Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 669-674
Received: 13-03-2019
Accepted: 15-04-2019

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Effect of heavy metals (Hg, As and La) on biochemical constituents of *Spinacia oleracea*

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Abstract
Toxic heavy metals contamination of soil, water and air by various human activities is a critical environmental problem. Due to increase in geological and anthropogenic activities soil polluted with heavy metals have become common throughout the world. Reduction in growth, performance, and yield is seen in plants growing in these soils. Growing medicinal plants in heavy metal polluted soils affects the biosynthesis of secondary metabolites, causing considerable changes in the quality and quantity of these compounds. The aim of this study was to investigate the effects of Mercury (Hg), Lanthanum (La), and Arsenic (As) on the biochemical constituents (Chlorophyll A and B, protein, phenols, flavonoids, metal chelators and radical scavengers respectively) of *Spinacia oleracea*. The experimental set-ups were prepared as per standard protocol for plant toxicity testing. Four concentrations of heavy metal salts viz., $HgCl_2$, $LaCl_3$ and As_2O_3 (25mM, 50mM, 75mM and 100mM) were tested for plant toxicity assessment. A set-up without metal was used as the experimental control. The set-up with Hg showed drastic decrease in vital biochemical constituents that contribute to the growth of the plant; La and as did not seem to have a considerable impact except for slight variations. The apparent ineffectiveness of as and La remains yet to be explored.

Keywords: *Spinacia oleracea*, heavy metals, mercury (Hg), lanthanum (La), Arsenic (As)

Introduction
Heavy metal compounds are used in many industrial and commercial processes for numerous reasons. They are also toxic to plants beyond a certain concentration. Due to their adverse effects on food quality, crop growth and environmental health, it is of great concern in agricultural production (Ma *et al*., 1994) [1]. Vegetative growth and crop productivity are reportedly affected due to high heavy metal concentrations (Liu *et al*., 2003) [2]; their potential for bioaccumulation and bio-magnification leads to relatively high exposure in some plants.
Hg can be easily modified into several oxidation states and spread through different ecosystems (Clarkson and Magos, 2006) [3]. Hg^0, Hg^{2+}, and CH_3Hg are the forms that are usually accumulated in plants. High levels of Hg^{2+} have phytotoxic effects. Hg^{2+} shows high affinity for thiol groups, especially for the anions $R–S^-$ (Iglesia-Turino *et al*., 2006) [4]; this can be fatal to the plant. However the extent of understanding about the mechanism of phytotoxicity imparted by Hg remains insufficient. Hg contamination of soils is mostly due to the administration of fertilizers, lime, manures and sledges which contain this heavy metal as a key constituent (Boening, 2000) [5]. Organic mercurials used as a seed-coating to prevent fungal diseases in seeds also contribute to contamination (Patra *et al*., 2004) [6]. Furthermore, coal-fired power plants (Li *et al*., 2017) [7], Mercury mines (Miklavčič *et al*., 2013) [8], fluorescent lamp factories (Shao *et al*., 2012) [9] have all been identified as the sources of mercury emissions and contamination.
La is one of the rare earth metals. Its main oxidation state is (+3). It is considered to be biologically inactive and non-essential. Depending on dosage; there are mostly beneficial effects with an exception of few harmful effects on the growth of the plant. A dose of around 50 mg/l of La increases the mass and height of citrus plant (Turra *et al*., 2015) [10], this suggests its potential role as a fertilizer in citriculture. Increase in germination of old rice seedlings (Hong *et al*., 2000) [11], increase in photosynthetic pigments concentration (Chaturvedi *et al*., 2014) [12], enhanced photosynthetic light reaction and growth promotion (Chen *et al*., 2001) [13], increased activities of Mg^{2+}-ATPase and Ca^{2+}-ATPase on the chloroplast membrane (Pan *et al*., 1998) [14], increased chlorophyll contents, photosynthetic rate and growth promotion in spinach (Hong *et al*., 2002) [15]. In the absence of Mg^{2+} in solution La^{3+} may substitute Mg^{2+} for chlorophyll formation in spinach. La^{3+} also improves PSII formation and increases electron transport rate of PSII (Hong *et al*., 2002) [15]. These studies attribute to the beneficial aspects of La. Nothing significant with regard to toxicity of

Lanthanum is known except for a decrease in chlorophyll a and b in rape (Zeng *et al*., 2001) [16]. La is found in soils near the mining area (Jinxii *et al*., 2010) [17], it is used for anodic material of nickel-metal hydride batteries is La; when these batteries are disposed in open fields La makes its way in soils and water leads to rising concentration in plants (Jinxii *et al*., 2010) [17].

As is a non-essential toxic element found naturally in all soils (Smedley and Kinniburgh, 2002) [18]. It has both harmful and beneficial effects on plants. The harmful effects are attributed to inhibition of root extension and proliferation, decrease in biomass accumulation and plant reproductive capacity in shoots (Garg and Singla, 2011) [19], disruption of cell membranes leading to electrolyte leakage (Singh *et al*., 2006) [20]. Conversely**,** increased ascorbate synthesis in roots (Khan *et al*., 2009) [21], increases anthocyanin accumulation in leaves (Catarecha *et al*., 2007) [22], decreased plant transpiration intensity (Stoeva and Bineva, 2003) [23]. The molecular mechanisms underlying these physiological responses to as exposure are not clear, but have recently attracted attention. Both the inorganic forms of arsenic, arsenate As^{5+} and arsenite As^{3+} are readily uptaken by the plant root cells. As^{5+} can be spontaneously converted to the more toxic As^{3+}, both of which are toxic. As^{5+} is a chemically analogous to phosphate; it can therefore be translocated across cell membranes with the help of phosphate transport proteins and result in imbalances in phosphate supply, this in turn affects some phosphate-dependent aspects of metabolism (Wu *et al*., 2011) [24]. Furthermore, during phosphorylation reactions, As^{5+} competes with phosphate, resulting in unstable, short-lived As^{5+} adducts -- the formation of As^{5+}-ADP and its instant auto-hydrolysis results in the uncoupling of reactions involved in photophosphorylation and oxidative phosphorylation; this decreases the cellular ability to synthesize ATP and carry out normal metabolism (Wu *et al*., 2011) [24]. As^{3+} on the other hand is a dithiol reactive compound which binds to and inactivates enzymes containing closely spaced cysteines and other dithiol co-factors (Patrick *et al*., 2012) [25]. Arsenic enters the agricultural lands through geochemical processes (Smedley and Kinniburgh, 2002) [18], As-based pesticides, mining, irrigation with groundwater contaminated with as, and municipal solid wastes (Meharg *et al*., 2009) [26] are the major sources of contamination of As in soils.

Spinacia oleracea of family Amaranthaceae is one of the most valuable green vegetables with essential nutrients. It is rich in vitamins A, E, K, C, and B complex, minerals such as Mg, Na, K, P, Ca, Cu, I, Zn, Mn and Fe and folic acid. Spinach benefits are its diuretic, soothing, calming, coagulant, demulcent, detoxifying, laxative, cooling, and other properties (Perez-Espinosa *et al.*, 1999; Tandi *et al.*, 2004) [27, 28]. *Spinacia oleracea* is studied due to its high amino acid content (Tandi *et al.*, 2004) [28] and numerous health benefits (Perez-Espinosa *et al.*, 1999) [27]. The objective of this study was to investigate the effects of Hg, La and as on the biochemical constituents of *S. oleracea.*

Materials and methods

Leafy vegetable of *S oleracea* was the experimental plant used in this study. The experiment was conducted on two month old plants grown in earthen pots. $HgCl_2$, $LaCl_3$ and As_2O_3 salts were selected. Four concentrations (25mM, 50mM, 75mM and 100mM) were prepared for metal salts. The metal salt solutions were poured homogenously. Control was without any heavy metal treatment. After two doses of heavy metal treatment the plants were taken for estimation of biochemical constituents. The fresh weight of plant was determined immediately after harvesting of plants with help of electronic balance. After taking fresh weight, plant samples were dried in a hot air oven at 45ºC for three days to record plant dry weight.

Total chlorophyll content

Total chlorophyll was estimated by Arnon's method with equal quantities of fresh leaves (Arnon, 1949) [29]. Chlorophyll was extracted in 80% acetone and the absorbance at 663 nm and 645 nm were read in a spectrophotometer. 0.5 g of leaf was taken and ground to a fine pulp with the addition of 10 ml of 80% acetone. The residue was taken in a centrifuge tube and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a test tube and the volume was made up to 10 ml with 80% acetone. The absorbance was read at 645 and 663 nm respectively against the solvent (80% acetone) blank.

$$\text{mg total chlorophyll/g tissue} = 20.2\ (A_{645}) + 8.02\ (A_{663})\ X\ \frac{V}{1000\ X\ W}$$

Where A is the absorbance at specific wavelength, V is the volume of chlorophyll extract in 80% acetone and W is the fresh weight of the tissue extracted.

Protein Estimation

Bradford reagent assay was used for protein estimation (Bradford and Marion, 1976) [30]. 0.5 g of plant material was taken and ground to a fine pulp with 10 ml of PBS. The residue was taken in a centrifuge tube and centrifuged at 5000 rpm for 5 min. The supernatant was transferred to a test tube and the volume was made up to 10 ml with PBS. Five dilutions of a protein standard (Bovine Serum Albumin) ranging from 100 µg to 500 µg of the protein in 10 ml of PBS were prepared in 5 test tubes. 30 µl each of standard solution and unknown protein sample was taken in appropriately labeled test tubes. 30 µl of distilled water was taken as a blank. 1.5 ml of Bradford reagent was added to each tube, mixed well and incubated at room temperature for at least 5 min. Absorbance was then read at 595 nm. The total amount of protein was then extrapolated to 1g of the plant material.

Total Phenols

Total phenol content was determined by Folin-Ciocalteu (FC) assay with Gallic acid as a standard. 0.5 g of the plant sample was taken and ground with a pestle and mortar in 5 ml of 80% ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min and the supernatant was collected. The supernatant was evaporated to dryness and the residue was dissolved in 5 ml of distilled water. 0.5 ml of aliquot of the unknown sample was pipetted in the test tubes and was made up to 3 ml with distilled water and 0.5 ml of FC reagent was added. After 3 min, 2 ml of 20% Na_2CO_3 solution was added to each tube. After thorough mixing, the tubes were placed in boiling water for exactly 1 min, cooled and absorbance was measured at 650 nm. Standard curve was prepared using different concentrations of Gallic acid. From the standard curve the concentration of the test sample was found out and expressed as mg/g of tissue.

Total flavonoid content was measured by aluminium chloride colorimetric assay with quercetin as the standard **(**Zhishen *et al*., 1999) [31].

10 mg of quercetin was dissolved in 100ml methanol and then diluted to 6.25, 12.5, 25, 50, 80, and 100 µg/ml using

methanol. 0.5 g of the dried powdered plant material was extracted using 5 ml of methanol in a test tube. The homogenate was filtered and its volume was made up to 5 ml with methanol. The assay was determined using 0.5 ml of each extract stock solution and each dilution of standard quercetin taken separately in test tubes. To each test tube 1.5 ml methanol, 0.1ml aluminum chloride solution, 0.1ml potassium acetate solution and 2.8 ml distilled water were added and mixed well. Sample blank was prepared in a similar manner by replacing aluminium chloride solution with distilled water. Absorbance was taken at 415 nm against the suitable blank.

Radical Scavenging Activity

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) (Shen *et al*., 2010) [32]. Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicates higher free radical scavenging activity. 0.5 g of the dried powdered plant material was extracted using 5 ml of methanol in a test tube. The homogenate was filtered and its volume was made up to 5 ml with methanol. 100 µl of the extract was taken in a test tube. 2.9 ml of methanol was added. I ml of DPPH was added and the tubes were allowed to stand for 20 minutes in the dark for 20 minutes. The control was without the extract. After 20 minutes the absorbance was read at 517 nm.

The capability of scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = {(A0 – A1)/A0)*100}

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Metal Chelating Activity

Metal chelating activity was measured as described by Chew *et al*., 2009 [33]. 0.5 g of the sample ground in a mortar. The powdered samples were then extracted with 25 ml methanol and were shook continuously for an hour at room temperature. 0.2 ml of the extract was taken in a test tube and 0.1mM $FeSO_4$ (0.2 ml) and 0.25mM ferrozine (0.4 ml) were added. After incubating at room temperature for 10 min, absorbance of the mixture was recorded at 562 nm. Chelating activity was calculated using the following formula:

Metal chelating activity = $(A_{control} - A_{sample})/A_{control}$ x 100

Where $A_{control}$ is the absorbance of control reaction (without plant extract), and A_{sample} is the absorbance in the presence of a plant extract.

Results

Hg resulted in a linear decrease in the total chlorophyll content. As (Arsenic) imparted a neutral effect and La caused a net increase in chlorophyll content with a slight dip at a concentration of 100mM (Fig 1).

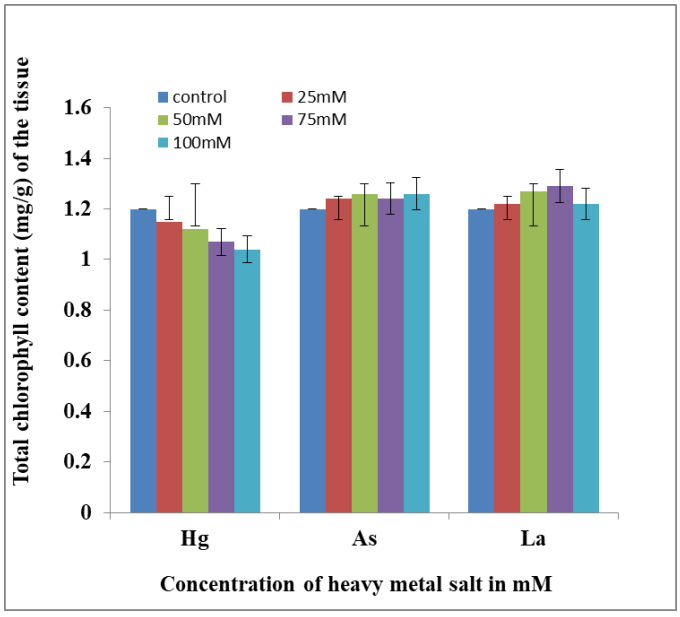

Fig 1: Effect of Hg, as and La on total chlorophyll content of *S. oleracea*

Hg had a net negative effect on the protein content. As resulted in a net increase in protein content in a dose dependent manner. The presence of La had a negative impact on the protein content, however with higher concentrations protein content increased in a dose dependent manner (Table 1).

Table 1: Effect of Hg, As and La on total protein content of *S. oleracea* (The values are expressed as mg/g of the tissue)

Cs	Control	25mM	50mM	75mM	100mM
Hg	0.21±0.002	0.204±0.003	0.222±0.003	0.199±0.002	0.192±0.003
As	0.21±0.002	0.257±0.02	0.220±0.001	0.234±0.004	0.2453±0.004
La	0.21±0.002	0.141±0.001	0.154±0.001	0.161±.003	0.188±0.002

Hg caused a net decrease in phenolic content in a dose dependent manner. As decreased the phenolic content however with a spike at a concentration of 100mM. La drastically decreased the phenolic content in a dose dependent manner.

Table 2: Effect of Hg, as and La on total phenolic content of *S. oleracea* (The values are expressed as mg/g of the tissue)

	Control	25mM	50mM	75mM	100mM
Hg	0.97±0.003	0.800±0.003	0.522±0.003	0.309±0.003	0.419±0.004
As	0.97±0.003	0.799±0.003	0.188±0.001	0.100±0.001	0.719±0.003
La	0.97±0.003	0.412±0.002	0.377±0.002	0.246±0.003	0.177±0.001

Hg imparted a positive impact on the flavonoids with a spike at the concentration of 50mM. As had a negative effect in a dose dependent manner. The presence of La had a net negative effect on total flavonoid.

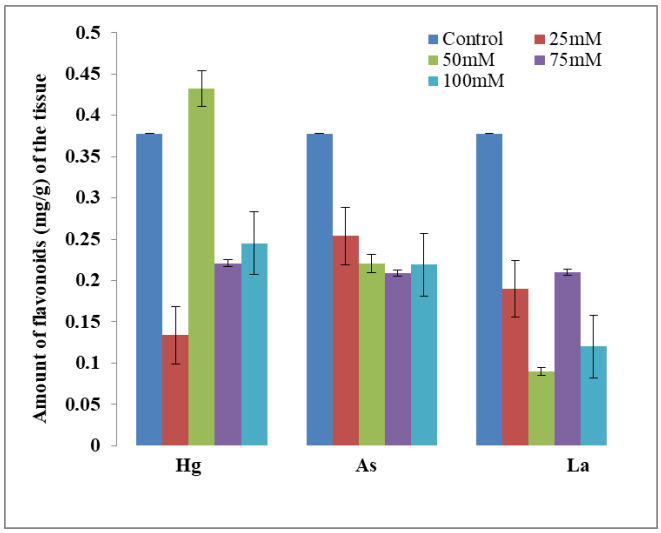

Fig 2: Effect of Hg, as and La on total flavonoid content of *S oleracea*

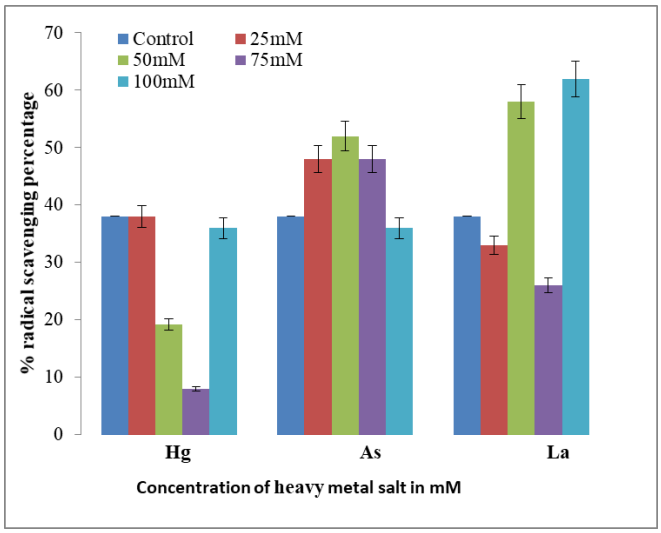

Fig 3: Effect of Hg, As and La on radical scavenging percentage of *S oleracea*

With increasing concentrations of Hg, there was a decrease in the radical scavenging potential, however at a concentration of 100mM there was a spike. There was an increase in radical scavenging potential up to the concentrations of 50 mM of As, higher concentration however decreased the radical scavenging potential in a dose dependent manner. La imparted a net positive effect.

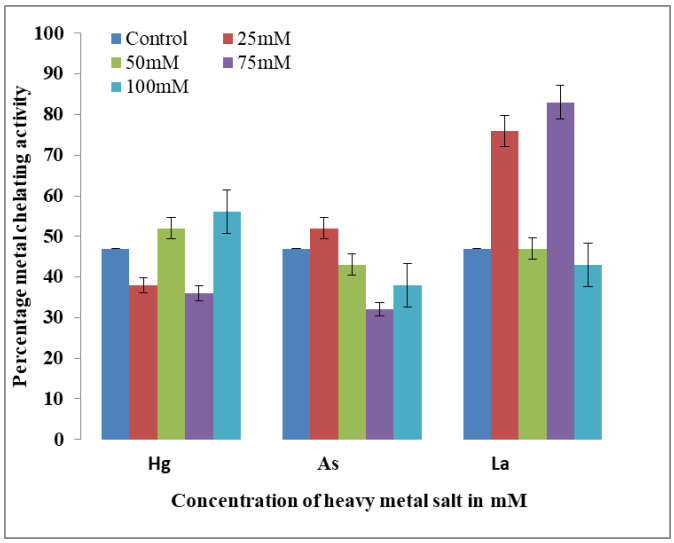

Fig 4: Effect of Hg, As and La on metal chelating percentage of *S oleracea*

Hg had a net positive impact on metal chelating activity. As negatively affected the metal chelating activity. There was an increase in metal chelating activity at the lower concentrations of La and at a concentration of 100mM the metal chelating potential comes down.

Discussion

Hg imparted an overall negative effect on total chlorophyll, protein, phenolic contents respectively and radical scavenging potential (except of spike at a concentration of 50mM). Positive effect was seen on total flavonoids and metal chelating activity. The negative effect can partly be attributed to Hg's affinity to sulfhydryl groups (Azevedo *et al*., 2011)[34] of important amino acids like cysteine and methionine which form a part of key enzymes involved in plant metabolic products. Hg's affinity to block the aquaporins (Savage and Stroud, 2007) [35] could also be the reason for the observed loss of biochemical constituents. Total phenolic content was affected with increasing Hg concentration, however, positive effect on flavonoid content points to its possible role in flavonoid biosynthetic pathway. The above observation leads us to hypothesize that the presence of Hg's influences the phenol-propanoid pathway to produce more flavonoids than phenolics. The mechanism, however, is not deciphered in this study and remains yet to be explored. The rise in radical scavenging and metal chelating activity correlates with the observed rise in flavonoids, which are potential metal chelators and radical scavengers too. This indicates the presence of mechanisms to control oxidative stress.

Arsenic had a negative impact on flavonoids and a positive impact on the total chlorophyll content. There was a decrease

in phenol content and metal chelating activity initially, however with a spike at higher concentrations. Radical scavenging activity increased initially, however a dip was seen at higher concentrations. Arsenic's negative effect could in part be explained by As^{3+}'s affinity to bind and inactivate enzymes containing closely spaced cysteine residues and other dithiol cofactors (Patrick *et al*., 2012) [25]; decrease in flavonoid content, points to Arsenic's role in affecting the enzymes involved in flavonoid biosynthetic pathway. Moreover a negative effect on metal chelators, flavonoids and radical scavengers hinders the plant's ability to scavenge free radicals and tackle oxidative stress; this points to its toxic potential and the plant's susceptibility.

La had a net positive effect on proteins, radical scavengers, metal chelators and total chlorophyll contents respectively. Negative effect was seen on phenols and flavonoids. The observed rise in total chlorophyll content of *S oleracea* could be due to La^{3+}'s affinity to substitute Mg^{2+} in its absence (Hong *et al.*, 2002) [15]. Reasons for the observed negative effect on other biochemical constituents like phenols and flavonoids could be due to La^{3+}'s affinity to compete with Ca^{2+} and bind with phosphate in ADP (Takashami *et al*., 2014) [36] and affect the biosynthetic pathways. This remains yet to be explored.

Conclusion

Mercury (Hg) imparted maximum adverse effect on the biochemical constituents studied; this attributes to its toxic potential. Arsenic (As) was not as toxic as Hg and the plant was able to control its toxicity through antioxidants like flavonoids, radical scavengers and metal chelators. Mercury was toxic enough to dismantle the oxidative stress responses as well. Lanthanum (La) largely had a positive effect.

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