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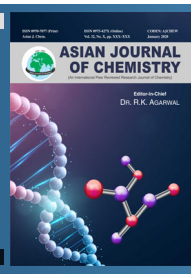

Effect of Heavy Metals on the Andrographolide Content, Phytochemicals and Antioxidant Activity of *Andrographis paniculata*

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Andrographis paniculata is a medicinal plant that has several medicinal properties and has been traditionally used in different medicinal preparations. The present study deals with the influence of heavy metals (lead, mercury and silver) on andrographolide, phytochemicals and antioxidant activity in *Andrographis paniculata*. Two months old saplings were subjected to heavy metal stress of two different concentrations (0.2 mM and 0.4 mM) for three different times at 3 day time interval. The results showed that the saplings treated with heavy metals showed increased concentration of andrographolide content. The saplings treated with 0.4 mM silver showed the highest increase in the andrographolide content (24.58 ± 2.85 mg/g of DW) compared with control (9.41 ± 1.26 mg/g of DW) and other treatments. Variations in the biochemical parameters like total phenolic content, total flavonoid content, *etc*. were also prominent with all the treated samples when compared to that of control.

Keywords: *Andrographis paniculata*, Andrographolide, Heavy metal stress, Antioxidants, Total phenolic, Total flavonoid.

INTRODUCTION

Andrographis paniculata commonly known as 'King of bitters' is a medicinal plant belongs to family Acanthaceae. Being native to India and Sri lanka, it is used in traditional medicines such as in Ayurvedic formulation [1]. It is a flowering herb that can be seen growing as a weed in some parts of India. The plant is used widely across the globe and phytochemicals like andrographolide, neo-andrographolide, deoxy-andrographolide, didehydro-andrographolide, *etc*. are largely responsible for the medicinal value of this plant [2,3]. Almost all parts of the plant, mainly leaves and roots are used in the remedial preparations for various ailments. Recently, *A. paniculata* extracts was also used in commercial preparations in some countries [4]. The plant is well-known for its therapeutic value as it has antiviral, anticancer, hepatoprotective, anti-hepatitis, cardiovascular, immunostimulant and anti-inflammatory activities [5].

During the plant growth, it come across many different types of stresses, which might negatively affect its growth. These stresses are caused by drought, change in temperature, humidity, soil salinity, heavy metals, *etc*. [3]. Heavy metals can be toxic at higher concentration, some of which can be toxic even at very low concentration. Lead is one among the most versatile and abundant heavy metal present in the earth. It can reduce the growth, biomass, protein content and even the germination capacity of the plant in its high concentration. Mercury can get collected in the shoots and roots of seedling and can cause decline in plant height, yield, germination percentage, fruit weight and can cause chlorosis [6-8]. Silver can form complex with purines, pyrimidines and amino acids and hence it is toxic to plants [9]. In response to the stress, plant produces secondary metabolites, which help them in their adaptation and defence mechanism [10]. Andrographolide is the key secondary metabolite present in *A. paniculata* and it possess a wide range of therapeutic properties [11]. The present work intends to study the effect of lead, mercury and silver on the andrographolide content, phytochemicals and antioxidant activity of the medicinal plant *Andrographis paniculata*.

EXPERIMENTAL

Treatment of *A. paniculata* with heavy metals: Two months old saplings of *Andrographis paniculata* were grown in the

poly house. Saplings were subjected to heavy metal stress of two different concentrations (0.2 and 0.4 mM) for three different times at 3 day time interval. The heavy metals used were lead acetate, mercuric chloride and silver nitrate. After heavy metal treatment, plants were harvested; leaves were washed thoroughly with tap water and then with distilled water. The leaves were dried under shade and then in hot air oven set at 40 ºC. Once the leaves were dried, it was grounded into fine powder form and stored for further experiments.

Estimation of chlorophyll: Chlorophyll was estimated by using 80% acetone. Total chlorophyll was calculated by the following formula:

$$\text{Total chlorophyll } (\text{mg/g}) = \frac{(20.2A_{645} + 8.02A_{663}) \times \text{Vol. (mL)}}{\text{Weight (g)}} \times 1000$$

where A_{645} and A_{663} are the absorbance at 645 and 663 nm, respectively [12].

Estimation of proteins: Protein estimation was performed following the method of Lowry *et al*. [13] with slight modification. The plant powder was homogenized using 0.05 M phosphate buffer followed by centrifugation for 10 min at 10,000 rpm at 4 ºC. Once centrifugation was done, 0.8 mL of supernatant was transferred into another test tube and made up using distilled water to a total volume of 1 mL followed by the addition of 5 mL of Biuret reagent and incubated at room temperature for 10 min. Folin-Ciocalteu reagent (0.5 mL) was gently poured into the mixture and followed by incubation for 30 min in dark. The absorbance was noted at 660 nm. Standard used for the experiment was bovine serum albumin (BSA).

Estimation of carbohydrates: Plant powder (0.1 g) was ground with 5 mL of 2.5 N HCl followed by 3 h incubation in boiling water bath. It was then cooled and the contents were neutralized by adding solid sodium carbonate powder until effervescence ceased. Centrifugation was then performed for 10 min at 10,000 rpm. To 1 mL of supernatant, 1 mL of 2% phenol solution was added and shaken well. About 5 mL of 96 % H_2SO_4 was poured gently followed by incubation at 25-30 ºC for 20 min. The mixture was cooled before taking reading at 490 nm and standard used for the experiment was D-glucose [14].

Quantification of andrographolide using HPLC: Extraction and HPLC analysis of andrographolide was performed according to the method of Praveen *et al*. [15] with slight modifications. Extraction of 0.5 g of dried powder was carried out using 25 mL of methanol. It was kept for overnight incubation at 20 ºC on a rotary shaker at 100 rpm followed by filtration of the extract. The filtrate was then allowed to air dry and 2 mL of HPLC grade methanol was used to redissolve the obtained residue. Before HPLC analysis, filtration of the sample using nylon membrane (0.45 µm pore size) was carried out. Authentic, HPLC grade (purity 96 %) andrographolide was obtained from Natural Remedies Pvt. Ltd. (Bangalore, India). Waters 510 series equipped with Waters 486 series detector with column (250 mm × 4.6 mm) was the HPLC system used. The mobile phase consists of acetonitrile:water (70:30 v/v) using isocratic program. Re-equilibrium was allowed for 10 min between the injections. Aliquots of 20 µL were injected in the HPLC at 26 ºC column temperature and the flow rate was 1.0 mL/min. The detector was set at a wavelength of 230 nm. HPLC was performed in triplicates for both standard and samples.

Preparation of extract: Methanol (10 mL) was used to suspend 2 g of the plant powder followed by incubation for 3 h and then filtered. The filtrate was kept in boiling water bath for the evaporation of the solvent and then diluted with methanol to attain a concentration of 30 mg/mL of the extract in all the samples [16]. The extract was used for the estimation of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant assays.

Determination of total phenolic content (TPC): Folin-Ciocalteu reagent (2.5 mL, 10%) was poured gently to 10 µL of the plant extract, which was initially made upto 0.5 mL using distilled water. Following this, 2 mL of 7.5% Na_2CO_3 was added and incubated in the absence of light at room temperature for 30 min. Absorbance was read at 760 nm and the standard used for the experiment was gallic acid [17].

Determination of total flavonoid content (TFC): The plant extract of 10 µL was made upto 1 mL using distilled water followed by the addition of 4 mL distilled water. It was then added with 5% $NaNO_2$ of 0.3 mL and incubated for 5 min at room temperature. Aluminum chloride (0.3 mL, 10%) was added to this mixture at room temperature and allowed to stand for 5 min. After incubation, 1 M NaOH of 2 mL was poured in the reaction mixture and with distilled water, the final volume was made upto 10 mL. The UV reading was taken at 510 nm and data is represented as mg/g of extract equivalent to quercetin [17].

Radical scavenging activity using 2,2-diphenyl-1-picryl-hydrazyl (DPPH): Plant extract (50 µL) was made upto 2 mL using methanol followed by the addition of 1 mL of 0.1 mM DPPH reagent. The mixture was allowed to stand in dark for 15 min and the wavelength was measured at 517 nm against the blank. Methanol (3 mL) served as the blank and ascorbic acid was used to prepare the standard. Radical scavenging activity was calculated using the following formula:

$$\text{Radical scavenging activity } (\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of control without plant extract and sample with plant extract, respectively [18].

Phosphomolybdate assay: Phosphomolybdate assay was performed following Prieto *et al*. [19] method with slight modification. Plant extract (10 µL) was made upto 0.3 mL using methanol and 3 mL of reagent was prepared by 0.6 M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate was added. The mixture was allowed to stand for 90 min at 95 ºC in hot water bath. Before taking the absorbance at 695 nm it was cooled down and standard used was ascorbic acid.

Metal chelating activity: Procedure of Wong *et al*. [20] was followed with slight modification for the determination of metal chelating activity. Plant extract (50 µL) was made upto 1 mL using methanol followed by the addition of 1 mL of 0.1 mM $FeSO_4$ and 2 mL of 0.25 mM ferrozine. It was incubated for10 min at room temperature and absorbance was measured at 562 nm. EDTA was used in standard preparation.

Metal chelating activity was calculated using the following formula:

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

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

RESULTS AND DISCUSSION

Effect of heavy metal stress on production of chlorophyll, protein and carbohydrate content: Chlorophyll is a compound present abundantly in both terrestrial and as well as aquatic life where it gets decomposed and reproduced continuously in adequate amounts [21]. Table-1 shows the effect of heavy metals (Pb, Hg and Ag) on chlorophyll, protein and carbohydrate content of *A. paniculata*. Among all the samples, the control (2.52 mg/g of fresh leaf) showed the highest concentration of chlorophyll followed by 0.4 mM Ag treated plants (2.41 mg/g of fresh leaf). Silver treated plants has shown good amount of chlorophyll while in mercury treated plants, a reduction in chlorophyll content was observed. The decline in the chlorophyll concentration of treated saplings can be due to the reduction in the action of plant enzymes that might have caused chlorophyll degradation. The changes in the chlorophyll content can be linked with the photosynthetic productivity [22].

TABLE-1
EFFECT OF HEAVY METAL STRESS (Pb, Hg AND Ag) ON THE PRODUCTION OF CHLOROPHYLL, PROTEIN AND CARBOHYDRATE CONTENT

Heavy metal concentration (mM)	Chlorophyll (mg/g) of fresh leaf	Protein (mg/g) of dry weight	Carbohydrate (mg/g) of dry weight
Control	2.52±0.02a	14.79±0.78b	134.83±2.45e
Pb 0.2	1.94±0.00d	18.12±0.11a	193.33±0.16ab
Pb 0.4	1.98±0.02d	11.02±0.00d	200.00±0.76a
Hg 0.2	1.83±0.01e	10.41±0.15de	138.50±7.91e
Hg 0.4	1.83±0.00e	10.55±0.10de	162.00±3.32d
Ag 0.2	2.12±0.03c	09.57±0.21e	172.66±1.92cd
Ag 0.4	2.41±0.01b	12.69±0.46c	185.00±7.52bc

All samples were analyzed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ where Duncan Multiple Range Test (DMRT) was used.

In the electron transport chain (ETC), photosystem appears to be the most sensitive site to metal ions. Almost all the metals studied till now are found to be inhibitors of photosystem II whereas photosystem I was found to be less sensitive [23]. Amino acids are precursors of protein and they act as signalling molecule in plants. Change in amino acid spectra was observed to be one of the symptoms of various stresses [24]. The total protein content from the leaves of *A. paniculata* was determined by Lowry's method. The highest concentration of protein was observed for 0.2 mM Pb treated plants (18.12 mg/g of DW) followed by control (14.79 mg/g of DW) that is followed by all other heavy metal treated extracts while 0.2 mM Ag (9.57 mg/g of DW) treated plants has shown the lowest protein content.

In the study of Anju *et al*. [25], the level of protein in three weeks old seedlings of *Carica papaya* after heavy metal treatment (cadmium chloride) was observed to be increased with increasing time exposure. Decline in the protein content in the plant due to heavy metal stress can be attributed to increased protease activity [26]. Carbohydrates are produced by photosynthesis in plants and they serves as an important source of energy. Sugars are required in communication system of plants, plant defence and helps in immunity [27]. Phenol-sulphuric acid method was used in the determination of carbohydrate content using D-glucose as the standard. As the concentration of heavy metals increased, carbohydrate content also increased and the highest carbohydrate content was observed in 0.4 mM Pb treatment (200 mg/g of DW) followed by 0.2 mM Pb treated plants (193.33 mg/g of DW). The control (134.83 mg/g of DW) had the lowest carbohydrate content followed by 0.2 mM Hg treated plants (138.5 mg/g of DW). It was observed that the carbohydrate concentration of 3 week old seedlings of *Carica papaya* was decreased with increasing concentration of cadmium when compared with the control [25].

Effect of heavy metal stress on the andrographolide content: Andrographolide is the most prominent and important secondary metabolite present in *Andrographis paniculata*. It is a di-terpenoid which is colourless, bitter in taste and possesses a range of pharmaceutical applications [28]. The andrographolide content of the plants treated with heavy metals was determined by HPLC analysis (Fig. 1). All the heavy metals treated plants showed a drastic increase in the andrographolide content from the control. Silver (0.4 mM) treated saplings showed the highest andrographolide concen-tration of about 24.58 mg/g of DW followed by 0.2 mM Hg (19.14 mg/g of DW) and 0.4 mM Pb (18.21 mg/g of DW) treated plants, respectively. The lowest concentration of andrographolide was observed for the control (9.41 mg/g of DW). The present results were compared

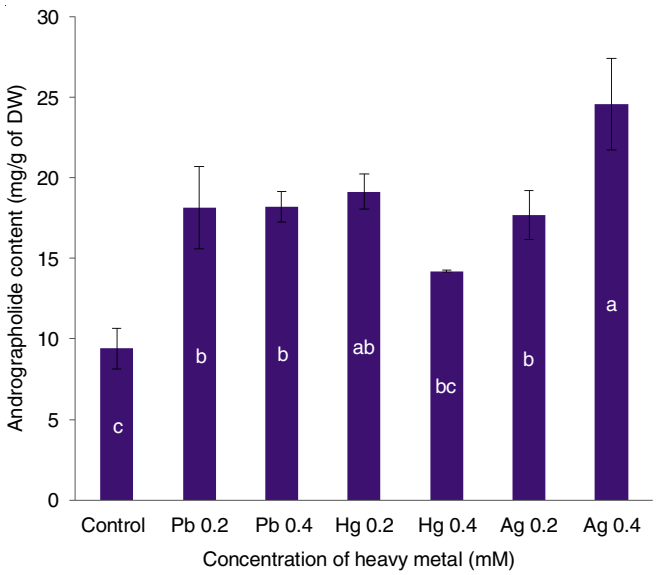

Fig. 1. Effect of heavy metal stress (Pb, Hg and Ag) on the andrographolide content in *A. paniculata.* All samples were analysed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ where Duncan multiple range test (DMRT) was used

with the results obtained by Das and Bandyopadhyay [29], where the andrographolide production was enhanced by the treatment using abiotic elicitor, $AgNO_3$ while $HgCl_2$ reduced the andrographolide content in *in vitro* culture of *A. paniculata*.

An increase in the andrographolide production was observed in the suspension culture of *A. paniculata* after treatment with heavy metals ($AgNO_3$ and $HgCl_2$) [5]. Similarly, an increase in the rosmarinic acid accumulation was also observed in *Salvia miltiorrhiza* after Ag^+ treatment in the hairy root [30] while, a decline in atropine concentration in the hairy roots of *Datura metel* was observed when compared with the control after $AgNO_3$ treatments [31].

Effect of heavy metal stress on the total phenol and flavonoid contents: Phenolic compounds are secondary metabolites which are important in the plant reproduction and growth [32]. Total phenolic and flavonoid contents of the methanolic extracts of *A. paniculata* under different heavy metal treatments are shown in Table-2. The highest concentration of TPC was observed in Pb treated plants (18.96 and 18.73 mg/g of extract) followed by control (15.9 mg/g of extract) which is followed by Ag and Hg treatments, respectively. In comparison with the control, Pb treatments showed an increase in the TPC while Hg and Ag treatments showed a decline in the TPC as compared to the control. The increase of TPC due to heavy metal stress can be a protective function to chelate the metals and reactive oxygen species scavenging [26]. Flavonoids are also secondary metabolites produced by plant that possess antioxidant property. These compounds help to reduce cancer, diabetes, stroke and heart failure [32]. TFC was determined by $AlCl_3$ method, observed to be highest for the control (3.28 mg/g of extract) and lowest for 0.4 mM Hg treated plants (1.06 mg/g of extract). Dutta and Maharia [33] reported that total flavonoid content of *A. paniculata* in copper contaminated soil was found to be lower than the control, however, Ibrahim *et al.* [22] reported an increased TPC and TFC values in *G. procumbens* when treated with copper and cadmium in comparison to control.

TABLE-2
EFFECT OF HEAVY METAL STRESS (Pb, Hg AND Ag) ON TOTAL PHENOL AND FLAVONOID CONTENT

Heavy metal concentration (mM)	Total phenolic contents (mg/g) of extract	Total flavonoid contents (mg/g) of extract
Control	15.90 ± 0.31b	3.28 ± 0.02a
Pb 0.2	18.73 ± 0.77a	2.63 ± 0.11b
Pb 0.4	18.96 ± 0.05a	2.71 ± 0.08b
Hg 0.2	5.83 ± 0.81de	1.58 ± 0.09c
Hg 0.4	4.11 ± 0.81e	1.06 ± 0.30d
Ag 0.2	6.86 ± 0.98cd	2.37 ± 0.18b
Ag 0.4	8.42 ± 1.00c	1.20 ± 0.12cd

All samples were analyzed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ where Duncan multiple range test (DMRT) was used.

Effect of heavy metal stress on DPPH radical scavenging, phosphomolybdate activity and metal chelating activity in *Andrographis paniculata*: Antioxidants are the chemical compounds which destroy or neutralize the action of free radicals or reactive oxygen species. DPPH is a stable radical that helps in stabilization and it can easily trap other radicals [34]. The DPPH activity of leaf extracts of *A. paniculata* under different concentration of heavy metal treatment is shown in Fig. 2. At 0.4 mM Ag treated plants (72.6%) showed the maximum DPPH radical scavenging activity and the lowest was shown by 0.4 mM Hg treated plants (37.2%). In comparison with the control, Pb and Ag treatment has shown increased antioxidant activity whereas Hg treatment has shown a lower antioxidant activity. Phosphomolybdenum assay is a quantitative method where the degree of reduction of Mo(VI) to Mo(V) can be measured and gives an estimate reducing capacity of antioxidants [35]. Table-3 shows the antioxidant activity of *A. paniculata* extract by phosphomolybdate assay. The highest antioxidant activity was observed for 0.4 mM Pb treated plants (21.47 mg/g equivalent to ascorbic acid). It was followed by 0.2 mM Pb treated plants (24.63 mg/g equivalent to ascorbic acid) while the lowest was observed for the plants treated with 0.4 mM Ag (74.54 mg/g equivalent to ascorbic acid) which is followed by control

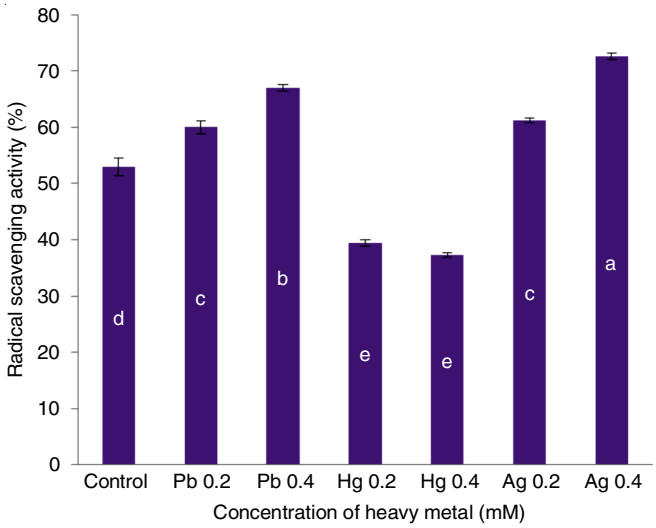

Fig. 2. Effect of heavy metal stress (Pb, Hg and Ag) on DPPH radical scavenging activity in *A. paniculata.* All samples were analysed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ 0.05 where Duncan multiple range test (DMRT) was used

TABLE-3
EFFECT OF HEAVY METAL STRESS (Pb, Hg AND Ag) ON PHOSPHOMOLYBDATE ACTIVITY IN *A. paniculata*

	Phosphomolybdate assay (mg/g equivalent to ascorbic acid)
Control	66.76 ± 1.63b
Pb 0.2	24.63 ± 2.20d
Pb 0.4	21.47 ± 2.08d
Hg 0.2	46.17 ± 1.54c
Hg 0.4	50.40 ± 1.39c
Ag 0.2	62.73 ± 1.76b
Ag 0.4	74.55 ± 1.51a

All samples were analyzed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ where Duncan multiple range test (DMRT) was used.

(66.76 mg/g equivalent to ascorbic acid). Ferrous ions chelating assay chelates transition metals and disrupts the formation of complex and thus causes colour reduction [34]. The Fe^{2+} chelating activity was determined by Fe^{2+}/ferrozine method. The highest metal chelation activity was observed for 0.2 mM Pb treated plants (65.9%) and the lowest was observed for 0.2 mM Hg treated plants (40.68%) (Fig. 3). Antioxidant activity of the plants after Hg treatment was found to be lower than the control.

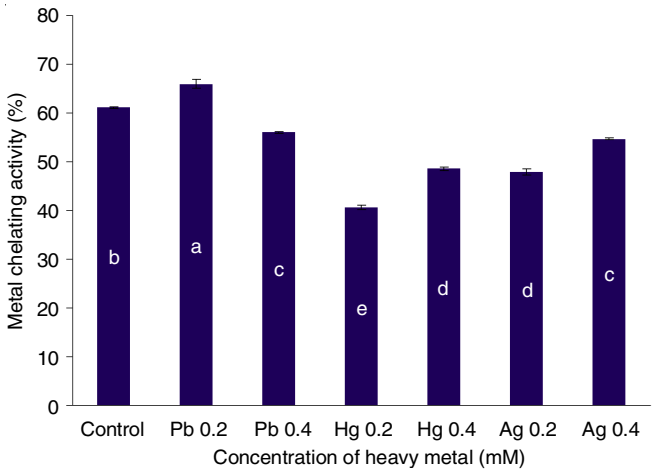

Fig. 3. Effect of heavy metal stress (Pb, Hg and Ag) on metal chelating activity in *A. paniculata.* All samples were analyzed in triplicates and the results are represented as mean ± standard error. Means not sharing a common single letter found to be significantly different at $p \leq 0.05$ 0.05 where Duncan multiple range test (DMRT) was used

Conclusion

In this study, variations in the andrographolide content, biochemical parameters like total phenolic, total flavonoid contents and antioxidant levels were studied in saplings treated with heavy metals when compared with control saplings. An increase in the andrographolide content of the plant extract justify that secondary metabolite production increases in response to heavy metal stress. This idea can be implemented in heavy metal contaminated soil for the increased production of andrographolide. This study may open up a new area where the regulation of genes involved in the andrographolide biosynthesis can be studied.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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