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Growth response of *Vernonia amygdalina* Del planted in soil treated with a combination of cadmium, lead and zinc.

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Abstract
Vernonia amygdalina Del. was grown (from stem cuttings) in soil polluted with a mixture of heavy metals; cadmium, lead and zinc. The mixture was in the ratio 1:1:1. The experiment consisted of control and four treatment concentrations (25, 50, 75 and 100 mg/kg) in three replicates. The cuttings were planted in untreated soil for one month prior to treatment. Data for growth parameters (plant height, number of leaves, leaf area, number of branches and girth) were collected monthly for 12 months, while physico- chemical and heavy metals analyses were carried out on soil samples at the end of the experiment. Plant samples were also analyzed for heavy metals content at this time. The results showed adverse effects of Cd+Pb+Zn treatment on the plant as manifested in the growth parameters, except leaf area which was enhanced. At the end of the study, control and the 25, 50, 75 and 100 mg/kg treatments had height values of 77.43±1.02, 40.27±0.75, 34.67±0.77, 26.23 ± 0.91, and 0.00 cm respectively. There was increase in soil acidity and soil carbon, with decreases in the soil nutrient element composition. The uptake of the metals was in the order Pb > Zn > Cd.

Keywords: Pollution, Cuttings, Mixture, Analyses

Introduction
The age long belief that mankind is endowed with an inexhaustible expanse of fertile and clean rich soil is fast being eroded as the world is faced with the reality of dwindling resources and scarcity of arable land. Soil is a natural body that consists of layers, composed primarily of minerals that differ from the parent materials in their texture, structure, consistency, colour, chemical, biological and other physical characteristics (1). Soil life plays a major role in many natural processes that determine nutrient and water availability for agricultural productivity. The primary activities of all living organisms are growth and reproduction. There is a mutual interdependence in every ecosystem. By-products from growing roots and plant residues feed soil organisms. In turn, soil organisms support plant health as they decompose organic matter, cycle nutrients, enhance soil structure and control the populations of soil organisms, both beneficial and harmful (pests and pathogens) in terms of crop productivity.

The accumulation of heavy metals in agricultural soils is of increasing concern due to food safety and potential health risks as well as its detrimental effects on soil ecosystems. The dangers this pose are even more relevant to humans owing to our indecent and indiscriminate disposals of heavy metal containing materials and equipment. The increasing influx of heavy metals into water bodies from industrial, agricultural, and domestic activities is of global concern because of their well-documented negative effects on human and the ecosystem (2). Metals such as aluminium, arsenic, cadmium, cobalt, chromium, copper, lead, manganese, mercury, nickel, selenium and zinc have been considered as the major environmental pollutants and their phytotoxicity has already been established (3, 4, 5).

Cadmium (Cd), Copper (Cu), Lead (Pb) and Zinc (Zn) are among the most common heavy metals in agricultural soils (6). Metals like Pb, Hg, Cd, Ar, and Cr have no known biological function and are toxic to life even at very low concentration (7). Different plants absorb toxic and non-toxic metals from soil and water to varied extent and accumulate in different body parts (8).

Absorption, translocation and accumulation of heavy metal ions of Hg, Pb, Cr, and Cd by plants, reduce qualitative and quantitative productivity of the species and cause serious health hazards through the food chain to other life forms (9; 10; 11). Breckle and Kahile reported that a combined treatment of 20ppm lead and 50ppm cadmium greatly reduced the growth of young roots of *Fagus sylvatica* than in treatment where metals were applied separately (12). Edegbai and Anoliefo observed that when *V. amygdalina* was treated with 25, 50, 75 and 100 mg/kg cadmium; the 100mg /kg and the 75mg /kg treated soils lost all plants by 4 MAT and 5 MAT respectively (13).

V. amygdalina (the bitter leaf plant) is a highly cherished vegetable in West and Central Africa and is consumed in various dishes. In Nigeria, it is a major vegetable in the soup of some ethnic groups; where it is called 'ewuro' by the Yoruba and onugbu by the Igbo. Leaves of the bitter leaf are sometimes sold in the market after being shredded, parboiled and made into fist-sized balls. Practically every part of the bitter leaf plant is consumed or used for various purposes including medicine production. The present study aims at investigating the toxicity of selected heavy metals (cadmium, lead and zinc) to *Vernonia amygdalina.*

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Materials and Method

Study Area: The study was carried out in the experiment plot of the Department of Plant Biology and Biotechnology, University of Benin, Edo State, Nigeria which lies within the humid Tropical Vegetation. ($6^0$23'50"N and $5^0$37'23"E)

Collection of Plant Materials and Soil Samples

Stem: Stem cuttings of *Vernonia amygdalina* used in the study were obtained from a hedge composed primarily of the plant, in 'clean' soil within the Senior Staff Quarters of the University of Benin, Benin.

Soil: Soil samples were collected from the old Botanic Garden of the Department of Plant Biology and Biotechnology, University of Benin, Edo State; a site which had remained undisturbed for over fifteen (15) years. Top soil (0-10 cm), was collected from the plot and dried. Thereafter, 5 kg soil was placed into 15 pieces of labeled and bottom – perforated- 8 –litre - buckets.

Heavy Metals: Three heavy metals namely cadmium (Cd), lead (Pb) and zinc (Zn) were used in this study. They were obtained from their soluble salts; cadmium sulphate ($3CdSO_4 + 8H_2O$,), lead nitrate ($PbNO_3$) and zinc sulphate [$Zn(SO_4).7(H_2O)$]. The quantities of the heavy metals corresponding to the various treatments were then calculated.

Preparation of Stems: Uniform (30cm long, similar girth with 3 - 4 buds), young and freshly collected stem cuttings of *V. amygdalina* in preparation for planting were kept partially submerged in water for about one hour before planting. Three stems were planted in each bucket.

Preparation of Site: The site used for the experimental layout was properly weeded and the surface covered with black cellophane to confine the roots to the soil within the buckets.

Methodology

The buckets were laid out on site in a completely randomized design. Three stem cuttings of *V. amygdalina* were planted in each bucket containing 5 kg soil and later thinned to one (01) after fourteen (14) days of sprouting. The stands were allowed to stabilize for one (01) month before being exposed to treatment with the selected heavy metal mixture (Cd+Pb+Zn). There were four concentrations (25, 50, 75, 100 mg/kg) with a control and in 3 replicates. The pollutants were measured and dissolved in distilled water and dispensed.

After the soil treatment, data were collected on a monthly basis for 12 months (MAT - Months After Treatment) while soil and plant analyses were done at the end of the 12 month period.

Field Data Collection

Plant Height: For plant height measurements, previously identified plant stands were tagged and growth followed to ensure progressive appraisal and uniformity.

Number of leaves: The total number of leaves was taken by visual counting of the leaves on the plants.

Leaf area: Leaf area measurements of the study plants were obtained from the previously tagged plants or their branches and determinations done using the proportional method according to (14)

Number of branches: The number of branches for V. *amygdalina* was taken by visual counting of branches on the tagged plants at given interval.

Girth: The girth of V. *amygdalina* was taken monthly. The diameter of the shoot was obtained using the Esal vernier caliper, (Girth = πd).

Soil Physicochemical Analyses

Soils were dried at ambient temperature (22-25°C), crushed in a porcelain mortar and sieved through a 2-mm (10 meshes) stainless sieve. Air-dried and less than 2 mm samples were stored in polythene bags for subsequent analysis. The fraction was used for the determination of selected soil physicochemical properties and the heavy metal fractions.

pH and Electrical Conductivity: Twenty grammes (20 g) of fine soil was placed in a container and 50 ml of distilled water added. The suspension was shaken for 30 min and allowed to settle. Electrical conductivity and pH of the solution were then measured using a pH meter **(**Model 215**)** and conductivity meter. The pH meter was first standardized using a buffer solution.

*Nitrogen***:** One gramme (1.0 g) of the soil sample was placed into Kjedahl digestion flask. One table spoon of a catalyst and 20 ml concentrated tetraoxosulphate acid was added and the mixture was hand shaken to ensure mixing. At completion of digestion, 10 ml distilled water was added and the solution was filtered through a Whatman filter paper. Nitrogen was determined colorimetrically at 625 nm.

Organic Carbon: One gramme (1.0 g) of the soil sample was placed in a 250 ml conical flask. Then 10 ml of $K_2Cr_2O_7$ and 20 ml conc. H_2SO_4 were added and the mixture was hand shaken for 30 minutes. Distilled water was then added to make the volume up to 150ml. Ten (10) ml of phosphoric acid and 8 drops of diphenylamine solution were then added. A blank determination was done by using 10ml $K_2Cr_2O_7$ and 20 ml concentrated H_2SO_4 solution and titrated to a green colour with Ferrous Ammonium sulphate solution.

The total organic carbon (T O C) was calculated as:

$$\% \text{ T O C} = \frac{\text{Titre value of blank- titre value of sample} \times 0.3 \times \text{M}1.334}{\text{Weight of sample}}$$

Available Phosphorus: One gramme of soil was shaken for 5 minutes with 10 ml of extracting solution containing 0.03 N NH_4F and 0.1 N HCl. The solution was filtered through Whatman filter paper and 3 ml of the filtrate was transferred into a test tube and 3ml of ammonium molybdate was added. Thereafter, 5 drops of mixture of boric acid, sodium sulphite and sodium sulphate were added. The Phosphorus content was determined colorimetrically at 645 nm.

Cation Exchange Capacity: Five grammes (5 g) of soil were placed into sterile conical flasks and 20 ml of extracting solution (NH_4OAc) was added into the 250 ml volumetric flask containing the soil samples. Whatman filter paper was then used to filter the solution. Also 0.1 ml of the filtrate was transferred to a test tube and diluted with 10 ml 0.015 % stronium chloride solution. The sample was analyzed for sodium (Na) and potassium (K) by flame emission and for Ca and Mg by Atomic Absorption Spectrophotometry (AAS).

Sample Preparation for Analysis of Metals: Both plant and soil samples were ground into fine powder. Two (2) g portions of the samples were weighed accurately and 10.0ml of concentrated HNO_3 was added to each. The samples were digested on a hot plate for 15 minutes. The digest was cooled and 5 ml of concentrated nitric acid was added and heated for additional 30 minutes. The later step was repeated and the solution was reduced to about 5 ml without boiling. The sample was cooled again and 5ml of concentrated hydrochloric acid and 10 ml of distilled water was added and the sample was heated for additional 15 minutes without boiling. The sample was cooled and filtered through a whatman No.42 ashless filter paper and diluted to 60 ml with distilled water. Lead content in the digested samples was analyzed for using the Atomic Absorption Spectrophotometer.

***Statistics*:** Statistical analysis was carried out by determining the mean of three replicates and standard deviation (15).

Results

Plant height for *V. amygdalina* grown in soil treated with various levels of Cd + Pb + Zn mixtures and control is presented in Figure 1. Values recorded show that at the end of the experiment, control treatment grew highest while the 25, 50 and 75mg/kg had significantly different ($P < 0.05$) lower values. At this time the 100 mg/kg treated soils had lost all plants. All the treatments and control values were significantly different from one another with adverse effect along the concentration gradient. The values recorded were 77.43±1.02, 40.27±0.75, 34.67±0.77, 26.23cm ± 0.91, and 0.00 for control and the 25, 50, 75 and 100 mg/kg treatments respectively.

The result for the effect of treatment with Cd + Pb + Zn mixtures on number of leaves of *V. amygdalina* is shown in Figure 2. Control plants recorded higher number of leaves compared to the treatments. At 12 MAT, control plants recorded significantly higher number of leaves with adverse effect of treatment increasing along the concentration gradient. At this time, control mean number of leaves was 35.67±7.53, while the 75mg/kg and the 100mg/kg treatments recorded 8.67±1.20 and 0.00 numbers of leaves respectively.

Mean leaf area results for *V. amygdalina* sown in control and Cd + Pb + Zn mixture treated soil are shown in Figure 3. Control values were consistently lower than values recorded for the 25mg/kg, 50mg/kg and 75mg/kg treatments while the 100mg/kg treatment had lost all plant stands at 3 MAT. There was no significant difference ($P < 0.05$) between control and all the treatments at the end of the experiment. The highest leaf area value of 32.21 cm^2 ± 13.84 was recorded for the 50mg/kg treatment at 12 MAT.

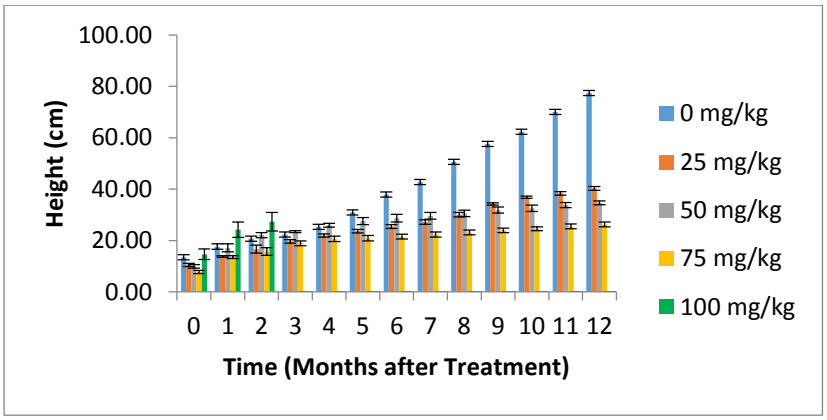

Figure 1: Effect of Cd + Pb + Zn mixture on the height (cm) of *V amygdalina*

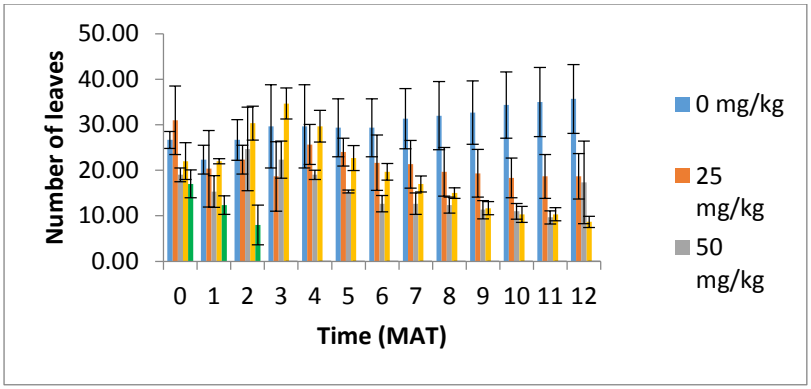

Figure 2: Effect of Cd + Pb + Zn mixture on the number of leaves of *V amygdalina*

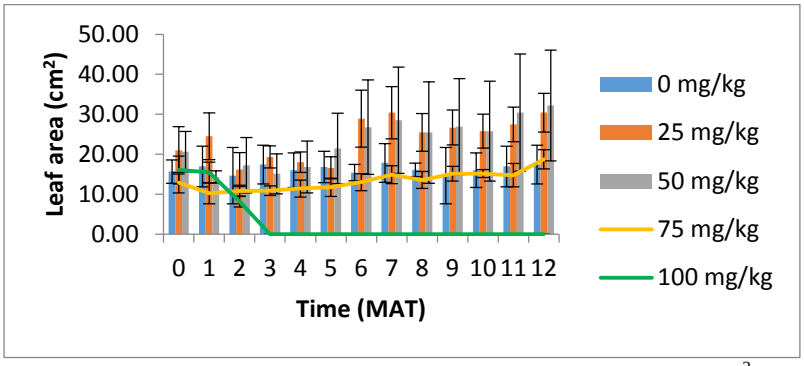

Figure 3: Effect of Cd + Pb + Zn mixture on the leaf area (cm^2) of *V amygdalina*

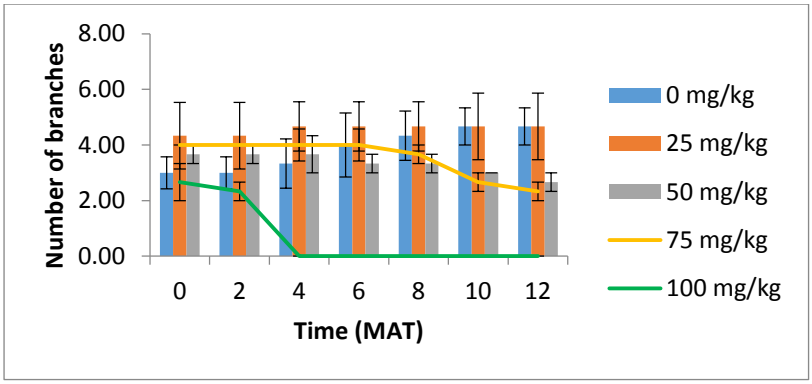

Figure 4: Effect of Cd + Pb + Zn mixture on the number of branches of *V amygdalina*

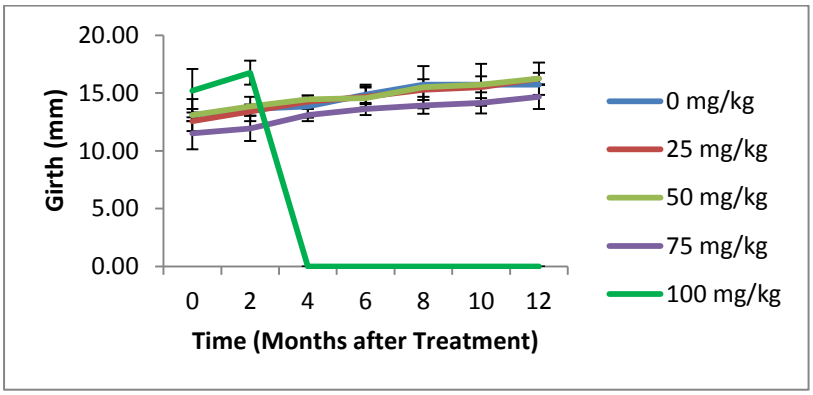

Figure 5: Effect of Cd + Pb + Zn mixture on the girth of *V amygdalina*

Figure 4 shows the effect of Cd + Pb + Zn mixture on the number of branches of *V. amygdalina* from 0 to 12 MAT. There was a consistent increase in control and the 25mg/kg values until 10MAT. There was however a decrease in values recorded for the 75mg/kg treatment at 10 MAT while 100mg/kg lost all branches at 3 MAT.

The values 4.67±0.66, 4.67±1.20, 2.67±0.33, 2.33± 0.33 and 0.00 were recorded for the control, the 25mg/kg, 50mg/kg, 75mg/kg and the 100mg/kg treatments respectively at 12MAT. There was significant difference ($P < 0.05$) between control mean and those of the 75mg/kg and the 100mg/kg treatments respectively. Adverse effect of treatment increased along the concentration gradient.

Figure 5 shows the mean girth values for *V. amygdalina* sown in control and the various treatments with Cd + Pb + Zn mixture. The 100 mg/ kg treatment lost all plant stands at 3 MAT. Control value was significantly different ($P < 0.05$) from the 100 mg/kg treatment only. Values of 15.71 ± 0.00, 16.23 ± 0.52, 16.23 ± 1.38, 14.66 mm ± 1.04 and 0.00 mm were recorded for the control, the 25 mg/kg, 50 mg/kg, 75 mg/kg and the 100 mg/kg treatments respectively at 12 MAT.

The Cd + Pb + Zn treatment caused a decrease in soil pH values along the concentration gradient (Table 1). The quantities of nitrogen, phosphorus, calcium and magnesium also decreased as the treatment concentration increased. Soil available carbon however increased along this line.

Table 1: Physicochemical properties of post *V amygdalina* cultivated soil at the end of the experiment (12 MAT)

Concentration (mg / kg)	pH	Carbon (%)	Nitrogen (%)	Phosphorus (%)	Ca (ppm)	Mg (ppm)
0	81	082	029	371	126	082
25	6	157	012	267	084	052
50	56	168	009	214	076	048
75	51	175	007	146	073	044
100	48	187	004	139	06	035

Table 2: Composition of heavy metals in *V amygdalina* at the end of the experiment (12 MAT)

Concentration (mg / kg)	Cd (ppm)	Pb (ppm)	Zn (ppm)
0	ND	ND	0.014
25	0.033	0.056	0.052
50	0.053	0.081	0.07
75	0.078	0.159	0.105
100	0.131	0.222	0.192

ND- Not Detected

Table 3: Comparative effect of various concentrations of Cd + Pb + Zn on the growth of *V. amygdalina* at 12 Months after Treatment

	Height	Number of leaves	Leaf area	Number of branches	Girth
0mg/kg	77.43 ± 1.03^{a}	35.67 ± 7.54^{a}	17.45 ± 4.85^{ab}	4.67 ± 0.67^{a}	15.71 ± 0.00^{a}
25mg/kg	40.27 ± 0.76^{b}	18.67 ± 4.98^{ab}	30.39 ± 4.83^{a}	4.67 ± 1.20^{a}	16.23 ± 0.52^{a}
50mg/kg	34.67 ± 0.78^{c}	17.33 ± 9.06^{bc}	32.21 ± 13.84^{a}	2.67 ± 0.33^{ac}	16.23 ± 1.39^{a}
75mg/kg	26.23 ± 0.92^{d}	8.67 ± 1.20^{bc}	18.76 ± 2.42^{ab}	2.33 ± 0.33^{c}	14.66 ± 1.05^{a}
100mg/kg	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}

Values are expressed as Mean ± SD (n = 3)
Values in the same column, with the same superscript are not statistically different ($P < 0.05$)

The amounts of the heavy metals found in *V. amygdalina* plants (Table 2) at 12 MAT shows that there was increased uptake of the metals as the concentration of the treatment increased. The order of uptake by quantity was Pb > Zn > Cd. Table 3 shows the comparative effect of various concentrations of Cd + Pb + Zn on the growth of *V. amygdalina.* There was significant difference between the heights of the various concentrations.

Discussion

The treatment with Cd + Pb + Zn, depressed the height (Figure 1) and the number of leaves (Figures 2) of *V. amygdalina* significantly compared to control but the 100 mg/kg concentration recorded death to plants at 3 MAT. There was increased effect along the concentration gradient. Cadmium is as one of the most dangerous elements to all kinds of organisms (16). Although it is considered to be a non-essential element for metabolic processes, it is easily absorbed by plants and even in small amount, it causes toxicity symptoms (16, 17). Lead also has been implicated in causing depressed growth of *Zea mays*, (18) and *Avena sativa* (19). It has been reported that yield of *Brassica* sp. was adversely affected by high Zn and Cr contents in soils (20). The combination of these heavy metals could result in either antagonistic or synergistic effect between the metals. Edegbai and Anoliefo observed that *V. amygdalina* plants died when treated with 100 mg /kg and 75 mg /kg cadmium at 4 and 5 MAT respectively (13). In this study, the 100 mg/kg treatment only, lost all plants at 3MAT, an indication that there could have been an antagonism between the metals.

The leaf area (figure 3) appeared to have been enhanced by the treatments except the 100mg/kg treatment though not significantly throughout the experiment, while the number of branches (Figures 4) appeared to have been enhanced at the early stages of the experiment. Beyond 5 MAT most of the plants raised in treated soil started losing most branches compared to control. The values recorded for 25 mg/kg treatment with Cd + Pb + Zn and control for number of branches at the end of the experiment were not significantly different. Similar results were recorded for girth (Figures 5) of stem in this experiment. There was significant difference ($P > 0.05$) between values recorded for control and the treatment concentrations at 12 MAT.

For growth to take place in any living organism there must be cell division. This cell division is primarily mitosis. Heavy metals affect the cell division of plants` (21), and the effects are different and depend on the concentration. Mo and Li studied the effects of Cd on the cell division of root tips in beans (22). Duan and Wang treated beans by using Cd, Pb and Zn (23). Their results showed that the cell division was extended under a low concentration of 0.01, 1.0 and 10 ppm of Cd, Pb and Zn, respectively, while cell division was shortened but the cell cycle was extended by increasing the dose. (24) on his part investigated the effects of Cd, Hg and Pb on the cell division of barley *(Hordeum vulgaris* Linn.) and also showed the trend of cell cycle extension under the 0.01 mol/L concentration treatment. These observations corroborate the results of this study.

In this study, the 100mg/kg treated plants died at 3 MAT. In their study with Cd, Hg and Pb, Duan and Wang also observed that the heavy metals combined with nuclear acid and damaged the structure of the nucleolus after 24 hours of treatment with a 0.005-0.0005 mol/L dosage and inhibited the DNase and RNase activities , thus resulting in the interruptive synthesis of DNA to affect cell division(25,26).

The various treatments with heavy metals resulted in increased acidity. There was a consistent drop in pH values along the concentration gradient. Soil pH is a very important factor that controls the mobility and availability of metals and soil nutrients. It has been reported that the solubility of heavy metals was significantly related to their total concentration, together with soil pH. The chemical properties of metals in soil and their retention in the solid phase of soil is affected by pH, quantity of the metal, cation-exchange capacity, content of organic matter and mineralogy of soil(27,28). Changes in chemical properties of soils affect concentration of free metals and result in changes in their availability for plants (29). With increasing pH, content of organic matter and clay, the solubility of most metals decreases due to their increased adsorption. The bioavailability of zinc, lead and copper from soil decreases with increasing pH. The decreased availability of metals is affected by higher adsorption and precipitation in alkaline and neutral environments (30, 31). The increased acidity in this study may have caused an increase in the solubility of the metals with the consequent adverse effects manifested.

Zhang and Wang studied the accumulation and mineralization of soil organic matter under impact of heavy metals pollution. Their results showed that high amount of heavy metals in polluted soil could slow down the mineralization rate of soil organic C, and increase the amount of hardly biodegradable organic C. With increasing soil heavy metals pollution, the particulate organic matter and its proportion in total soil organic C increased, while the microbial biomass C and its proportion in total organic C decreased (32). Heavy metals were largely enriched in particulate organic matter, which could impact the further mineralization of soil organic matter. In summary, soil heavy metals pollution could change the mineralization rate of soil organic matter, and affect its accumulation and distribution. In this study, it was observed that apart from % C which increased as heavy metal concentration increased, % N, % P, % Ca, % Mg, % K and % Na constituents of the soil decreased along the concentration gradient. The results showed that there was less competitive effect between the nutrient elements and the heavy metals when the concentration of the heavy metals was less. The more heavy metal species in the soil and the higher the concentration of the metals, the more they compete with the binding sites for these nutrient elements.

The ranking order of accumulation of Cd, Pb and Zn in *V. amygdalina* (Table 2) in the present study was Pb > Zn > Cd. The present concentrations of Cd, Pb and Zn 0.131, 0.222, 0.192 mg kg^{-1} are lower compared to the recommended tolerable levels proposed by joint FAO/WHO Expert Committee on Food Additives for leaves, stem and root of different vegetables, which are 0.3, 5.0 and 60.0 mg kg^{-1} for Cd, Pb and Zn respectively (33, 34). Edegbai and Anoliefo observed that when *V. amygdalina* was treated with 25, 50, 75 and 100 mg/kg

cadmium, 0.068, 0.094, 0.185 and 0.283 mg/kg was taken up by treated plants respectively. The highest amount of cadmium taken up by *V. amygdalina* in this study (0.192 mg/kg) was much less (13). Of the various growth parameters investigated, the height of *V. amygdalina* plants was significantly different between the various concentrations.

Conclusion: The present study investigated the toxicity of selected heavy metals to *Vernonia amygdalina.* There was competitive interaction between the heavy metals and which could have resulted in a reduction in their uptake compared to when individual metals were involved in the treatments. This was observed with cadmium. There interaction between these metals, though antagonistic, resulted in adverse effects to *V. Amygdalina.*

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