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Teratogenic Effects of *Carissa spinarum* and *Azadirachta indica* Aqueous Extracts in Mice

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ABSTRACT

Carissa spinarum (mũkawa in Kikuyu, lamuriak in Maasai) and *Azadirachta indica* (neem, mwarubaini in Kiswahili) are widely used in African communities. *C. spinarum* is used as food and as treatment for gonorrhoea, cancer, and abnormal pain in pregnancy. *A. indica* is used as a mosquito-repellent and as treatment for malaria and dental carries. Both plants have broad biological activities including anticancer, hypoglycaemic, antinociceptive, and anti-inflammatory activity. However, data on their safety for use during pregnancy is scarce. This study aimed to determine the teratogenic effects of *C. spinarum* and *A. indica* in mice. FDA guidelines for reproduction studies were used. Pregnant mice were randomly divided into 8 groups (n=5) for the assay. Plant extracts were prepared in the doses 100, 250, and 500mg/kg body weight. Phenytoin sodium 100mg/kg body weight was used as the positive control and distilled water was used as the negative control. Treatments were administered orally and began from gestation day 6 and ended on gestation day 15. On gestation day 18, the mice were weighed and euthanized and the pups were recovered. Weights of the gravid uteri, number of pups and their body measurements, and incidences of foetal resorption were also recorded. Data were expressed as means and their standard errors and analysed using one-way ANOVA/Dunnett's *post hoc* test. The significance level was set at $p<0.05$. Heavy metal concentrations in the plant samples were determined using flame photometry and atomic absorption spectrophotometry. All doses of *C. spinarum* and *A. indica* caused significant ($p<0.001$) weight loss and foetal resorption in the gravid mice. Reduced head sizes (microcephaly) and elongated limbs were observed in the *C. spinarum* 100mg/kg and *A. indica* 500mg/kg groups. Premature birth and high birth weights were also observed in the latter group. The plant samples were found to have high concentrations of lead, cadmium, iron, chromium, manganese, and zinc and this may have added to the teratogenicity observed. *C. spinarum* and *A. indica* exhibited significant teratogenicity and should be used with caution during pregnancy. In addition, the level of heavy metal contamination in Kenya should be assessed.

Keywords: *Carissa spinarum*, *Azadirachta indica*, Neem, Teratogenicity, Foetal resorption, Microcephaly.

INTRODUCTION

Carissa spinarum and *Azadirachta indica* are important plants in African societies. The bark of *C. spinarum* is boiled with meat to make soup and the fruits are eaten ripe or fermented to make wine [1]. The plant is also used to treat a myriad of illnesses including but not limited to cancer, dysentery, gonorrhoea, cataracts, malaria, stomach aches, herpes, and abnormal pain during pregnancy [1]. *C. spinarum* has exhibited numerous biological activities including anticancer [2, 3], anti-inflammatory [4], antipyretic and antinociceptive [5], and hypoglycaemic [6] activity. It has also exhibited antiviral, antioxidant, antirheumatic, and erythropoietic effects, among others [7]. High doses of *C. spinarum* methanol extracts caused foetal resorption in mice [8]. *A. indica* is used as a mosquito-repellent and as treatment for malaria, diabetes, psoriasis, and dental carries [9, 10]. *A. indica* has exhibited antiplasmodial [11] and hypoglycemic [12] activity. It also has immunomodulatory, antitumor, antioxidant, antifungal, spermicidal, anti-inflammatory, and antimicrobial biological activities, among others [13]. While these plants are widely used, information on their safety for use during pregnancy is limited. This study aimed to determine the teratogenic effect of *C. spinarum* and *A. indica* in mice.

MATERIALS AND METHODS

Preparation of the plant materials

Carissa spinarum root bark was collected from Nanyuki and *Azadirachta indica* stem bark was collected from Mombasa. The plants were separately ground using an electric grinding mill. Approximately 200g of the powder was weighed and put in a conical flask that was topped up with two litres of distilled water and stirred. The mixture was then incubated in a water bath at 60˚C for 4 hours then cooled, decanted,

filtered using Whatman No. 1 filter papers, and freeze-dried to obtain the extract which was stored at -20˚C. The yield was 15g for *C. spinarum* and 20g for *A. indica*. Doses of the plant extracts were prepared at 100, 250, and 500mg/kg body weight.

Drugs and chemicals

Distilled water, phenytoin sodium 100mg/kg body weight, chloroform, selenium, sulphuric acid, salicylic acid and soluble salts of sodium, potassium, calcium, chromium, cadmium, lead, iron, manganese, zinc, and copper were used.

Experimental animals

Virgin female Swiss albino mice weighing about 20g each were procured. They were housed in cages maintained at room temperature for a week prior to the study for acclimatization. Standard feed and water were provided *ad libitum*.

Assay

Virile male mice were introduced to females in oestrus for mating at a male-to-female ratio of 1:3 using 12-hour mating periods (evening to morning). Mating was confirmed by the presence of a copulatory plug in the early morning (gestation day 0). Pregnancy was confirmed by palpation of the abdomen, abdominal bulge, and protrusion of the nipples. Pregnant mice were randomly divided into 8 groups each consisting of five mice to be used in the assay. Distilled water was used as the negative control and phenytoin sodium was used as the positive control. Treatments were administered from gestation days 6 to day 15. On gestation day 18, the mice were weighed, euthanized and the pups recovered. Maternal weights, weights of the gravid uterus, number of the pups recovered and their body measurements, and incidences of foetal resorption were recorded. These experiments were conducted in accordance with FDA guidelines for single-generation reproduction studies for the evaluation of the safety of drugs for human use [14, 15] and the guidelines for the care and use of laboratory animals [16].

Analysis of heavy metal concentrations in the plant samples

A digestion mixture of selenium, sulphuric acid, and salicylic acid was prepared by dissolving 3.5g of selenium powder in 100ml concentrated sulphuric acid, heating to 300º C until the black colour of selenium turned light yellow, after which 7.2g of salicylic acid was added. Plant powder weighing 0.3g was then placed in a clean and dry digestion tube and 2.5ml of the digestion mixture was added and allowed to react at room temperature for 2 hours before addition of three successive portions of 1ml hydrogen peroxide with 10 seconds of wait time between each addition. This mixture was then heated to 330º C until the colour changed to brown-yellow. The tubes were removed from the digester when the mixture turned light yellow and they were allowed to cool to room temperature. The mixture was transferred to a 50ml volumetric flask and distilled water was added to the 50ml mark. This procedure was repeated for both *C. spinarum* and *A. indica*. Serial dilutions of the selected metal ions were then prepared along with blank samples to be used in making calibration curves. A flame photometer was used to determine the concentrations of sodium, potassium, and calcium while an atomic absorption spectrophotometer was used to determine the concentrations of lead, zinc, cadmium, iron, manganese, copper, and chromium.

Data analysis

The data was expressed as means and standard error of the means (SEM). One way ANOVA was performed followed by Dunnett's *post hoc* test. The level of significance was set at $p<0.05$.

RESULTS

All doses of *C. spinarum* and *A. indica* caused significant ($p<0.001$) weight loss in the gravid mice that was dose dependent. The weight loss was comparable to that of the standard drug phenytoin. The highest weight loss was observed with *C. Spinarum* at the dose 500 mg/kg.

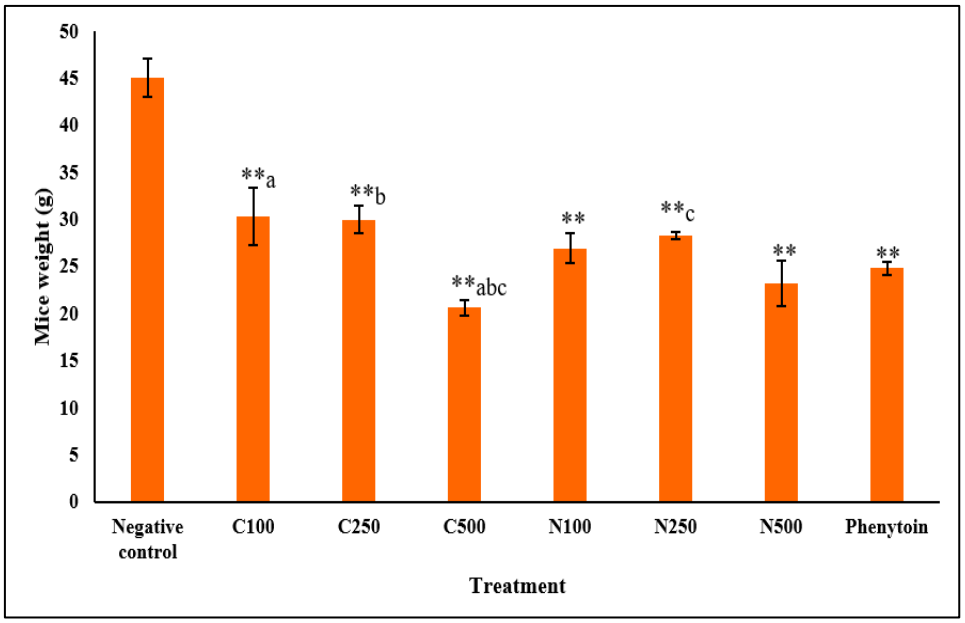

Figure 1: Effect of *Carissa spinarum* (C) and *Azadirachta indica* (N) on the weights of gravid mice. ** $p<0.001$ against negative control, a $p<0.05$ between C100 and C500, b $p<0.001$ between C250 and C500, c $p<0.001$ between C500 and N250

All plant doses significantly ($p<0.001$) decreased the weight of the gravid uteri. This was due to foetal resorption that occurred in each group. The effect was comparable to that of phenytoin. The occurrence rate for foetal resorption was 100% in the groups administered *C. Spinarum* 250mg/kg, *C. Spinarum* 500 mg/kg, *A. indica* 100mg/kg, *A. indica* 250mg/kg, and phenytoin 100mg/kg and 80% in the groups administered *C. spinarum* 100mg/kg and *A. indica* 500mg/kg. There was no case of foetal resorption in the negative control group (0% occurrence rate). The foetal resorption caused by *C. spinarum* and *A. indica* was comparable at each dose level.

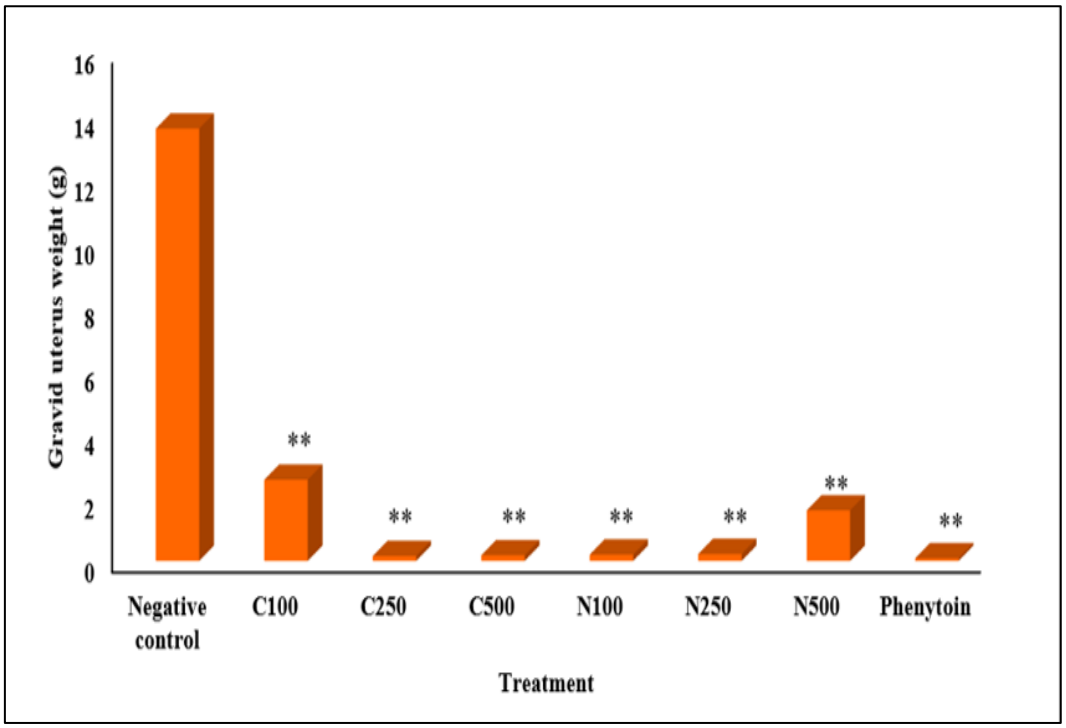

Figure 2: Effect of ***Carissa spinarum* (C)** and ***Azadirachta indica* (N)** on the weight of gravid uteri. ** $p<0.001$ against negative control

Extracts of both plants significantly ($p<0.001$) decreased the number of pups in each litter in a dose dependent manner. This was due to foetal resorption that occurred in each group. The effect was comparable to that of phenytoin. There were no pups recovered from the groups administered *C. Spinarum* 250mg/kg, *C. Spinarum* 500 mg/kg, *A. indica* 100mg/kg, *A. indica* 250mg/kg, and phenytoin 100mg/kg due to the 100% occurrence rate of foetal resorption in these groups. Although pups were recovered from the group administered a high dose of *A. indica* (500mg/kg), these pups were birthed prematurely and had more discernible malformations.

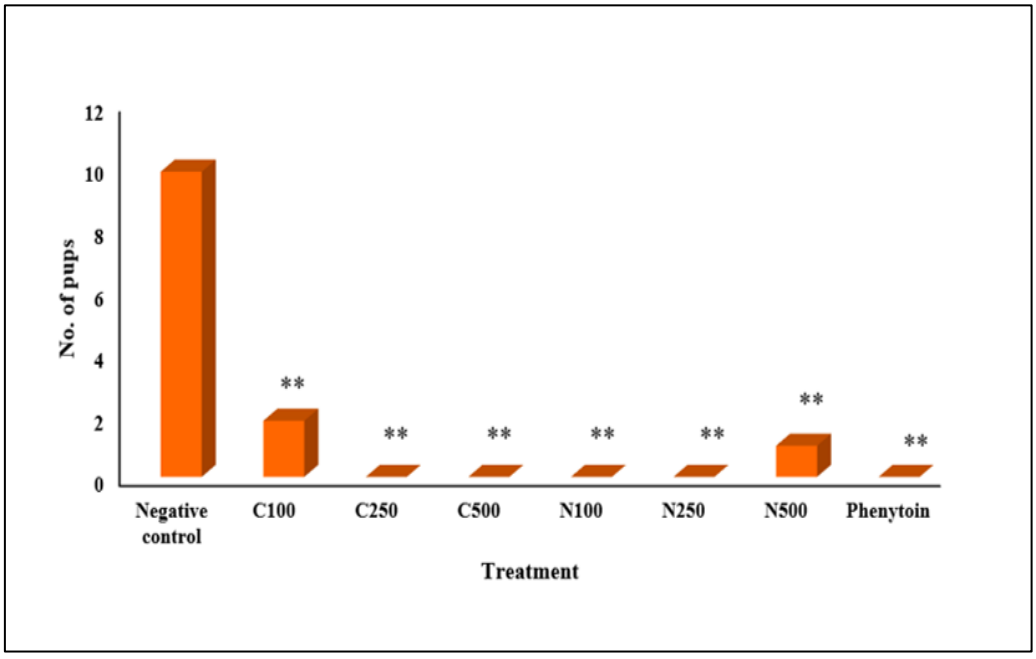

Figure 3: Effect of *Carissa spinarum* (C) and *Azadirachta indica* (N) on the litter size. ** $p<0.001$ against negative control

The pups recovered from the group that received *A. indica* 500mg/kg had significantly ($p<0.05$) higher birth weights. The birth weights of pups from the negative control group and the group administered *C. spinarum* 100mg/kg were unaffected.

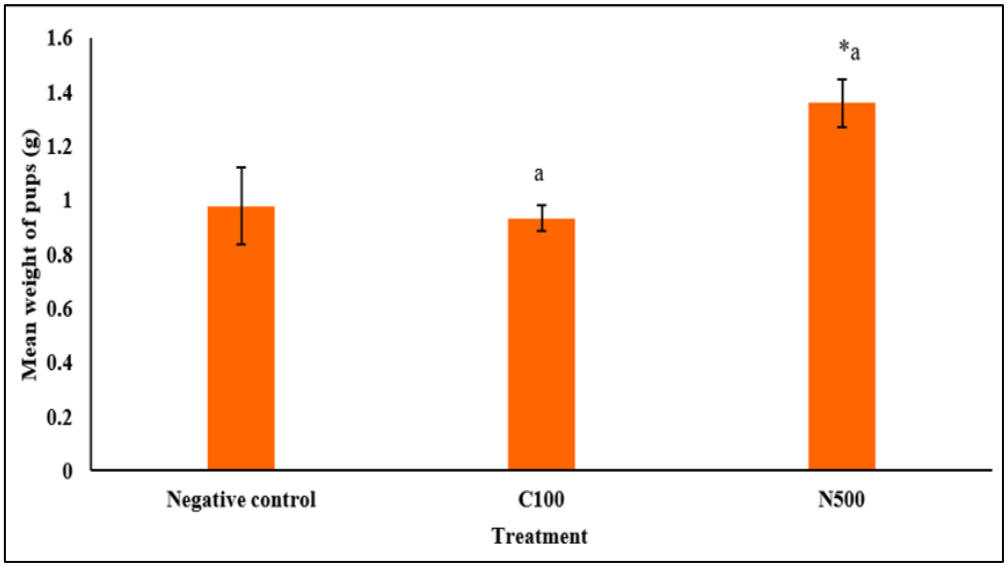

Figure 4: Effect of *Carissa spinarum* (C) and *Azadirachta indica* (N) on the mean weight of pups. * p<0.05 against negative control, a p<0.001 between C100 and N500

Pups from the group that received *C. Spinarum* 100mg/kg had significantly (p<0.001) smaller heads (microcephaly) and significantly (p<0.001) elongated hindlimbs while pups from the group that received *A. indica* 500 mg/kg had significantly (p<0.001) smaller heads and significantly (p<0.001) elongated forelimbs and hindlimbs. *C. spinarum* 100mg/kg caused an 11.4% reduction in head size while *A. indica* 500mg/kg caused a 15% reduction in head size. In both groups, the body length and the tail length were unaffected. There were no gross deformities observed in the pups recovered from the negative control group.

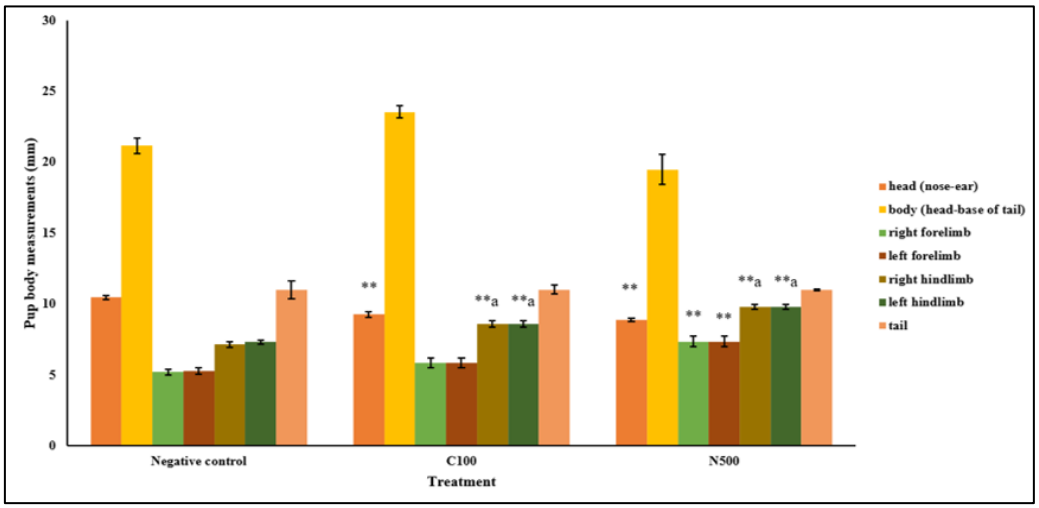

Figure 5: Effect of *Carissa spinarum* (C) and *Azadirachta indica* (N) on the pup body measurements. **p<0.001 against negative control, a p<0.05 between C100 and N500

Metal concentrations in the plant samples

C. spinarum had elevated levels of iron, chromium, manganese, lead, and zinc metals. *A. indica* had elevated levels of cadmium, iron, chromium, manganese, lead, and zinc metals. These metals exceed the recommended levels as per the limits set by the Food and Nutrition Board, Institute of Medicine [17] and the Scientific Committee of Food [18].

Table 1: Concentrations of metal ions in the plant samples in ppm

Metal element	*C. spinarum*	*A. indica*
Calcium	13392.86	5952.382
Potassium	11687.94	12689.76
Sodium	1200.828	1035.197
Cadmium	0	6.666667
Iron	501.7922	161.2903
Copper	10.89333	10.89333
Chromium	26.66667	13.33333
Manganese	428.2297	204.9442
Lead	77.957	104.8387
Zinc	370.3704	185.1852

DISCUSSION

Both *C. spinarum* and *A. indica* exhibited significant teratogenicity by causing extreme weight loss in the gravid mice, foetal resorption, and gross malformations in the pups. These results concur with earlier observations whereby methanol-extracted *C. spinarum* caused extreme weight loss in the gravida and foetal resorption [8]. Previous studies of *A. indica* have also shown comparable results vis a vis weight loss, whereby the hypoglycaemic mechanisms of *A. indica*, in addition to the high level of tannins found in the extract, cause weight loss [19]. Weight loss in pregnancy, especially extreme weight loss, is disadvantageous and poses a myriad of dangers to both the mother and foetus. These dangers include pre-eclampsia, low birth weight, delivery complications, miscarriage, intra-uterine growth restriction, and preterm delivery [20, 21, 22, 23]. Women who are underweight and/or don't meet weight targets during pregnancy have a higher risk of having underweight babies who have greater risks of morbidity and mortality than neonates of normal weight [23, 24]. The mother, if at low maternal weight during pregnancy, is at increased risk of toxaemia [23]. Low weight during gestation may be caused by stress, malnutrition, illness, environmental conditions such as drought and famine, or pharmaceuticals [24]. In mice, miscarriages are indicated by incidences of resorption. This is because in rodents, a lost pregnancy is reabsorbed [25]. Therefore, it can be hypothesized that phytochemicals in *C. spinarum* and *A. indica* led to weight loss in the gravid mice which resulted to the adverse pregnancy outcomes observed. In addition, the hypoglycaemic mechanisms of *A. indica* may have led to the higher birth weights of the mice pups as insulin is a key hormone in foetal growth and excessive insulin secretion leads to foetal overgrowth [26, 27] and *A. indica* stimulates the beta cells of the pancreas thus increasing endogenous insulin production [12, 28, 29].

The pups were observed to have significantly reduced head sizes (microcephaly) which indicates a small brain that may not have developed properly [30]. Microcephaly is caused by exposure to teratogens during pregnancy and severe maternal malnutrition during pregnancy [30]. The condition usually results from a reduction in the size of the cerebral cortex caused by inadequate production of stem cells, impaired neurogenesis, and the death of neurons and neural stem cells [31]. The cerebral cortex performs many functions including sensor, motor, and association functions, and thus its malformation results in the impairment of these functions. This can be seen in microcephaly as the issues associated with the condition include seizures, speech difficulties, developmental delay, intellectual disabilities, feeding problems, poor motor control, and sensory difficulties [30]. Most full-term new-borns with a head circumference smaller than two standard deviations and who are neurologically typical go on to have ordinary intelligence by age seven [30]. However, those with head circumferences smaller than three standard deviations usually experience intellectual disabilities [30]. The pups were also observed to have significantly longer limbs. In humans, this condition is known as dolichostenomelia and it is commonly found in disorders that affect the connective tissues such as Marfan syndrome, a genetic disorder whose patients are very tall and have disproportionately long and thin limbs and digits, abnormal spinal curvature, abnormal joint flexibility, and cardiovascular defects [32].

C. spinarum and *A. indica* both showed elevated levels of heavy metals. Heavy metals in the environment are absorbed by plants and may exact their effects alongside the effects of plant phytochemicals. Zinc was found in high concentrations in both *C. spinarum* and *A. indica*. However, its absorption may have been hindered by the high iron levels in both plant extracts as high iron levels reduce zinc absorption and cause adverse gastrointestinal effects including nausea, epigastric pain, and diarrhoea [17, 18]. Iron consumption causes adverse effects at 15mg/day and it is highly toxic if consumption exceeds 20mg/day [17,18]. Zinc is important for enzyme function and it is required for foetal cell growth and brain development [17, 18] and its hindered absorption may have contributed to the microcephaly observed. Mice treated with *C. spinarum* and *A. indica* were also observed to have diarrhoea, and this may be explained by the elevated levels of iron in the extracts. It may also explain why mice in *C. spinarum* groups lost more weight compared to those in *A. indica* groups as *C. spinarum* had higher iron levels. Lead was found in high concentrations in both *C. spinarum* and *A. indica* and this may be due to environmental lead pollution which is a big problem in Kenya [33, 34, 35]. Lead is an extremely toxic heavy metal that causes devastating effects in all parts of the body [36]. There is no safe amount of lead to consume, but levels exceeding 5ug/dl are toxic to children and levels exceeding10ug/dl are toxic to adults [36]. Sources of lead in the environment include leaded fuel, lead paints, and lead mining. Lead persists in the environment and thus its levels build up over time. Harmful effects of lead in pregnancy include preterm birth, spontaneous abortion (miscarriage), low birth weight, and developmental problems in childhood [36]. Lead may thus have contributed to the toxicity and teratogenicity observed. Cadmium is toxic even in trace amounts due to its low permissible exposure [37]. Like lead, cadmium persists in the environment and builds up with time. Sources of cadmium include industrial waste, electronic waste, and fertilizers [37]. Cadmium is a carcinogen, and it also impairs renal function [37]. Chromium is toxic in all its forms but its toxicity varies across different speciations. Chromium III is a trace element essential to the human diet and most dietary chromium is excreted in urine [38] giving this form of chromium less opportunity to exert its toxic effects. However, chromium VI is genotoxic, hemotoxic, carcinogenic, and has a median lethal dose of 50-150mg/kg [38]. Though manganese is an essential element, it is also a powerful neurotoxin [17, 18]. The upper tolerable intake for manganese in adults is 11mg/day and excessive manganese exposure may lead to the neurodegenerative disorder called manganism [17, 18].

Conclusion and recommendations

The extracts of *Carissa spinarum* and *Azadirachta indica* exhibited significant teratogenic effects. In addition, the elevated levels of heavy metals in the plant samples may have contributed to the teratogenicity observed. Therefore, *C. spinarum* and *A. indica* should be used with caution during pregnancy and further studies should be carried out to identify and isolate the teratogenic phytochemicals in the plants as well as to assess the level of heavy metal contamination in the country.

Author contributions

Yvonne W. Wabai conducted the research and prepared the manuscript under the supervision of Dr John K. Mwonjoria and Prof. Joseph N. Ngeranwa. Dr. Charles G. Githinji reviewed the research. All authors reviewed and edited the manuscript.

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Conflict of Interest

None declared.

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