Annals of Biology **38** (2) : 268-273, 2022

Impact Score : 0.32 (Scopus)

Risk Assessment of Cadmium and Lead in Herbal Decoction of *Tinospora cordifolia* Leaves and their Antibacterial Activity on Pathogenic Gram-negative Bacteria

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(Received : April 5, 2022; Accepted : May 5, 2022)

ABSTRACT

Tinospora cordifolia possesses antimicrobial, anti-inflammatory, vaso-relaxing activity and phytoremediation properties. The present study focused on the toxicological risk assessment of herbal decoction. Two prominent heavy metals viz., cadmium and lead were analyzed using inductively coupled plasma- optical emission spectroscopy (ICP-OES). A comparative study between hot-plate microwave digestion of the sample was also performed. The outcomes of the study showed a higher amount of targeted metals but a little lesser than the permissible limit of WHO. Further the efficiency of decoction gram-negative pathogenic bacteria strain with multiple drug resistance (MDR) was checked by estimating its antibacterial activity. The results of the study provided its authenticity as herbal remedy with effective antibacterial properties. Further *in situ* hazard assessment of heavy metals in *T. cordifolia* cell lines on various micro-organisms is required.

Key words : *Tinospora cordifolia,* decoction, inductively coupled plasma-optical emission spectroscopy, multiple drug-resistant

INTRODUCTION

From ancient times, plants were used for the treatment of different body ailments. Almost 80% world's population relies on herbal products even now. Herbal decoctions are used as the main home remedy as traditional medicines. *Tinospora cordifolia* a member of the Menispermaceae family is among these home remedial plants commonly known by different names like "Gudhuchi" in Sanskrit, "Amrutha" and "Gurcha" in Hindi. Plant and their parts are used for the preparation of decoction that provides relief for multiple problems like general weakness, gonorrhoea, urinary disease, anemia, fever, viral hepatitis and dyspepsia (Ruthiran *et al.*, 2016). Decoction contains major photo-active constituents that are used in treatment due to their anti-periodic, anti-microbial, anti-allergic, anti-diabetic, anti-inflammatory, anti-osteoporotic, anti-spasmodic, anti-cancerous and cardio-protective properties (Ilamkar *et al.*, 2020). Agricultural crop residues, emissions of the vehicles industry and other anthropogenic activity act as a potent source of heavy metal contamination. Municipal and industrial sewage waste water is used with or without treatment for irrigation purposes in agricultural land under less or low water availability providing these a suitable path to enter the food chain showing a harmful impact on human health (Bara *et al.*, 2020). Sequestration of heavy metals in productive land resulted in poor productivity and conversion of fertile land into infertile ones (Mahurpawar, 2015). Some metals showed accumulation of toxic content in a selective manner (Meseret *et al.*, 2020). In developing countries like Bangladesh, India, Afghanistan and Pakistan this situation is becoming worst due to the continuous use of untreated water in agricultural fields. The organism body shows impairment of vital organs due to biochemical changes that happened in presence of toxic elements (Rajtor and Piotrowska-Seget, 2016). Not only this but other anthropogenic activities create changes in the ecosystem several thousand folds become the main reason attracting global attention to this situation.

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Heavy metals remain for a prolonged life in the soil up to 100 years; thus behave as a potential threat to human health showing a negative impact on environmental micro flora. Due to phyto-remediation activity, dependency of plants on soil for micronutrients leads this problem to the next level. Atmospheric pollutants, fertilizers and pesticides in crop, dust, burning of fossil fuels and rainfall become the additional source that is causing enhancement of such factors in this problem (Dghaim *et al.*, 2015). Piles of the earth's crust consist of trace elements like Cr, Pb, Zn, Co, Mg, Hg, Ni, Mn, etc. as its natural component. Some of these behave as micro-nutrients; others play an unknown role in the biological system. Trace metals like Hg, As, Pb and Cd if enter in living system cause toxicity. Uptake of micro-nutrients from soil or use of plants for remediation of contaminated reported a high level of toxic elements in plant parts like stem, root, leaf and fruit. Field studies in *Populus* sp., *Brassica computers, Salix viminalis, Helianthus annus, Brachythecium populeum* and *Pteris vittata* represented a significantly higher concentration of Pb and Cd in leaves. Greenhouse pot study also revealed similar results in a laboratory experiment conducted on plants like *Portulaca griflora, Raphanus sativus, Sanvitalia procumbens, Alternanthera phyloxeroides, Brassica juncea, Brassica rapa, Brassica napus, Agrostis capilaris, Leersia oryzoides* and *Salix alba* (Kite *et al.*, 2017). The main purpose of this study was to estimate the toxicological risk associated with lead and cadmium in the herbal decoction of *Tinospora cordifolia* (Jurowski *et al.*, 2022). Greenockite or cadmium mineral is generally used to form coloured pigments like yellow and red during mid 19th century then used in conjugation with nickel for rechargeable batteries. Now-a-days it is mostly used in batteries, alloys, solar cells and neutron absorbers also. Developed countries like North America, Japan, China and South Korea are major cadmium producers all over the world. Cadmium is highly toxic due to its serious impact on humans causing gastrointestinal, reproductive and neurological disorders. Unlike cadmium, lead also expresses some devastating effects on humans. Lead can easily resist high atmospheric pressure and boiling temperature. It is present in almost all sources in different forms (Bruno *et al.*, 2018). Car exhaust, industrial gases, leaded fuel and plumbing old pipes are the main agents of lead in soil and plants. The soil's upper layers up to 8 inches showed immovable activity of lead until it's microbial or phyto-remediation occurs. Even in very negligible concentration, Pb showed acute toxicity in human being (Wilschefski and Baxter, 2019). To estimate the hazardous effects of these heavy metals inductively couple spectroscopy was used. This instrument provides better detection in comparison to Atomic Absorption Spectroscopy (AAS) (Didukh-Shadrina *et al.*, 2019). For elemental analysis, specific lamps provide a less time-consuming approach. To check the antibacterial activity of decoction *Aeromonas hydrophila* sub-sp. *Hydrophila* was used. Isolated bacterial strain possessed multiple drug resistance activity against commonly used antibiotics like ampicillin, streptomycin, rifampicin and tetracycline (Mulaudzi *et al.*, 2017, Sun *et al.*, 2019).

MATERIALS AND METHODS

Tinospora cordifolia fresh leaves were collected from the medicinal and aromatic plants section, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana, India. For proper cleaning and removal of dust and sand particles, leaves were washed with running tap water and then with the help of double-distilled water. After proper washing, leaves were dried at room temperature (25±2°C) at shady place to avoid loss of photo-active constituents. The grinding of dried leaves was done by using a grinder. The obtained leaf powder was used for the preparation of herbal decoction. 100 ml of water and 5 mg of leaves were boiled for 15 min then the supernatant was filtered and stored for estimation of heavy metals and their antibacterial activity.

Digestion of plant leaf powder and decoction was performed in triplicates. US EPA protocol was used for the digestion of samples. Two different methods were used for the digestion of samples for heavy metal analysis (1) closed digestion and (2) hot plate digestion. Both digestion processes were completed using US EPA protocol 3050B. Digested samples were subjected to a filtration using Whatman No. 42 filter paper and 50 ml total volume was made up using triple distilled water. Filtered samples were stored for quantification of cadmium and lead using ICP-OES. Antibacterial activity tests were performed using Mueller-Hinton broth for this media by autoclaving and pouring into Petri-plates. Simultaneously fresh culture of *Aeromonas hydrophila* (Accession No. CP000462) was prepared using nutrient broth and kept in an incubator at 37°C for 24 h. Meanwhile, Mueller-Hinton plates were placed in incubator for proper checking of contamination. If Petri-plates appear transparent after 24 h then these were used for antibacterial activity testing using the Kirby-Baurer Disk Diffusion method. Contamination free culture plates were kept in a Laminar Air Flow cabinet for further spreading and well formation. The antibacterial activity was performed using three different concentrations of herbal decoction viz., 10, 50 and 100 µl. The bacterial culture plates placed on Laminar Air Flow were used in triplicates and (10)6 CFU of fresh microbial culture was used for spreading. 20 min were provided for drying of bacterial culture and then 5 mm well was prepared using a sterilized cork borer. Three different concentrations of herbal decoction were added to different wells and then again culture plates were transferred back to the incubator for incubation at 37°C. Antibacterial activity results were checked at different intervals of time and obtained results were used for plotting of graph.

MARS Closed vessel classic XP Microwave Digester (CEM, MATTHEWS, NC and USA) using 3150W max power and Tarsons Hot-plate were used for microwave and Hot-Plate digestion of leaf and decoction sample. Inductively Coupled Plasma-Optical Emission Spectrometer 5110 (Agilent Technologies, Australia) coupled with ultrasonic Nebulizer-Conikal U-series (Agilent, Australia), ICP-double pass S/C helix+UFT Spray Chamber+Ball joint and SPS4 Auto-sampler was used for quantification of cadmium and lead. 99.995% purity Argon gas was used to sustain the activity of plasma. ACS grade 65% nitric acid SUPRAPUR (Merk, Germany) was used for standard preparations and volume makeup. Standard solutions of cadmium and lead from Environmental Calibration Standard, Agilent Technologies were used. All culture media were purchased from HiMedia for culturing micro-organisms.

Daily ingestion of heavy metals through medication was calculated as :

$$C = A/B$$

Where, A – Concentration of heavy metal in a sample (µg/g), B – Capsule/tablet weight in gram and C – Concentration of heavy metal in capsule/tablet (µg). Daily ingestion of heavy metal = C × Prescribed in capsules/tablets per day.

All experimental work was performed in triplicates and statistical analysis was performed using the statistical tool SPSS-22. Origin Pro 8.5 win 10 was used for plotting of graphs.

RESULTS AND DISCUSSION

Herbal decoction showed very low levels of cadmium and lead value defined according to ICP-OES results. Cadmium hot plate digested plant powder consisted of 0.54±0.03 and 0.36±0.01 ppb in herbal decoction. Similarly, microwave digested plant powder showed 0.72±0.04 and 0.47±0.02 ppb in decoction (Fig. 1). Lead hot plate digested samples showed 161.56±4.42 and 136.38±4.19 ppb, concentration in plant powder and decoction. Lead showed the following outcomes in microwave digested samples in plant powder 180.09±4.37 and 146.56±1.94 ppb, respectively, in herbal decoction, in a single dose (Fig. 2). All results obtained were below the permissible limit set according to FAO and WHO.

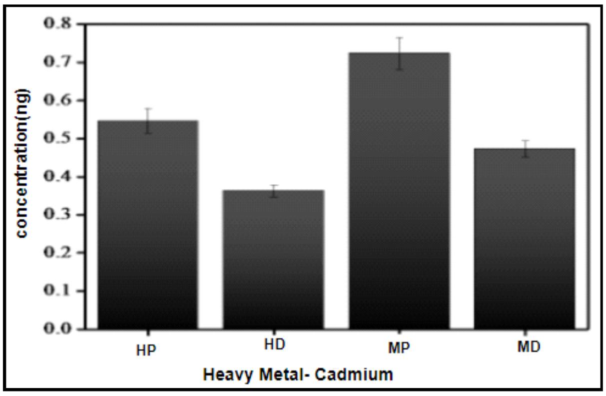

Fig. 1. Microwave digested sample analysis of Cd and Pb level in plant powder decoction.
HP–Cadmium in hot plate digested *T. cordifolia* leaves powder, HD–Cadmium in hot plate digested leaves decoction, MP–Cadmium in microwave digested plant leaves powder and MD–Cadmium in microwave digested leaves decoction.

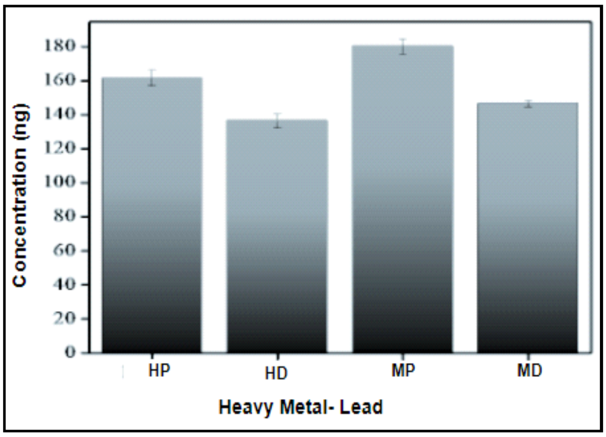

Fig. 2. Hot plate digested sample analysis of Cd and Pb in plant powder decoction.

HP–Cadmium in hot plate digested *T. cordifolia* leaves powder, HD–Cadmium in hot plate digested leaves decoction, MP–Cadmium in microwave digested plant leaves powder and MD–Cadmium in microwave digested leaves decoction.

Indigestion of both heavy metals caused toxicity in their own ways if entered the body. At a higher level, cadmium showed toxic carcinogenic activity. Continuous intakes of cadmium lead to accumulation which caused lung damage, fragile bone and impairment of vital organ functioning like the kidneys and renal tract. If body was continuously or prolonged exposed to lead this may lead to loss of vision and hearing impairments and also showed some adverse effects on the brain, kidney, reproductive defects, gastro-intestinal problems and weak coordination between muscles and bone. Thus, it was a highly toxic heavy metal and its prolonged exposure may cause vision and hearing impairments, gastro-intestinal symptoms, kidney and brain damage, reproductive defects, and weak muscle coordination. Even low concentration accumulation of lead in living tissue or if it got bio-accumulated at a low level from prolonged duration showed adverse effects. Sometimes this activity was favoured due to the consumption of herbal decoction or herbal medicines containing heavy metals.

Toxicological risk assessment was identified on the basis of single time or one-time administration of herbal decoction of *T. cordifolia*. For a 10 ml dose of decoction, it showed 0.00000072 ng/g of cadmium accumulation in hot plate digestion while 0.0000094 ng/g in the microwave digested sample. Similarly, a single dose of decoction when administrated showed 0.00027 ng/g of Pb from the hot plate digested sample and the microwave digested sample showed 0.00029 ng/g. Oral administration of herbal decoction showed below the permissible unit of heavy metal impurities.

The risk assessment approach was summarized by specific data of component or product combined with knowledge and information obtained across the product. It was as processed or in raw form (Table 1). Raw data provided the Permissible Daily Exposure (PDE). Cadmium's permissible limit for herbal traditional products was 0.3 mg/kg and for lead it was 10 mg/kg. The permissible daily exposure limit of heavy metals in raw and finished herbal products was used to study its safer use for different purposes.

Table 1. PDE of heavy metal

Heavy metal	Permissible limit of heavy metal in raw/finished products (ng/g)		
	WHO PIWI	India	USFDA
Cd	0.3 0.8 bw/day	0.3	-
Pb	10.0 0.02-3.0 bw/day	10.0	10.0

A. hydrophila is a gram-negative pathogenic bacteria strain that is isolated from the sewage treatment plant. This bacterial strain possesses multiple antibiotic-resistant genes like Agar. Well diffusion method showed effective antibacterial activity of *T. cordifolia* at different concentrations specifically 10, 50 and 100 µl. *A. hydrophila* showed a 23 mm zone of inhibition in 10 µl decoction petri plates at 24 h. While 50 and 100 µl showed almost similar results at 18 and at 24 h. These concentrations provided 30 and 32 mm diameter of zone of inhibition. After 24 h of incubation, bacteria showed negligible growth and almost all colonies vanished out at 30 h and colour of culture medium also changeed from pale yellow to light brown. Fig. 3 provides diameters of zone of inhibition activity obtained from herbal decoction at various time intervals.

ICP-OES provides better detection of trace or heavy metals even at very low concentrations as compared to that of Atomic Absorption Spectroscopy (AAS). In the case of AAS, separate lamp is required for the analysis of individual heavy metal and it takes a lot of

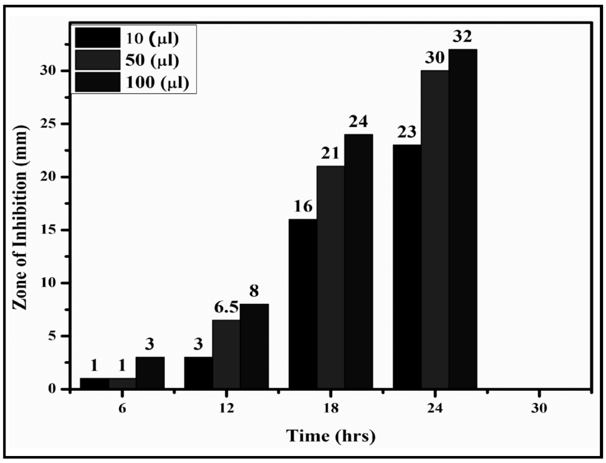

Fig. 3. Zone of inhibition activity using herbal decoction on *A. hydrophila*.

time during analysis. Digestion of samples is the main step in the detection of metals. Microwave digestion provided following advancements over hot plate digestion. In short interval of time and with less volume of reagents, better results were obtained. Loss of volatile compounds, like cadmium and lead, were easily prevented using microwave digestion and chances of contamination like dust particles was easily avoided. Microwave digestion had a disadvantage also : it included a costly microwave that was not required in hot plate digestion. AAS separate lamp was required to detect specific metal impurities. A major limitation of this was to detect all heavy metal impurities in samples. With the help of the risk assessment approach, one easily estimated the use of herbal products or decoction and extracts were safe for use. Results from instrumental analysis helped in estimation of their use and outcomes on human health. Pharmaceutical and drug manufacturers got documented data for selection. The use of herbal decoction on micro-organisms helped in understanding the prior knowledge of listed compounds or chemicals with their application. Prior knowledge of different properties of herbal decoctions, like antiviral, anti-spasmodic, antibacterial, anti-allergic, cardio-protective and anti-cancerous published literature was used during synthesis and manufacturing. It also helped in drug testing tasks. Plants provided variety of bioactive compounds showing bioactive activity against pathogenic bacterial strains. These bioactive compounds helped in combating serious issues like multiple drug resistance. The research provided multiple drug resistant bacteria. Plant bioactive compounds provided better co-evolution with pathogenic organisms that was not present in case of synthetic drugs. To remove adulterations of heavy metals which were not showing any degradation activity, multiple techniques were employed including extraction, dilution, diafiltration and ultra-filtration.

ACKNOWLEDGEMENTS

One of the authors would like to thank Dr. Ms Saloni Gupta and Dr. Promila Bishnoi for their support in experimentation.

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