100 ppm Hg treated plants. The reducing power of 0.782Abs (highest) and 0.314Abs (lowest) were observed in plants treated with 100 ppm Hg and 400 ppm Pb respectively. The initial increase in non-enzymatic antioxidant activity can be correlated with the total phenol and flavonoid content produced in heavy metal-treated plants.

The enzymes such as SOD, CAT and peroxidase are involved in antioxidant activity by scavenging ROS. The SOD enzymes convert superoxides into H_2O_2. The catalase enzyme lowers H_2O_2 by scavenging it into H_2O and O_2 molecules. The peroxidase enzyme catalyzes oxidoreduction of H_2O_2 (37). The highest APX activity of 0.14µmoles min^{-1} mg^{-1} protein, catalase activity of 31.09µmoles min^{-1} mg^{-1} protein and SOD activity of 75.39 units mg^{-1} protein was obtained in plants treated with 200 ppm Cd, 100 ppm mercury and 2000 ppm lead respectively The lowest APX activity of 0.05µmoles min^{-1} mg^{-1} protein, catalase activity of 1.74µmoles min^{-1} mg^{-1} protein in untreated control plants and the lowest SOD activity of 18.24units mg^{-1} protein was obtained in control plants (Table 3). The increase in enzymatic antioxidants is due to de-novo synthesis of the enzymatic proteins and induction of the expression of genes encoding APX, CAT and peroxidase enzymes in response to ROS accumulation under heavy metal stress. However, it has been observed that CAT activity has decreased under higher doses of Cd and Pb. This could be due to proteolytic degradation, enzyme inactivation, disturbance in CAT subunits assembling and/or proteolytic degradation by peroxisomal protease (37).

Accumulation of heavy metals in W. somnifera

The uptake of heavy metals by the seeds during the germination can be quantified using AAS. The amount of metal in the plant indicates the plant's ability to uptake the metals. The metal uptake varies with the plant species exhibiting different morphologies and the metal to which the plant is exposed (48). The metal accumulation increased in a dose-dependent manner in all the 3 metal-treated plants. The highest Cd accumulation of 14.30mg $kg^{-1}$and Hg accumulation

Table 3. The effect of heavy metals on enzymatic antioxidants in *Withania somnifera*

Cd conc (ppm)	APX (µmoles min^{-1} mg^{-1} protein)	CAT (µmoles min^{-1} mg^{-1} protein)	SOD (units mg^{-1} protein)	Hg conc. (ppm)	APX (µmoles min^{-1} mg^{-1} protein)	CAT (µmoles min^{-1} mg^{-1} protein)	SOD (units mg^{-1} protein)	Pb conc (ppm)	APX (µmoles min^{-1} mg^{-1} protein)	CAT (µmoles min^{-1} mg^{-1} protein)	SOD (units mg^{-1} protein)
Control	0.05±0.01^{f}	1.74±0.12^{c}	21.65±0.17^{j}	**Control**	0.05±0.01^{d}	1.74±0.12^{g}	21.65±0.17^{k}	**Control**	0.05±0.01^{c}	1.7±0.11^{c}	21.65±0.17^{g}
20	0.03±0.01^{ef}	5.85±0.49^{b}	23.26±0.45^{i}	**10**	0.05±0^{d}	3.24±0.62^{g}	23.74±0.32^{j}	**200**	0.02±0^{d}	1.95±0.33^{c}	18.85±0.29^{g}
40	0.05±0.01^{e}	5.57±0.44^{b}	25.26±0.28^{h}	**20**	0.06±0.01^{cd}	5.71±0.73^{f}	28.82±0.33^{i}	**400**	0.02±0^{d}	2.83±0.44^{c}	18.24±0.27^{g}
60	0.06±0.01^{def}	6.13±0.48^{b}	32.05±0.31^{g}	**30**	0.06±0.01^{cd}	6.14±0.72^{f}	33.24±0.77^{h}	**600**	0.05±0.01^{c}	7.9±1.54^{ab}	27.23±0.31^{g}
80	0.06±0.01^{cdef}	7.75±0.69^{b}	36.39±0.34^{e}	**40**	0.08±0.01^{c}	6.62±0.72^{f}	35.76±0.69^{g}	**800**	0.04±0.01^{cd}	9.42±1.9^{a}	30.95±0.64^{f}
100	0.08±0.01^{bcde}	10.83±1.16^{a}	38.96±0.34^{d}	**50**	0.08±0.01^{c}	7.75±0.79^{ef}	40.41±0.7^{f}	**1000**	0.06±0.01^{bc}	11.69±2.42^{a}	39±0.59^{e}
120	0.09±0.01^{bcd}	12.46±1.39^{a}	42.86±0.36b	**60**	0.08±0.01^{c}	9.13±0.82^{de}	44.4±0.61^{e}	**1200**	0.07±0.01^{b}	10.73±2.17^{a}	41.16±1.06^{de}
140	0.08±0.01^{bcde}	11.33±1.21^{a}	47.29±0.36^{a}	**70**	0.13±0.01^{b}	10.95±0.88^{cd}	49.45±0.8^{d}	**1400**	0.11±0.01^{a}	4.42±0.64^{bc}	43.52±0.76^{d}
160	0.1±0.01^{bc}	7.69±0.65^{b}	43.53±0.36^{b}	**80**	0.15±0.01^{b}	12.61±0.92^{c}	52.01±0.31^{c}	**1600**	0.11±0.01^{a}	3.12±0.39^{c}	47.97±0.4^{c}
180	0.11±0.01^{ab}	7.92±0.67^{b}	40.16±0.37^{c}	**90**	0.14±0.01^{b}	20.5±0.95^{b}	56.22±0.43^{b}	**1800**	0.11±0.01^{a}	3.34±0.42^{c}	56.8±0.57^{b}
200	0.14±0.01^{a}	7.64±0.6^{b}	35.09±0.39^{f}	**100**	0.25±0.01^{a}	31.09±0.99^{a}	60.49±0.45^{a}	**2000**	0.11±0.01^{a}	2.24±0.56^{c}	75.39±0.96^{a}

Data represent mean values±SE of three replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). Cd: Cadmium, Hg: Mercury, Pb: Lead

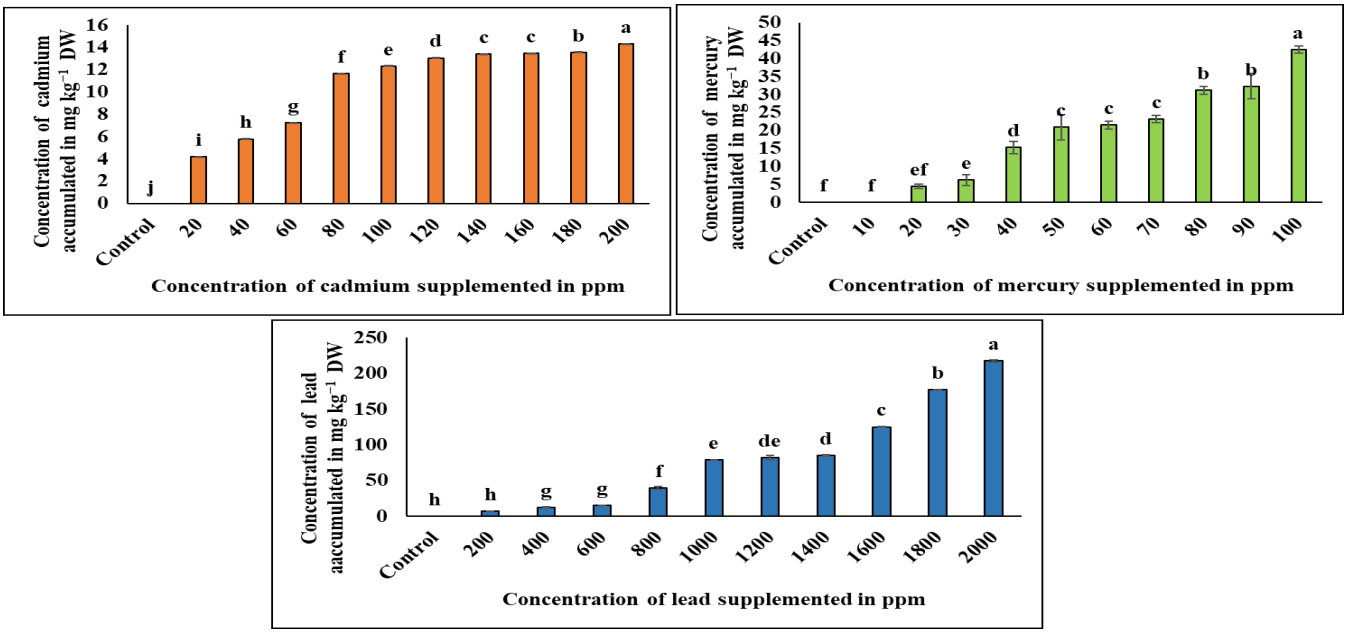

Fig.5. The bioaccumulation of heavy metals in (A) Cadmium treated, (B) Mercury treated,(C) Lead treated *Withania somnifera*. Data represent mean values ± SE of 3 replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P ≤ 0.05 according to Duncan's multiple range test (DMRT).

of 42.45mg kg^{-1},and Pb accumulation of 217.46mg kg^{-1} was observed and the lowest metal accumulation of 4.19mg kg^{-1} Cd, 4.53mg kg^{-1} Hg and 7.44mg kg^{-1} Pb (Fig. 5). The uptake of heavy metal ions from soil and their translocation within the plant would have been facilitated by specialized transporters such as metal ion transporters and complexing agents in the plasma membrane of root cells (14).

Withanolide content in heavy metal-treated W. somnifera

Withanolides are one of the major secondary metabolites found in *W. somnifera.* The heavy metals that gain entry into the plant disturb not only the overall plant growth but also the secondary metabolite production(10). In the present study, the withanolide A and withaferin A content increased at lower doses of metal; however, at higher metal concentrations, the withanolide content decreased. The highest withanolide A content of 1.7mg g^{-1} and highest withaferin A content of 3.2mg g^{-1} of dry weight were obtained in plants treated with 1200 ppm Pb and 80 ppm Cd treated plants respectively. The lowest withanolide A content of 0.2mg g^{-1} and withaferin A content of 0.19mg g^{-1} in 100 ppm Hg treated plants (Table 4). Similar to our studies, metal toxicity has been evaluated on

Table 4. The effect of heavy metals on withanolides in *Withania somnifera*

Cd Conc in ppm	Withaferin A (mg g^{-1})	Withanolide A (mg g^{-1})	Hg Conc in ppm	Withaferin A (mg g^{-1})	Withanolide A (mg g^{-1})	Pb Conc in ppm	Withaferin A (mg g^{-1})	Withanolide A (mg g^{-1})
Control	0.54±0.002^{i}	0.97±0.002^{j}	**Control**	0.54±0.002^{g}	0.97±0.002^{c}	**Control**	0.54±0.002^{h}	0.99±0.017^{i}
20	0.67±0.001^{g}	1.33±0.002^{g}	**10**	0.52±0.001^{h}	0.55±0.003^{g}	**200**	0.54±0.001^{h}	0.97±0.002^{j}
40	1.13±0.001^{e}	1.79±0.002^{e}	**20**	0.56±0.002^{f}	0.55±0.002^{g}	**400**	0.71±0.004^{g}	1.58±0.001^{g}
60	1.35±0.002^{b}	2.5±0.002^{b}	**30**	0.7±0.002^{c}	0.79±0^{d}	**600**	1.21±0.006^{d}	2.16±0.002^{e}
80	1.85±0.002^{a}	3.22±0.001^{a}	**40**	0.57±0.003^{e}	0.63±0.001^{f}	**800**	1.35±0^{c}	2.15±0.001^{f}
100	1.27±0.002^{c}	2.38±0.002^{c}	**50**	0.96±0.003^{b}	1.02±0.001^{b}	**1000**	1.53±0.001^{b}	2.98±0.004^{b}
120	1.22±0.001^{d}	2.15±0.001^{d}	**60**	1±0.001^{a}	1.09±0.002^{a}	**1200**	1.66±0.003^{a}	3.31±0^{a}
140	0.63±0.002^{h}	1.31±0.003^{h}	**70**	0.6±0.001^{d}	0.76±0.002^{e}	**1400**	1.35±0.003^{c}	2.22±0^{c}
160	0.52±0.001^{j}	0.98±0.001^{i}	**80**	0.4±0.002^{i}	0.41±0.002^{h}	**1600**	1.12±0.003^{e}	2.21±0.002^{d}
180	0.3±0.001^{k}	0.55±0.001^{k}	**90**	0.37±0.003^{j}	0.38±0.002^{i}	**1800**	0.75±0.002^{f}	1.45±0.001^{h}
200	0.9±0^{f}	1.42±0.004^{f}	**100**	0.19±0.001^{k}	0.2±0.001^{j}	**2000**	0.3±0.001^{i}	0.58±0.001^{j}

Data represent mean values±SE of three replicates; each experiment was repeated thrice. Means with common letters are not significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). Cd: Cadmium, Hg: Mercury, Pb: Lead.

andrographolide production in *Andrographis paniculata* where concentration dependent metal toxicity was observed for andrographolide production (49). The heavy metal stress condition induces the defense mechanism in plants which in turn alters the genes' transcription of the enzymes involved in the biosynthesis and accumulation of secondary metabolites. However, the increase in stress and time of exposure to stress, damages the plant and reduces the accumulation of secondary metabolites as the plants tend to invest the energy to stay alive instead of synthesizing secondary metabolites (50).

Conclusion

In conclusion, the plants treated with heavy metals (Cd, Hg and Pb) have shown variations in vegetative and biochemical parameters such as protein, carbohydrate, chlorophyll and MDA content. The accumulation of the heavy metal in plants was dose-dependent. The plants could adapt to stress conditions by combating the toxic effect caused by ROS molecules produced under stress. The heavy metal-treated plants could survive the stress by showing enhanced proline production, increased enzymatic antioxidant activity and non-enzymatic antioxidants. The withanolides like withanolide A and withaferin A increased at lower metal concentrations. Even though the *W. somnifera* plants could tolerate heavy metal stress at a lower metal concentration of Cd, Hg, and Pb, the important plant metabolites decreased and the plants' efficacy reduced at higher metal concentrations. Also, the metals accumulated increasingly, making them unfit for direct consumption. However, the withanolides increased at lower metal concentrations which could be purified and commercialized.

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Authors' contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Compliance with ethical standards

This study does not involve experiments on animals and/or humans.

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

References

1. Afewerky HK, Ayodeji AE, Tiamiyu BB, Orege JI, Okeke ES, Oyejobi AO et al. Critical review of the *Withania somnifera* (L.) Dunal: Ethnobotany, pharmacological efficacy and commercialization significance in Africa. Bull Natl Salmon Resour Cent. 2021;45(1):176. https://doi.org/10.1186/s42269-021-00635-6.
2. Kulkarni SK, Dhir A. *Withania somnifera*: an Indian ginseng. Prog Neuropsychopharmacol Biol Psychiatry. 2008;32(5):1093-05. https://doi.org/10.1016/j.pnpbp.2007.09.011.
3. Balkrishna A, Sinha S, Srivastava J, Varshney A. *Withania somnifera* (L.) Dunal whole-plant extract demonstrates acceptable non-clinical safety in rat 28-day subacute toxicity evaluation under GLP-compliance. Sci Rep. 2022;12(1):11047. https://doi.org/10.1038/s41598-022-14944-x.
4. Saggam A, Limgaokar K, Borse S, Chavan-Gautam P, Dixit S, Tillu G et al. *Withania somnifera* (L.) Dunal: Opportunity for clinical repurposing in COVID-19 management. Front Pharmacol. 2021;12:623795. https://doi.org/10.3389/fphar.2021.623795.
5. Mukherjee PK, Banerjee S, Biswas S, Das B, Kar A, Katiyar CK. *Withania somnifera* (L.) Dunal - Modern perspectives of an ancient Rasayana from Ayurveda. J Ethnopharmacol. 2021; 264:113157. https://doi.org/10.1016/j.jep.2020.113157.
6. Kalra R, Kaushik N. *Withania somnifera* (Linn.) Dunal: a review of chemical and pharmacological diversity. Phytochem Rev. 2017;16(5):953-87. https://doi.org/10.1007/s11101-017-9504-6.
7. Newswire CDN, Sandle T. Ashwagandha market future demand, business strategies, industry growth, regional outlook, challenges and forecast by 2029 [Internet]. Digital Journal. Digital Journal Inc; 2022 [cited 12 Jan 2023]. Available from: https://www.digitaljournal.com/pr/ashwagandha-market-future-demand-business-strategies-industry-growth-regional-outlook-challenges-and-forecast-by-2029.
8. Ahlawat S, Saxena P, Ali A, Khan S, Abdin MZ. Comparative study of withanolide production and the related transcriptional responses of biosynthetic genes in fungi elicited cell suspension culture of *Withania somnifera* in shake flask and bioreactor. Plant Physiol Biochem. 2017;114:19-28.https://doi.org/10.1016/j.plaphy.2017.02.013.
9. Mareri L, Parrotta L, Cai G. Environmental stress and plants. Int J Mol Sci. 2022; 23(10):5416. https://doi.org/10.3390/ijms23105416.
10. Asgari Lajayer B, Ghorbanpour M, Nikabadi S. Heavy metals in contaminated environment: Destiny of secondary metabolite biosynthesis, oxidative status and phytoextraction in medicinal plants. Ecotoxicol Environ Saf. 2017;145:377-90. https://doi.org/10.1016/j.ecoenv.2017.07.035.
11. Haider FU, Liqun C, Coulter JA, Cheema SA, Wu J, Zhang R et al. Cadmium toxicity in plants: Impacts and remediation strategies. Ecotoxicol Environ Saf. 2021;211:111887. https://doi.org/10.1016/j.ecoenv.2020.111887.
12. Gworek B, Dmuchowski W, Baczewska-Dąbrowska AH. Mercury in the terrestrial environment: a review. Environ Sci Eur. 2020;32(1):1-19. https://doi.org/10.1186/s12302-020-00401-x.
13. Pourrut B, Shahid M, Dumat C, Winterton P, Pinelli E. Lead uptake, toxicity and detoxification in plants. Rev Environ Contam Toxicol. 2011;213:113-36. https://doi.org/10.1007/978-1-4419-9860-6_4.
14. Yan A, Wang Y, Tan SN, Mohd Yusof ML, Ghosh S, Chen Z. Phytoremediation: A promising approach for revegetation of heavy metal-polluted land. Front Plant Sci. 2020;11:359. https://doi.org/10.3389/fpls.2020.00359.
15. Behera B, Bhattacharya S. The importance of assessing heavy metals in medicinal herbs: A quantitative study. Cell Med. 2016;6(1):31-34. https://doi.org/10.5667/tang.2015.0029.
16. Saidulu C, Venkateshwar C, Rao SG. HPLC analysis of withanolides in heavy metal treated plant drug parts (leaves and roots) of *Withania somnifera* (L.) Dunal. World J Pharm

Res. 2014;3(5):657-61.
17. Gupta A, Rai S, Bano A, Khanam A, Sharma S, Pathak N. Comparative evaluation of different salt-tolerant plant growth-promoting bacterial isolates in mitigating the induced adverse effect of salinity in *Pisum sativum*. Biointerface Res Appl Chem. 2021;11(5):13141-54. https://doi.org/10.33263/BRIAC115.1314113154.
18. Pavel VL, Sobariu DL, Diaconu M, Stătescu F, Gavrilescu M. Effects of heavy metals on *Lepidium sativum* germination and growth. J Environ Eng Landsc Manage. 2013;12(4): 727-33. https://doi.org/10.30638/eemj.2013.089 .
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193(1):265-75. https://doi.org/10.1016/S0021-9258(19)52451-6.
20. DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956;28(3):350-56. https://doi.org/10.1021/ac60111a017
21. Arnon DI. Copper enzymes in isolated chloroplasts. polyphenoloxidase in *Beta vulgaris.* Plant Physiol. 1949;24 (1):1-15. https://doi.org/10.1104/pp.24.1.1.
22. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39(1):205-57. https://doi.org/10.1007/BF00018060.
23. Sembiring EN, Elya B, Sauriasari R. Phytochemical screening, total flavonoid and total phenolic content and antioxidant activity of different parts of *Caesalpinia bonduc* (L.) Roxb. Pharmacogn J. 2017;10(1):123-27. https://doi.org/10.5530/pj.2018.1.22.
24. Chandana C. Heavy metal accumulation and the biochemical responses in selected medicinal plants. School of Bio Sciences [thesis]. Athirampuzha: Mahatma Gandhi University; 2018.
25. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181(4617):1199-200. https://doi.org/10.1038/1811199a0.
26. Chew YL, Goh JK, Lim YY. Assessment of in vitro antioxidant capacity and polyphenolic composition of selected medicinal herbs from Leguminosae family in Peninsular Malaysia. Food Chem. 2009;116(1):13-18. https://doi.org/10.1016/j.foodchem.2009.01.091.
27. Chung IM, Ali M, Praveen N, Yu BR, Kim SH, Ahmad A. New polyglucopyranosyl and polyarabinopyranosyl of fatty acid derivatives from the fruits of *Lycium chinense* and its antioxidant activity. Food Chem. 2014;151:435-43. https://doi.org/10.1016/j.foodchem.2013.11.061.
28. Uarrota VG, Moresco R, Schmidt EC, Bouzon ZL, Nunes E da C, Neubert E de O et al. The role of ascorbate peroxidase, guaiacol peroxidase and polysaccharides in cassava (*Manihot esculenta* Crantz) roots under postharvest physiological deterioration. Food Chem. 2016; 197(Pt A):737-46. https://doi.org/10.1016/j.foodchem.2015.11.025.
29. Varshney DS. Effect of some heavy metals on the growth and development of some medicinally important Plants. Botany [Thesis]. Aligarh: Aligarh Muslim University; 2014.
30. Turek A, Wieczorek K, Wolf WM. Digestion procedure and determination of heavy metals in sewage sludge-an analytical problem. Sustain Sci Pract Policy. 2019;11(6):1753. https://doi.org/10.3390/su11061753.
31. Praveen N, Murthy HN. Production of withanolide-A from adventitious root cultures of *Withania somnifera*. Acta Physiol Plant. 2010;32(5):1017-22. https://doi.org/10.1007/s11738-010-0489-7.
32. Zhang H, Zhang G, Lü X, Zhou D, Han X. Salt tolerance during seed germination and early seedling stages of 12 halophytes. Plant Soil. 2015;388(1):229-41. https://doi.org/10.1007/s11104-014-2322-3.
33. Sethy SK, Ghosh S. Effect of heavy metals on germination of seeds. J Nat Sci Biol Med. 2013;4(2):272-75. https://doi.org/10.4103/0976-9668.116964.
34. Liu JG, Zhang YX, Shi PL, Chai TY. Effect of cadmium on seed germination and antioxidative enzymes activities in cotyledon of *Solanum nigrum* L. Sci China Life Sci. 2012;55(9):793-99. https://doi.org/10.1007/s10653-017-0005-8.
35. Seneviratne M, Rajakaruna N, Rizwan M, Madawala HMSP, Ok YS, Vithanage M. Heavy metal-induced oxidative stress on seed germination and seedling development: a critical review. Environ Geochem Health.;41(4):1813-31. https://doi.org/10.1007/s10653-017-0005-8.
36. Singh S, Parihar P, Singh R, Singh VP, Prasad SM. Heavy metal tolerance in plants: Role of transcriptomics, proteomics, metabolomics and ionomics. Front Plant Sci. 2015;6:1143. https://doi.org/10.3389/fpls.2015.01143.
37. Parmar, Dave, Sudhir, Panchal. Physiological, biochemical and molecular response of plants against heavy metals stress. Afr J Curr Med Res. 2013;5(1):80-89.
38. Prasad TK. Mechanisms of chilling-induced oxidative stress injury and tolerance in developing maize seedlings: Changes in antioxidant system, oxidation of proteins and lipids and protease activities. Plant J. 1996;10(6):1017-26. https://doi.org/10.1046/j.1365-313X.1996.10061017.x
39. Choudhary M, Jetley UK, Abash Khan M, Zutshi S, Fatma T. Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. Ecotoxicol Environ Saf. 2007;66(2):204-09. https://doi.org/10.1016/j.ecoenv.2006.02.002
40. Palma JM, Sandalio LM, Javier Corpas F, Romero-Puertas MC, McCarthy I, del Río LA. Plant proteases, protein degradation and oxidative stress: Role of peroxisomes. Plant Physiol Biochem. 2002;40(6):521-30. https://doi.org/10.1016/S0981-9428(02)01404-3.
41. Lin YC, Kao CH. Proline accumulation induced by excess nickel in detached rice leaves. Biol Plant. 2007;51(2):351-54. https://doi.org/10.1007/s10535-007-0071-3.
42. Küpper H, Küpper F, Spiller M. Environmental relevance of heavy metal-substituted chlorophylls using the example of water plants. J Exp Bot. 1996;47(2):259-66. https://doi.org/10.1093/jxb/47.2.259.
43. Trivedi LD. A study on interactive effects of heavy metal on growth and biochemical changes in medicinal plants. Botany [thesis]. Ahmedabad: Gujarat University; 2005.
44. Michalak A. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Pol J Environ Stud. 2006;15(4):523-30.
45. Dursun K, Kayir O, Sağlam N, Şahin S, Lokman O, Mahfuz E. Changes of phenolic compounds in tomato associated with the heavy metal stress. J Nat Agents Mol Ther. 2019;2(1):35-43.
46. Gupta A, Bano A, Rai S, Kumar M, Ali J, Sharma S et al. ACC deaminase producing plant growth promoting rhizobacteria enhance salinity stress tolerance in *Pisum sativum*. 3 Biotech. 2021;11(12):514.https://doi.org/10.1007/s13205-021-03047-5.
47. Gupta A, Dubey P, Kumar M, Roy A, Sharma D, Khan MM et al. Consequences of arsenic contamination on plants and mycoremediation-mediated arsenic stress tolerance for sustainable agriculture. Plants. 2022;11(23):3220. https://doi.org/10.3390/plants11233220.
48. Schreck E, Foucault Y, Sarret G, Sobanska S, Cécillon L, Castrec-Rouelle M et al. Metal and metalloid foliar uptake by various plant species exposed to atmospheric industrial fallout: mechanisms involved for lead. Sci Total Environ. 2012;427-428:253-62. https://doi.org/10.1016/j.scitotenv.2012.03.051.
49. Antony A, Nagella P. Effect of heavy metals on the andrographolide content, phytochemicals and antioxidant activity of *Andrographis paniculata*. Asian J Chem. 2020;32 (11):2748-52. https://doi.org/10.14233/ajchem.2020.22831
50. Li X, Wang S, Guo L, Huang L. Effect of cadmium in the soil on growth, secondary metabolites and metal uptake in *Salvia miltiorrhiza*. Toxicol Environ Chem. 2013;95(9):1525-38. https://doi.org/10.1080/02772248.2014.887717.