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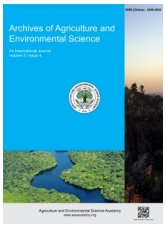

ORIGINAL RESEARCH ARTICLE

Preliminary toxicity assessment of chromium (Cr) and lead (Pb) on terrestrial snail (*Helix aspersa*)

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ABSTRACT

Chromium and lead are among the major toxicants that have their own environmental concerns and are very harmful for living organisms even in small concentrations. The present study is performed to evaluate the acute toxicity potential of chromium and lead on terrestrial garden snail (*Helix aspersa*) at different concentration ranges from 1.0 mg/L to 20 mg/L at different exposure time. In determining acute toxicity, LC_{50} values after 24, 48, 72 and 96 h for both the metals were determined by probit statistical analysis method. Graphs also plotted between percent mortality and used concentrations to obtain regression equation and R^2 values. The obtained median lethal concentration (LC_{50}) for chromium were 15.13 mg/L, 12.88 mg/L, 6.76 mg/L and 4.027 mg/L for 24, 48, 72 and 96 h period of exposure, respectively. Similarly, for lead obtained LC_{50} values were 15.282 mg/L, 6.095 mg/L, 2.094 mg/L and 1.352 mg/L for 24, 48, 72 and 96 h period of exposure, respectively. The study signifies the role of these metals in causing metal stress which leads to variation in electrolytes and A/G (Albumin to Globulin) ratio. The analysis of obtained results revealed that chromium is less toxic to *H. aspersa* in comparison to lead at similar concentrations and exposure time. It is concluded that the present study is one of its own kind of approach which may contribute in developing standard protocols for determining preliminary toxicity assessment of different heavy metals.

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INTRODUCTION

Majority of the known heavy metals and their metalloids are found to be highly toxic to the living organisms (Singh *et al.*, 2011). Exposure to minute concentration causes anomalies in the normal metabolic functioning of the animals and plants. Chromium (Cr) and lead (Pb) are one of the known toxic metals existed in various forms in the terrestrial and aquatic ecosystem influences the local flora and fauna (Wilbur, 2000; Ab Latif Wani and Usmani, 2015). Studies signifies the role of chromium in maintaining the carbohydrate and enzymatic metabolism in animal bodies (Havel, 2004) while lead is highly toxic in even parts per billion level of concentration therefore considered as non-essential metal (Ab Latif Wani and Usmani, 2015). These metals are comparatively highly toxic as compared to the essential metals that positively bind with number of enzymes as their co-factors. Besides their natural origin, heavy metals are always remains a matter of serious concern due to their toxic actions on our environment (Jaishankar *et al.*, 2014). Terrestrial ecosystem is facing huge concern of heavy metal contamination (Tovar-Sánchez *et al.*, 2018). Anthropogenic activities such as mineral mining, electronic waste dumping and use of plastics, releases the heavy metals in the terrestrial environment involve the phenomenon of leaching (Tchounwou *et al.*, 2012).This directly contaminates the terrestrial ecosystem. Non-biodegradable nature of these metals is the main issue associated with them (Igiri *et al.*, 2018). By following dfferent exposure routes like inhalation, ingestion and dermal absorption, they find their place

in the soft tissues of the organisms and initiate the process of bio magnification (Jakimska *et al.*, 2011). Through food chain they enter in the trophic levels. The risk assessment of these heavy metals is basically analyzed on the basis of dose-response relationship (Zhang *et al.*, 2007). Body of living organisms is unable to metabolize these metals which ultimately causes toxicity in them and finally leads to death (Jan *et al.*, 2015). Therefore, toxicity test and assays have been conducted to explore the toxic effects of these metals.

Garden snail, *Helix aspersa* is among the terrestrial biotic community which is very useful for pollution biomonitoring (Berger and Dallinger, 1993). Their soft tissues and shells are highly susceptible towards any kind of contamination. They have the capability to bio accumulate these metal contaminants (Rać, Stachowska and Machoy, 2005). Moreover, Biochemical examinations prove them as potential bio monitoring agents for heavy metals at particular site. Terrestrial garden snail, *H. aspersa* is used in the toxicity assay during the present study. This snail species is found to be abundant and highly distributed along the moist places, home gardens and agricultural field's of shahpur area of Himachal Pradesh, India. They live in colonial culture (Selander and Kaufman, 1975) and love to eat calcium rich food, fresh twigs and leaves of various cereals and vegetables such as cabbage, lettuce, bamboo twigs etc. (Iglesias, 1999). This research aims to study the preliminary toxic effects of chromium and lead on adult garden snail, *Helix aspersa* to determine the electrolyte variations, changes in A/G protein ratio and mortality rate for each metal exposure at different concentrations.

MATERIALS AND METHODS

Samples collection

Adult snails were collected from the surrounding area and within the campus of central university of Himachal Pradesh, Dharamshala during rainy season in the month of September. Animals were transported to the lab in plastic boxes to the laboratory and placed in glass chambers (20×20×20cm) containing moistened soil, organic matter from the same habitat. These snails were acclimatized to laboratory conditions by providing suitable habitat to them for fifteen days under room temperature with 12 hours light and 12 hours of dark period using fluorescent lamps.

Sample preparation and metal concentration

Crude extract is prepared by the physical sonication of snails (Figure1) viscera for the absolute homogenization of the extract. Both snails from metals treated and control chamber are being used in preparation of homogenized crude samples for further biochemical analysis. Before physical sonication, visceral parts are treated with 4N H_2SO_4. The prepared extract is filtered out using Whatman filter paper no. 42. The samples were stored in 50 ml glass vials at -20°C in a deep freezer until further analysis.

Chromium and Lead salts used are of AR grade. Test concentrations were prepared from the stock solutions of both the heavy metals using calibrated pipettes and graduated cylinders. In this experiment, the used Chromium concentrations were control, 1.0, 5.0, 10.0 and 20.0 mg/L that was sprinkled on per 100 g of littering soil where snails are placed. In case of Lead, used concentrations were similar to Chromium. These concentration ranges were chosen on the basis of previous hit and trial studies.

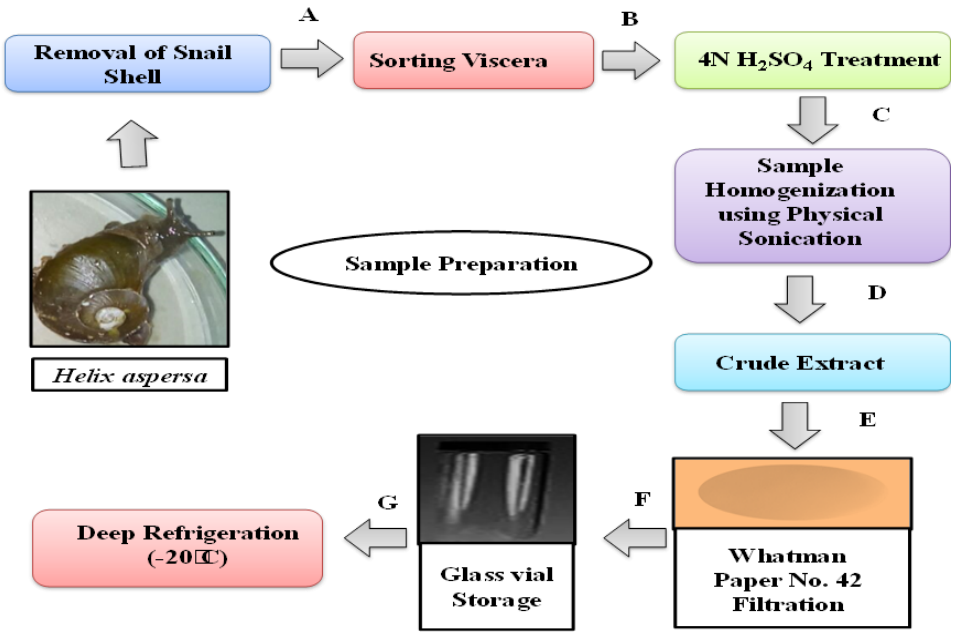

Figure 1. *Flow chart of sample preparation.*

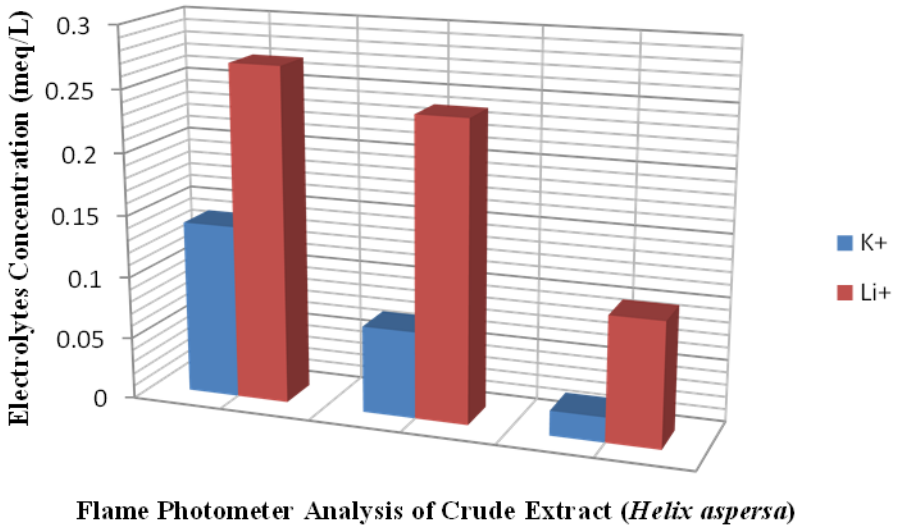

Figure 2. *Flame photometer analysis of potassium and lithium ions.*

Toxicity assay

In this preliminary study, two toxicity experiments were carried out in the departmental laboratory for determining toxic effects of Chromium and Lead on the terrestrial garden snail, *H. aspersa*. The snails for experiment are transferred to glass chambers. Each chamber contains suitable moisture level which is maintained by using wet cotton cloth. Twenty snails were put per container with two replicates including the control chamber. All the toxicity assay procedures are carried out in controlled conditions under room temperature of 28± 2°C with photoperiods of 12 hour's light and 12 hours dark. For carrying out the experiment, equal sized snails are preferred and exposed to the given concentrations. Counting of snails is carried out at every 12 hours and mortalities were recorded during the whole procedure (Tables 2 and 3). The alive and dead snails from control and treated glass chambers are taken away for further biochemical analysis.

Biochemical analysis

Biochemical analysis of crude extract of the snail's viscera involves the determination of biochemical changes in the electrolytes (Potassium and Lithium), variation in A/G protein ratio after metal exposure (Figure 2).

Flame photometer analysis

Crude extract is taken in four different beakers and mixed with 4N H_2S0_4 solution in equal proportion. Magnetic stirrer is used to mix the solution properly. The prepared solution is transferred to water bath until water has been evaporated from the solution and charred mass remains. After this process, remaining mass is transferred into muffle furnace at a temperature of 500 °C for the preparation of ash. The ash formed is diluted to 100 cc of distilled water and formed solutions are filtered out to carryout flame photometer analysis. The main purpose of ash preparation is to remove ron salts from the crude extract as they interfere with sodium ions during flame photometer analysis (Figure 2).

Percent mortality conversion and probit regression analysis for LC_{50} estimation

Probit method is useful in analyzing binomial response variables which involves regression analysis. Present study uses this approach to identify the dose-response relationship. For this purpose, the used concentrations of metals in the experiment were converted into log concentration values. Specially designated "Finney's table" is used for obtaining probit values of percent mortality of snails used in the experiment (Vincent, 2008; Finney and Stevens, 1948). For the calculation of LC_{50} values of different metals used in various concentrations, following mathematical equation is used:

$y = ax + b$

Where a is variable; y is dependent variable; x is independent variable and b is intercept. In regression analysis, x variable and intercept are substituted in the above formula and y probit value of 50 using Finney's table is substituted in the same equation. Here is the calculation procedure:

$y = ax + b$
y= variable x ± (intercept)
5= variable x ± (intercept)
5± intercept = variable x
x= (5± intercept)/ x variable
x= obtained value
LC_{50}= antilog of value of x
LC_{50}= 10^x

LC_{50}= Required result

Before the determination of probit values, the mortality rate of snails is calculated using the Abotte's formula (Rosenheim and Hoy, 1989). The LC_{50} values for different time intervals were taken from 5 probit value mentioned in Finney's table because this value corresponds to 50% of the mortality. The actual LC_{50} was calculated by taking inverse log of the used concentrations.

Table 1. Variations in i) Total protein; ii) Albumen level and iii) A/G ratio in metal (Pb, Cr) stress in comparison to control.

Glass Chambers	Total Protein (g/dL)	Albumen Level (g/dL)	A/G Ratio (g/dL)
Control Chamber	<3.00	0.02	-1.00
Pb Treated	<3.00	0.01	0.11
Cr Treated	<3.00	0.01	0.11

Table 2. Mortality recorded in snails at different Cr concentrations (different time intervals).

Exposure hours	No. of snails Per chamber	Cr concentrations (mg/L) in 100 g soil				
		Control	1.0	5.0	10.0	20.0
24 hr	20	0	0	03	06	09
48 hr		0	02	06	09	12
72 hr		0	02	09	12	15
96 hr		0	04	11	14	17

Table 3. Mortality recorded in snails at different Pb concentrations (different time intervals).

Exposure hours	No. of snails Per chamber	Pb concentrations (mg/L) in 100 g soil				
		Control	1.0	5.0	10.0	20.0
24 hr	20	0	02	05	08	12
48 hr		0	05	08	11	15
72 hr		0	08	12	15	18
96 hr		0	10	13	17	19

Table 4. Log concentrations and probit values when exposed to Chromium after 24 and 48 h.

Cr concentration (mg/L)	Log concentration	Number of snails used per chamber	24 h		48 h	
			% Mortality	Probit	% Mortality	Probit
1.0	0	20	0	0	10	3.72
5.0	0.698970004		15	3.96	30	4.48
10.0	1		30	4.48	45	4.87
20.0	1.301029996		45	4.87	60	5.25

Table 5. Log concentrations and probit values when exposed to Chromium after 72 and 96 h.

Cr concentration (mg/L)	Log concentration	Number of snails used per chamber	72 h		96 h	
			% Mortality	Probit	% Mortality	Probit
1.0	0	20	10	3.72	20	4.16
5.0	0.698970004		45	4.87	55	5.13
10.0	1		60	5.25	70	5.52
20.0	1.301029996		75	5.67	85	6.04

Table 6. Log concentrations and probit values when exposed to Lead after 24 and 48 h.

Pb concentration (mg/L)	Log concentration	Number of snails used per chamber	24 h		48 h	
			% Mortality	Probit	% Mortality	Probit
1.0	0	20	10	3.72	25	4.33
5.0	0.698970004		25	4.33	40	4.75
10.0	1		40	4.75	55	5.13
20.0	1.301029996		60	5.25	75	5.67

Table 7. Log concentrations and probit values when exposed to Lead after 72 and 96 h.

Pb concentration (mg/L)	Log concentration	Number of snails used per chamber	72 h		96 h	
			% Mortality	Probit	% Mortality	Probit
1.0	0	20	40	4.75	50	5.00
5.0	0.698970004		60	5.25	65	6.30
10.0	1		75	5.67	85	6.04
20.0	1.301029996		90	6.28	95	6.64

RESULTS AND DISCUSSION

Anthropogenic activities such as high rate of urbanization and industrialization are responsible for environmental pollution. Mainly release of heavy metals in the environment is a serious threat. By determining the changes in A/G protein ratio and electrolyte disturbance in the crude extract of Helix *aspersa*, we can easily predict the toxic potential of heavy metals and their harmful effects on the environment. Studies have been made to access changes in the protein content on exposure to different heavy metals which reveals the disturbed protein metabolism in different snail species (Waykar and Petare, 2018). Similarly, A study in year 2018 assess impacts of heavy metals in marine mollusk Mytilus *galloprovincialis*, reveals the typical fluctuations

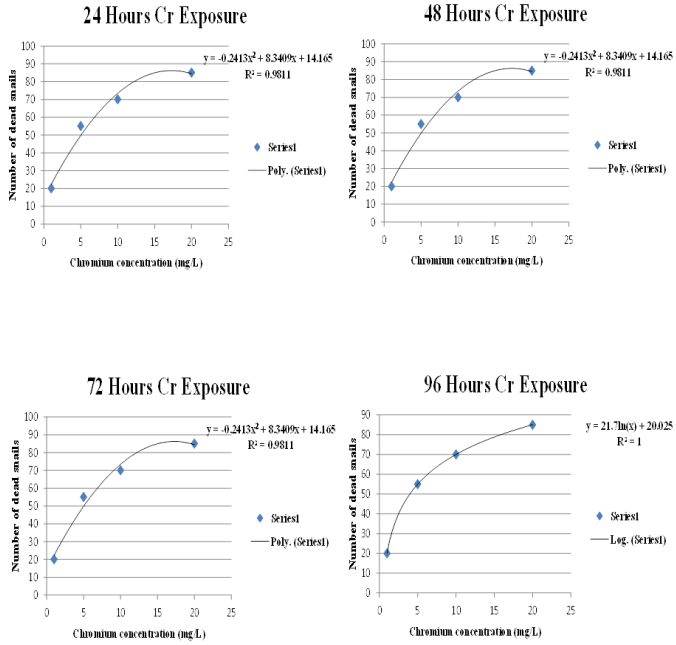

Figure 3. *Plot of metal concentrations (Cr) versus snail's mortality from table 4 and table 5 at different exposure periods.*

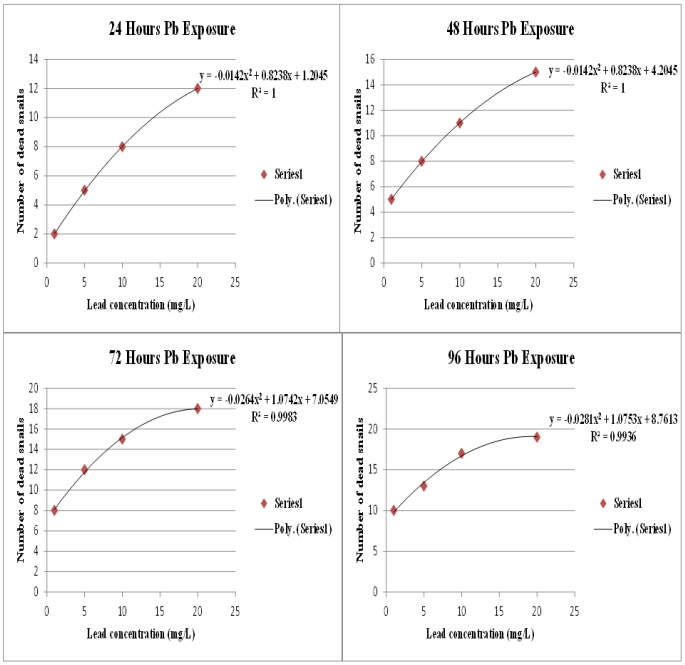

Figure 4. *Plot of metal concentrations (Pb) versus snail's mortality from table 6 and table 7 at different exposure periods.*

in its body elecrolytes (Capillo *et al.*, 2018). On comparison to other studies, the results of present study reveals the similar trend in changes in the A/G ratio and electrolytes of the target snail on exposure to different concentrations of chromium and lead. The obtained results discussed below:

A/G Protein ratio

On analyzing the total protein ratio of untreated and metal treated snails, it was found that the total protein content in all the snails was less than <3.00 g/dL while the albumen level decreases from 0.02 (control chamber) to 0.01 g/dL in both lead and chromium treated snails. A huge difference is recorded in A/G ratio in metal treated snails as compared to control. The A/G ratio is increases from -1.00 g/dL to 0.11 g/dL in both lead and chromium treated snails (Table 1) which highlighted that the rate of proteins in the snail's body increases in a manner in the presence of heavy metals. These results go in the same direction with a study carried out by Masaya and his associates in year 2000 which highlighted a significant increase in the protein rate under the effect of a chemical stress at different biological models (Masaya *et al.*, 2002).

Electrolytes disturbance

Flame photometer analysis shows significant variation in the electrolyte balance in the extract of snails in control and others facing metal stress. The analysis of lead and chromium treated snails extract shows decline in K^+ and Li^+ ions level.

LC_{50} and snail's mortality rate

In this experiment four concentrations of chromium and lead viz. 0.1 mg/L, 5.00 mg/L, 10.00 mg/L and 20.00 mg/L were taken and mortality rate was observed after 24, 48, 72 and 96 h exposure respectively (Tables 2 and 3). It was observed that, on increasing dose and exposure time, mortality rate was significantly increased. Lowest mortality was observed at 1.0 mg/L exposure for 24 hours while 85% mortality rate (Table 4 and Table 5) was observed in the snails for 96 hours for chromium exposure of varied concentrations.

Similarly, in case of lead exposure, higher mortality rate was observed as compared to chromium at similar concentrations. 60% and 95% of mortality was observed in 24 and 96 hours at 20 mg/L concentration exposure respectively (Table 6 and Table 7). The concentrations taken in the whole experiment were converted into log concentrations and their corresponding probit values.

By taking antilog, actual LC_{50} values obtained for chromium were 15.13 mg/L, 12.88 mg/L, 6.76 mg/L and 4.027 mg/L for 24, 48, 72 and 96 h period of exposure respectively. Similarly, for lead obtained LC_{50} values were 15.282 mg/L, 6.095 mg/L, 2.094 mg/L and 1.352 mg/L for 24, 48, 72 and 96 h period of exposure, respectively. Some physical and behavioral changes were also observed during the experiment. Avoiding behavior, random movements, mucus secretion from the body, Color of snails appeared dark brown, stop feeding and restlessness before death were some of the major observations. These changes can

be attributed to an increase in physiological stress due to metal intoxication. The Figure 3 and 4 shows the graphical representation of metal concentrations versus snail's mortality. By plotting the graph, regression and R^2 values were obtained for both the metals at different time intervals.

Conclusion

The present investigation is an attempt to measure variable toxic effects of chromium and lead on the garden snail, Helix *aspersa*. The mortality rate increases with the increase of metal concentrations in the soil and also with increasing the duration of exposure. General observation was made that, the Helix *aspersa* become more susceptible to the toxic effects of both metals by increasing their ambient concentrations. *H. aspersa* could be used as cheap living model bio indicator for heavy metals biomonitoring in terrestrial ecosystem by observing physiological and chemical changes occurred due to metal stress. Flame photometer analysis shows the deviation in normal trend of electrolytes concentration as compared to control. Also, changes have been observed in A/G ratio of the protein content due to these metals exposure. Regarding metal toxicity, chromium was found less toxic than lead to this snail species. This study is valuable and highly important for the determination of LC_{50} concentration and total mortality dose to terrestrial living communities. This study also proves to be fruitful in developing future understanding of ecological and environmental concerns associated with chromium and lead.

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Conflict of interest: The author declares that there is no conflict of interest.

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