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Cytotoxicity of *Heliotropium indicum* Methanolic Leaf Extract in Mice

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Abstract

Heliotropium indicum grows well in wet fertile soils often near rivers and lakes in tropical and subtropical countries. It contains phytochemicals with beneficial health effects. However, information is scarce on its cytotoxicity. The objective of this study was to determine cytotoxic effects of methanolic leaf extract of the plant in mouse organs. Mice were randomly divided into four groups: Control group A received distilled water 10 ml/kg. Groups B, C, and D received orally plant leaf extract at doses of 5 mg/kg, 10 mg/kg, and 15 mg/kg respectively per day for 7 days. At the end of day 7 mice were sacrificed, tissues from various organs dissected, processed into tissue blocks and sectioned followed by Haematoxylin and Eosin staining to examine histological organization of the tissues and immunohistochemical analysis for single stranded DNA (ssDNA) to determined cell death by apoptosis. The results showed that the extract at all doses in both male and female mice induced cell death. Affected cells include spermatogonia in testis leading to absence of spermatozoa in seminiferous tubules; stromal cells and cumurus oophorus of the ovary, epithelial cells lining glandular and non-glandular stomach; hepatocytes of the liver, collecting ductal cells in medullary rays in the kidney, pneumocyte type I, II and macrophages of the lungs and cells of red and white pulps of the spleen. It can be concluded that methanolic leaf extract of *H. indicum* is cytotoxic to cell of the vital organs of the body, suggesting that medicinal use of the plant leaf extract in any form should be taken with care.

Keywords: Mouse; body organs; apoptosis; traditional medicine

Introduction

Heliotropium indicum or commonly known as Indian heliotrope or Indian turnsole, belongs to the family Boraginaceae and is widely distributed in tropical and subtropical regions (Dash and Abdullah 2013). The plant is an annual erect ascending hirsute branched about 20 to 60 cm tall and coarse fetid herb. The leaves are opposite or sub-opposite, alternate or straight forward, ovate to obovate, hairy and acute, 5 to 10 cm long. The flowers are regular, sessile and arranged in two rows, flowering throughout the year (Dash and Abdullah 2013). Several compounds have been found in the plant including flavonoids, tannins and phytochemicals such as saponin, tannins, alkaloids, helotrine, phenols and steroids (Akinlolu et al. 2008, Roy 2015, Santhosha et al. 2015). Phytochemicals are reported to be

naturally produced in plants as protective molecules in response to biotic and abiotic stresses (Fokunang et al. 2019).

Heliotropium indicum is known for its traditional medicinal use in the treatment of arthritis, rheumatism, eye infections, diarrhea, dysentery, kidney stone, wounds and malaria (Dash and Murthy 2011, Yeo et al. 2011). The plant has also other biological activities including anti-inflammatory, anti-tumor, anti-microbial, anti-tussive, anti-nociceptive, anti-fertility, anti-tuberculosis and anti-anaphylaxis (Akinlolu et al. 2008, Ghosh et al. 2018, Rahman et al. 2011, Sivajothi et al. 2015, Villa et al. 2016). Despite the importance of *H. indicum* in traditional healing medicine (Dash and Abdullah 2013), it is also reported to be hepatotoxic due to the presence of pyrrolizidine alkaloids (Bayala et al. 2019, Reza et al. 2018).

Toxicity of *H. indicum* is thought to be associated with concentration of the phytochemical compounds in its parts: the roots stem and leaves. Physicochemical properties of soil, including pH, electrical conductivity, organic matter content, and cation exchange capacity, affect accumulation of compounds in plants (Iqbal et al. 2019, Li et al. 2019). Hence, a plant can have medicinal potential in one application but becomes toxic in another. Despite its wide use as medicinal plant on Indian subcontinent, leaf extract of *H. indicum* has been proved to be cytotoxic and genotoxic by inhibiting mitosis in Brine Shrimps and *Allium cepa* cells (Azeez et al. 2020) and inhibiting growth of Hela cell line (Sivajothi et al. 2015). Little knowledge is available on the medicinal use of the plant growing on African continent with different soils and climatic condition as well as its toxicity to various organs. The objective of this study was therefore to determine the cytotoxic effects of methanolic leaf extract of *H. indicum* found in Tanzania in various tissues in mice using immunohistochemistry for ssDNA which is known to be a specific marker for cells degenerating or dying by apoptosis.

Materials and Methods

Plant collection

Fresh leaves of *H. indicum* were collected at Kikundi river bank in Morogoro town (6.8278° S, 37.6591° E) during dry season in July, 2023. The plant was identified and authenticated at the Department of Crop Science, Sokoine University of Agriculture (Figure 1).

Figure 1: ***Heriotropium indicum*** **plant**. Image taken on habitat at Kikundi River in Morogoro town, Tanzania.

Preparation of plant extract

The leaves of *H. indicum* were air dried at room temperature for three weeks, ground into powder form using a laboratory blender and then extracted with methanol (1:5 w/v) with occasional stirring for a period of 48 hours. The extract was then filtered using Whatman filter paper. The filtrates obtained were evaporated to semisolid substance using rotary evaporator at 70ºC and concentrated using water bath at 46ºC to yield a solid mass, which was packed into separate air tight containers and stored in the refrigerator until use.

Study Animals

A total of twenty (20) mice (10 males and 10 females) were purchased from the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture (SUA). They were kept in stainless steel cages in animal laboratory house under controlled conditions of light (12-h light-dark cycles) and temperature (20-25°C) and fed standard laboratory chow and water *ad libitum*. The animals were quarantined for two weeks before the start of the experiments. All mice were handled in accordance with the standard guidelines for animal experimentation approved by Research and Ethical Committee of Sokoine University of Agriculture.

Experimental procedure and tissue sampling

The mice were randomly divided into 4 groups each consisting of five animals, as follows: Group A was given distilled water 10 ml/kg. Group B, C and D were respectively given 5mg/kg, 10mg/kg and 15mg/kg per day for 7 days by oral administration. At the end of the experiment, all mice were weighed and anesthetized by intra-muscular injection of ketamine hydrochloride (40 mg/kg) plus xylazine hydrochloride (5 mg/kg) followed by dissection to obtain tissue samples including testis, ovary, stomach, liver, kidney, lungs and spleen. Fixation of the tissues was done using Bouin's solution for 24 hours at room temperature. Tissue processing was performed as previously described by Slaoui and Fiette (2011) with minor modifications. Tissue blocks were prepared and cut using rotary microtome (Baird and Tatlock (London) Ltd; England) at 4 μm thickness to produce tissue sections. Some sections were used for Haematoxylin and Eosin (H and E) staining to examine histological organization and others for immunohistochemistry using 3, 3′-diaminobenzidine tetra-hydrochloride (DAB) for detection of binding sites for ssDNA.

Immunohistochemistry

Tissue sections were deparaffinized and rehydrated followed by incubation for 10 min. with hydrogen peroxide block (Abcam, ab64261, supplied ready to use) at room temperature (RT) to inhibit endogenous peroxidase activity, then washed (3 x 5 min.) in PBS. Sections were then incubated with protein block (Abcam, ab64261, supplied ready to use) for 10 min. at RT to block non-specific background binding. Tissue sections were then incubated with anti-single stranded DNA (ssDNA) rabbit IgG affinity purified (IBL, DAKO 1D114) primary antibody overnight in a dark, humid chamber at 4°C diluted at a ratio of 1:500 in PBS. For negative control, PBS was applied in place of the primary antibody. Sections were then washed (3x15 min.) in PBS followed by incubation with goat anti-rabbit HRP conjugate micro-polymer (Abcam, ab64261, supplied ready to use) for 60 min. at RT. Sections were then washed (3x15min.) in PBS before incubation for 3-5 min. with 50X DAB chromogen solution (Abcam, ab64261, supplied ready to use) to visualize binding sites, then rinsed in water for 10 min. to stop reaction followed by dehydration through a graded ethanol series, clearance and mounting by a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylene (DPX). Binding sites were evaluated using Olympus BH-2 microscope fitted with Olympus camera for image capturing.

Statistical analysis

Cell count for immunohistochemical expression of CTLA2-alpha in various tissues was performed using Image J bundled with 64-bit Java 8. The cell counts were recorded in Excel software, then analyzed for

statistical significance of means by two-way ANOVA using R statistical software version 4.3.1. P-value < 0.05 was considered to be significant.

Results

Immunohistochemistry for ssDNA was performed in order to localize cells which have been damaged by methanolic leaf extract of *H. indicum* upon administration by oral gavage. The sampled tissues from all mice (male and female) given 5mg/kg, 10mg/kg and 15mg/kg per day for 7 days of the plant extract were equally damaged.

Testis: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed strong staining for ssDNA in spermatogonia, the undifferentiated spermatogenic stem cells lying along the basal lamina at the periphery of seminiferous tubules. Moderate staining was observed in secondary spermatocytes. The primary spermatocytes, sertoli cells and Leydig cells were not stained, thus not damaged. The primary spermatocytes are usually found at the periphery of the lumen of seminiferous tubules closer to spermatogonia. The sertoli cells are localized from the basal lamina extending to the lumen. The Leydig cells are found in the interstitial area between the seminiferous tubules. Lumens of seminiferous tubules were empty which are normally occupied by spermatids in location closer to the lumen and mature spermatozoa at the center of the lumen, indicating that spermatogenesis ceased. Immunoreactivity was also not observed in sections made from mice that received distilled water and in sections not incubated with the anti-ssDNA primary antibody (Figure 2).

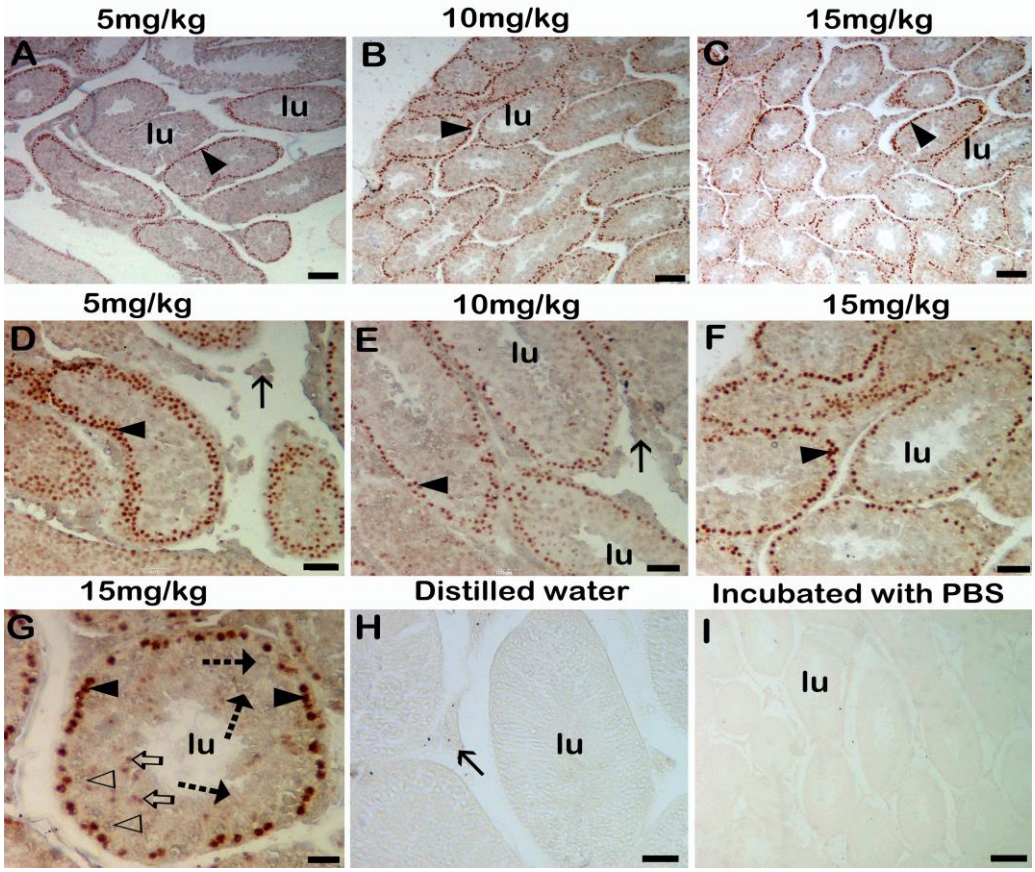

Figure 2: Immunohistochemical images showing immunoreactivity for anti-ssDNA in the testis. (A-G) images of testis sections made from mice treated for 7 days with

the plant leaf extract at doses of 5, 10 and 15 mg/kg per day showing strong similar pattern of immunoreactivity for ssDNA appearing as brown reaction product in spermatogonia (arrow heads); not present in primary (open arrow heads), sertoli cells (dotted arrows) and Leydig cells (arrows) but moderate in some secondary (open arrows) spermatocytes. The lumen (lu) of seminiferous tubules appear empty which is usually occupied by spermatozoa. Immunoreactivity is absent in control tissue section (H) from mice given distilled water and in (I) section not incubated with anti-ssDNA primary antibody. Scale bar: (A), (B), (C) and (I) 200 µm; (D, E, and F) 100 µm and (G) and (H) 50 µm.

Ovary: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed randomly distributed ssDNA positive cells with nuclei appearing as brown reaction product within stroma of the ovary as well as in cumulus oophurus and corona radiata. Moderate staining occurred in theca cells of secondary follicles, primordial and primary follicles and in the corpus luteum but was not observed in the antral follicles and in the oocytes as well as in the sections made from mice that received distilled water and in the sections not incubated with the anti-ssDNA primary antibody (Figure 3).

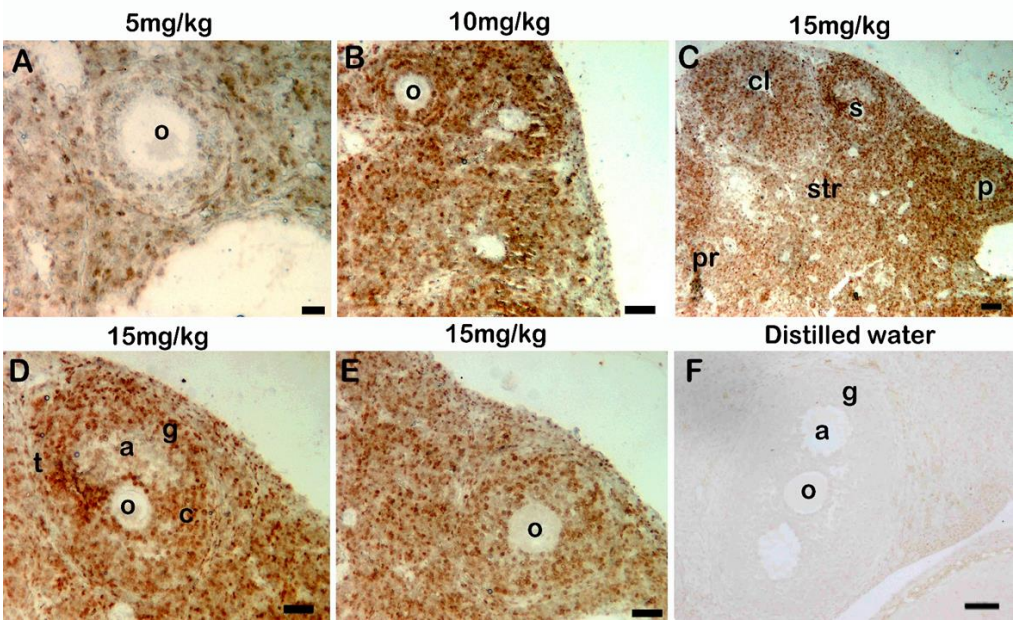

Figure 3: Immunohistochemical images showing anti-ssDNA immunoreactivity in the ovary. (A-C) images of ovary sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15 mg/kg body weight per day showing strong similar pattern of immunoreactivity for ssDNA appearing as brown reaction product. (D and E) higher magnification images of ovaries sections treated with 15mg/kg body weight. Immunoreactivity is not observed in the antral follicle **a**, and in oocyte **o**, as well as in the sections (F) made from mice that received distilled water. Note: Granulosa cells layer, **g**; cumulus oophorus, **c**; theca layer, **t**; and an antrum, **a**, surrounding the oocyte, **o**. Stroma, **str**. Primodial, **pr**; primary, **p**, secondary / antral, **s**, corpus luteum, **cl**. Scale bar: (B and C) 200 µm; (A, D, E and F) 100 µm.

Stomach: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed significant labeling for ssDNA in the stomach in tunica mucosa within epithelial cell lining and moderately in the lamina propria of the glandular and non-glandular parts of the stomach. Staining was not observed in the lamina muscularis, tela submucosa, tunica muscularis, and tunica serosa as well as in tissue sections made from mice that received distilled water and in sections not incubated with the anti-ssDNA primary antibody (Figure 4).

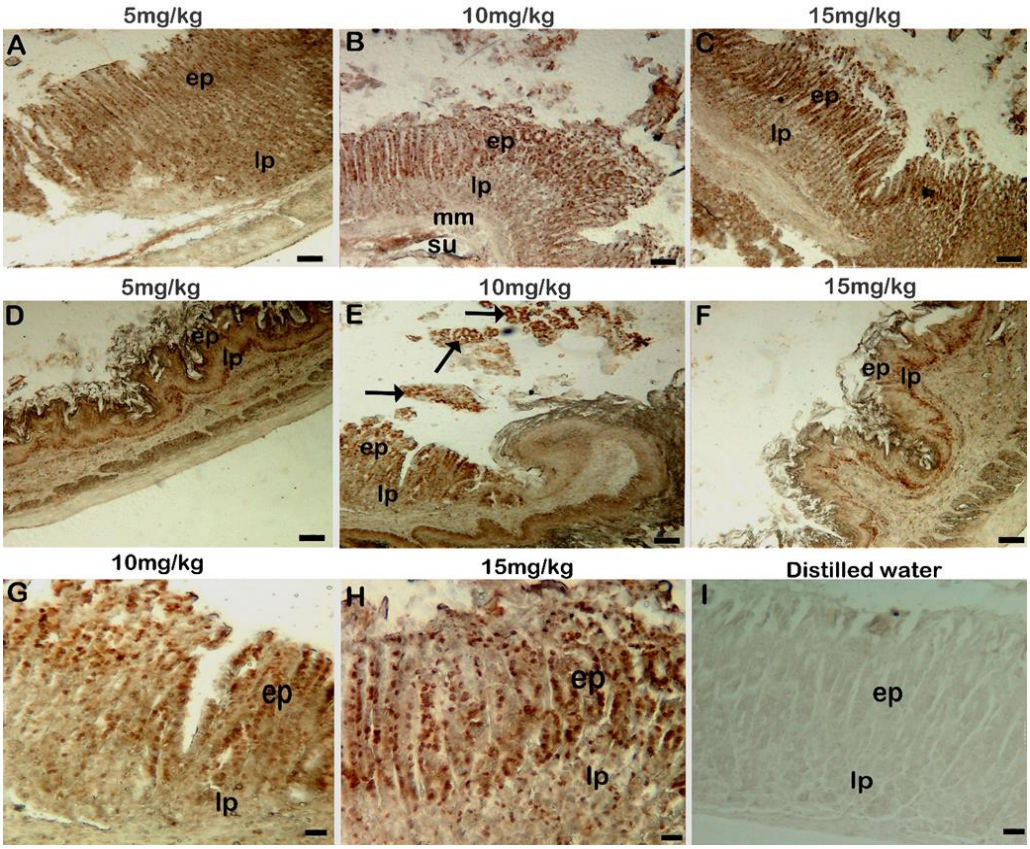

Figure 4: **Immunohistochemical images showing anti-ssDNA immunoreactivity in the stomach**. (A-C) Glandular, (D-F) non-glandular stomach and (G and H) large magnification of glandular stomach made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15 mg/kg body weight per day showing strong similar pattern of immunoreactivity for ssDNA appearing as brown reaction product in epithelial cells, some of which slough off in the lumen / stomach (arrows); moderately in lamina propria, **lp**, but not observed in lamina muscularis, **mm** and tela submucosa, **su,** as well as in tissue sections (I) made from mice that received distilled water. Scale bar: (A -F) 200 µm; (G-I) 50 µm.

Liver: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed strong labeling for ssDNA in hepatocytes which appeared randomly distributed throughout the liver. Apoptotic hepatocytes (Pyknotic nuclei) were manifested by dark brown deposit of DAB

chromogen. Immunoreactivity was not observed in sections made from mice that received distilled water and in the sections not incubated with the anti-ssDNA primary antibody (Figure 5).

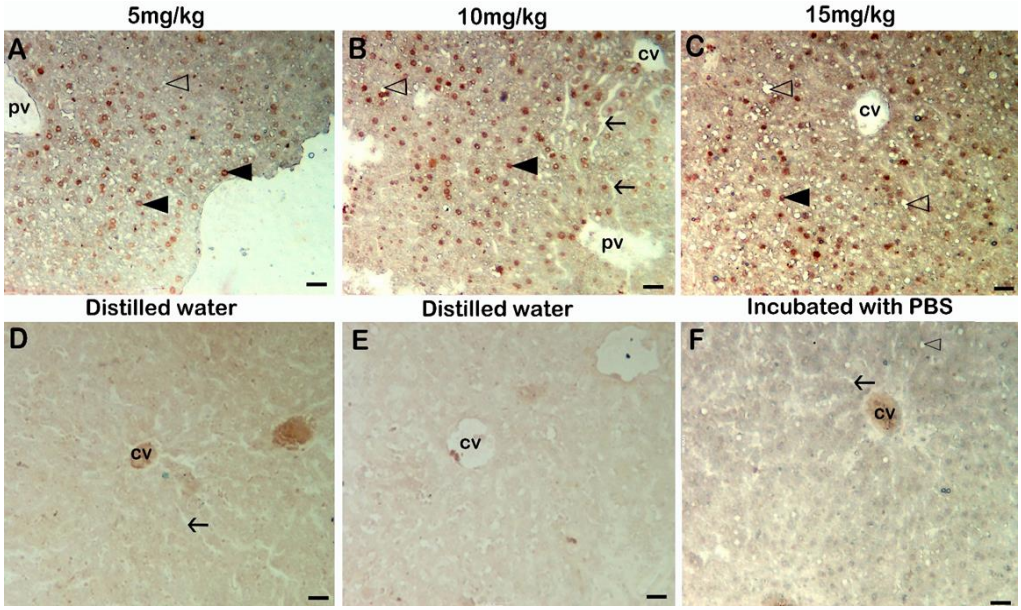

Figure 5: Immunohistochemical images showing anti-ssDNA immunoreactivity in liver. (A-C) images of liver sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15 mg/kg body weight showing strong similar pattern of immunoreactivity for ssDNA appearing as brown reaction product in hepatocytes. Note: apoptotic hepatocytes are manifested by dark brown (black arrow head) deposit of DAB chromogen, fat deposits (open arrow head) showing fatty change, dilated sinusoids (arrow). Immunoreactivity is not observed in (D and E) sections made from mice that received distilled water and in (F) section not incubated with the anti-ssDNA primary antibody. Sinusoids (arrows); Central vein, **cv**; Portal vein, **pv**. Scale bar: (A-F) 100 um.

Kidney: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed similar pattern of immunoreactivity for ssDNA in cuboidal cells lining the collecting ducts in medullary rays in the medulla as well as in the glomerular tuft in the cortical region. Immunoreactivity in the cortex was observed in glomerulus in podocytes, recognized by their large nuclei and bound the visceral layer of glomerular capsular space (Bowman's capsule) and mesangial cells characterized by their relatively small nucleus compared to podocytes. The proximal with microvilli and distal convoluted tubules were not labeled for ssDNA. Labelling was also not observed in tissue sections made from mice that received distilled water and in the sections not incubated with the anti-ssDNA primary antibody (Figure 6).

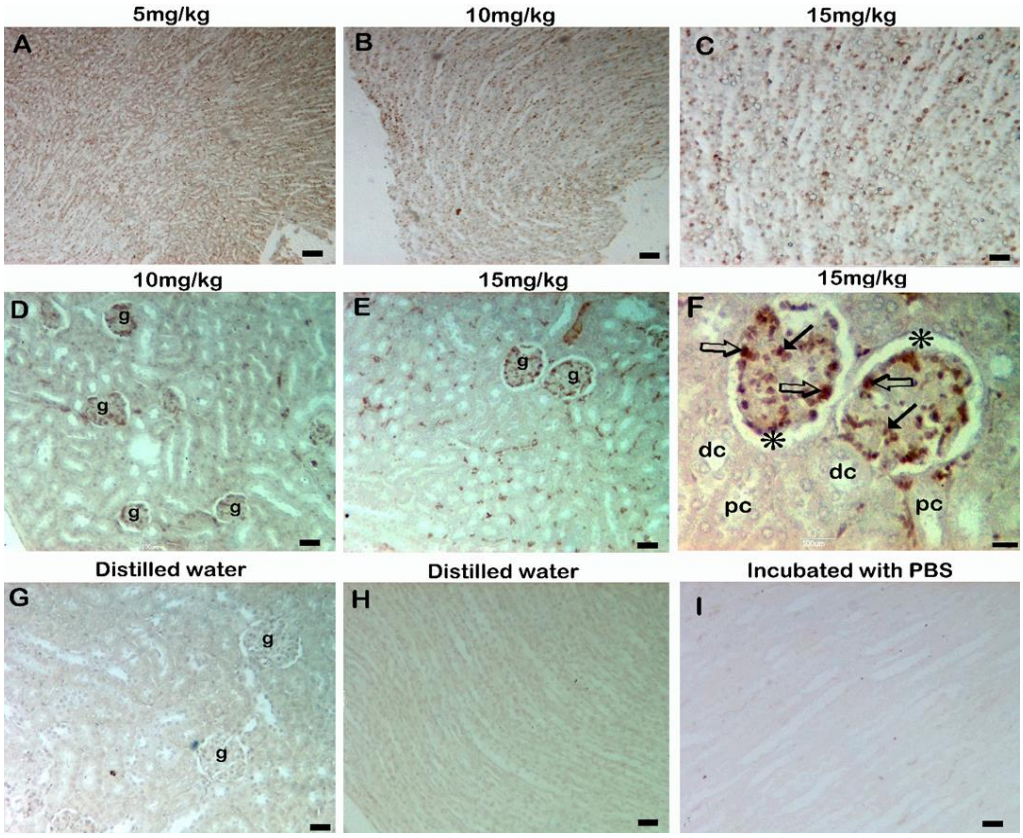

Figure 6: Immunohistochemical images showing anti-ssDNA immunoreactivity in kidney. (A-F) images of kidney sections made from mice treated with the plant leaf extract at doses of 5, 10 and 15 mg/kg body weight per day for 7 days showing strong similar pattern of immunoreactivity for ssDNA appearing as brown reaction product in cuboidal cells lining the collecting ducts (arrow heads) in medulla. In the cortex, immunoreactivity is seen in the glomerulus **g,** in podocytes (open arrows) which have large nucleus and bound the visceral layer of glomerular capsular space (Bowman's capsule) [asterisks] and mesangial cells (arrows) which have relatively small nucleus compared to podocytes. The proximal (**pc**) and distal (**dc**) convoluted tubules were not labeled for ssDNA. Note that proximal convoluted tubules are recognized by having brush border on their apical surface. Immunoreactivity is not observed in (G and H) sections made from mice that received distilled water and in (I) section not incubated with the anti-ssDNA primary antibody. Scale bar: (A, B, D, E, G and H) 200 μm; (C and I) 100 μm and (F) 50 μm.

Lungs: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed similar pattern of immunoreactivity for ssDNA in the lung parenchyma and in the wall of alveoli. Some of the cells were pneumocyte type I which appeared squamous in shape, pneumocyte type II that appeared large and cuboidal in shape as well as the alveolar macrophages which extruded in the lumen of alveoli. Others were cells of the interstitial space. Immunoreactivity was also not observed in tissue sections made from mice that received distilled water and in the sections not incubated with the anti-ssDNA primary antibody (Figure 7).

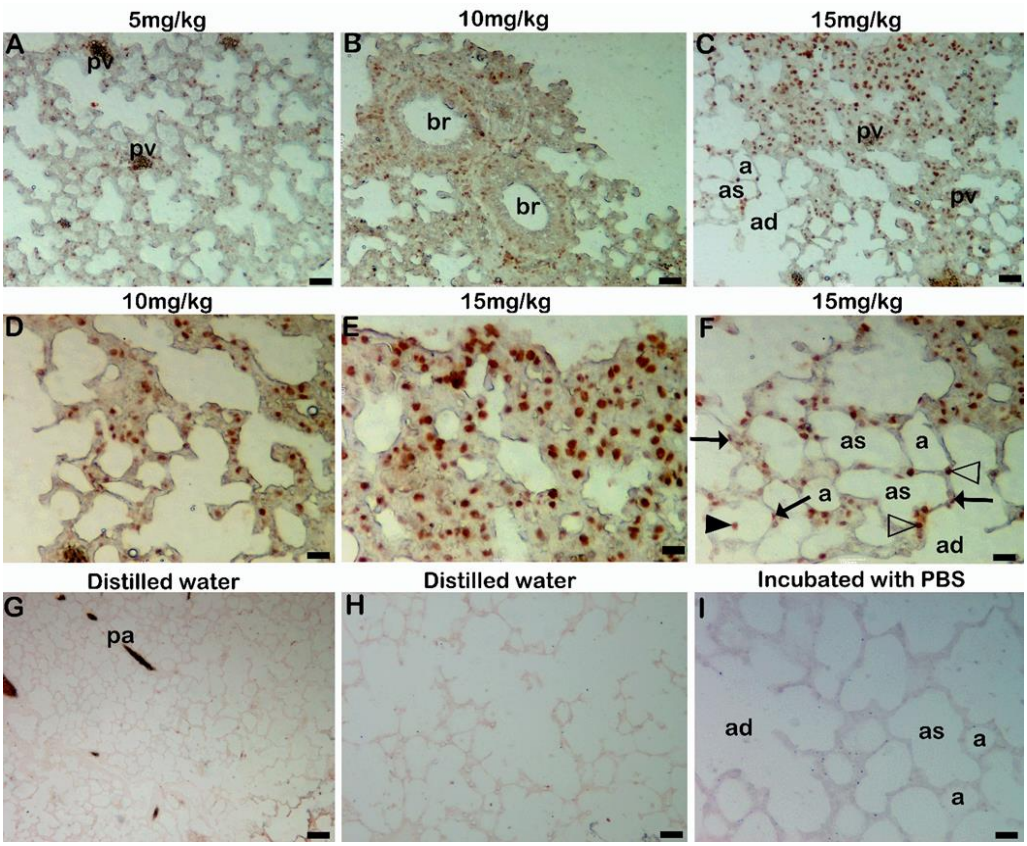

Figure 7: Immunohistochemical images showing Anti-ssDNA immunoreactivity in the lung. (A-C) Low and (D-F) high magnification images of lung sections made from mice treated with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day for 7 days showing strong similar pattern of immunoreactivityfor ssDNA in lung parenchyma. Immunoreactivity is seen in in alveolar penumocyte type I cells which are squamous in shape (arrows), alveolar pneumocyte type II which are cuboidal in shape (open arrow head) and alveolar macrophages (arrow heads) which protrude into alveoli or found in the lumen of alveoli. Immunoreactivity is not observed in (G and H) sections made from mice that received distilled water and in (I) section not incubated with the anti-ssDNA primary antibody. Note: Alveoli, **a**; alveolar sac, **as**; alveolar duct, **ad;** pulmonary vein, **pv**; pulmonary artery, **pa**. Scale bar: (A-C, and G and H) 200 µm; (F) 50 µm and (D, F and I) 100 µm.

Spleen: All tissue sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showed strong labeling for ssDNA in randomly distributed cells throughout the spleen appearing in the red and white pulps. Immunoreactivity was not observed in tissue sections made from mice that received distilled water and in the sections not incubated with the anti-ssDNA primary antibody (Figure 8).

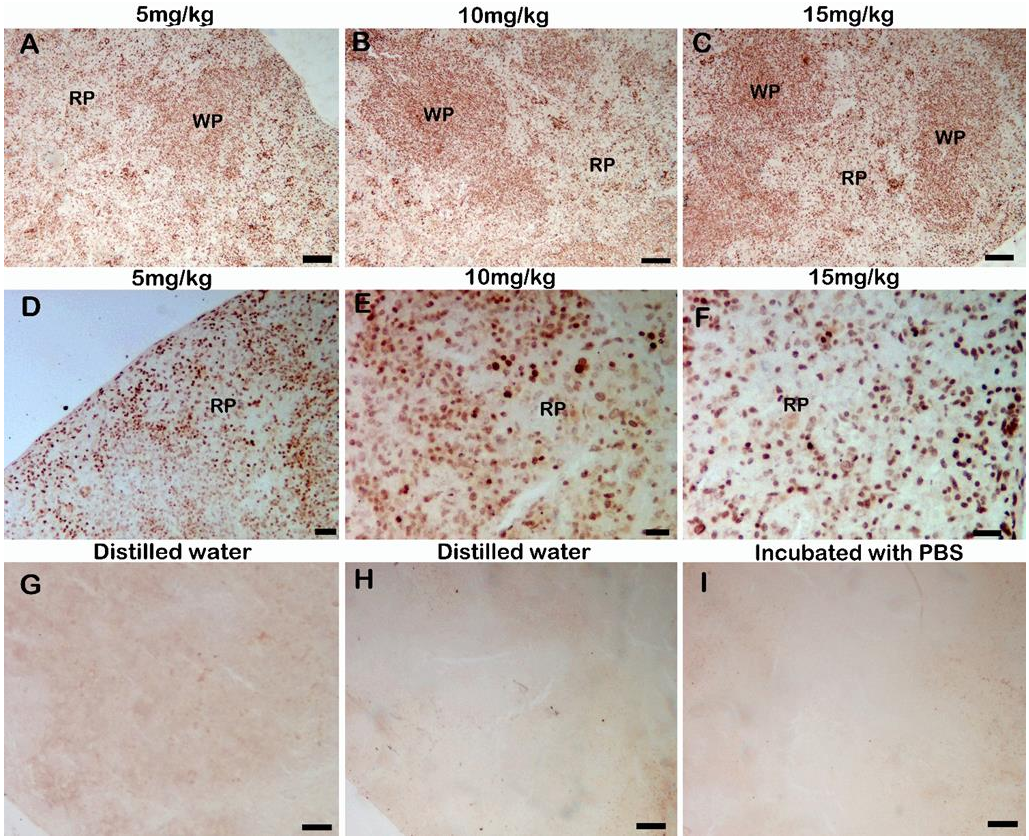

Figure 8: Immunohistochemical images showing Anti-ssDNA immunoreactivity in the spleen. (A-C) Low and (D-F) high magnification images of spleen sections made from mice treated for 7 days with the plant leaf extract at doses of 5, 10 and 15mg/kg body weight per day showing strong similar pattern of immunoreactivity for ssDNA in cells appearing as brown deposit of DAB chromogen distributed throughout the spleen within white (WP) and red (RP) pulps. Immunoreactivity is not observed in (G and H) sections made from mice that received distilled water and in (I) section not incubated with the anti-ssDNA primary antibody. Scale bar: (A-C): 200 µm; (D-I): 100 µm.

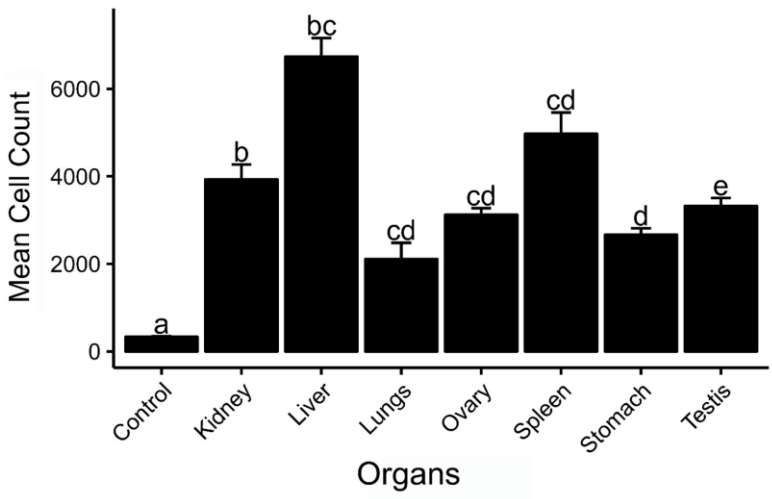

Figure 9: Cell count for ssDNA immunoreactivity in various tissues using Image J. Cell counts was performed at 1000µm^2 area for each organ in ten microscopic fields in 5 slides of control section (Mice given distilled water) and the rest were given the plant extract (10 mg/kg). Examined tissues include kidney, liver, lungs, ovary, spleen, stomach and testis. All data were averaged and analyzed for statistical significance of means by two-way ANOVA using R statistical software version 4.3.1. Bars with different letters were found to be statistically significantly different at $P < 0.05$ with highest apoptotic mean cell count seen in the liver and moderately in the spleen and kidney.

Discussion

Heriotropium indicum has its center of origin in the Asian continent and is reported to be used as a medicinal plant (Dash and Abdullah, 2013, Khurm et al. 2016, Roy 2015). The plant is also available in Tanzania, growing on different soils from that of native Asia. The objective of this study was to evaluate methanolic leaf extract of the plant indigenous to Tanzania so as to determine its cytotoxic effects in various mouse organs. Results obtained show that methanolic leaf extract of *H. indicum* effectively induced apoptosis to various cells in several organs including testis, ovary, stomach, liver, kidney, lung and spleen of mice. These findings extend previous observations on the cytotoxic and genotoxic effects of the plant to Brine Shrimps and *Allium cepa* cells by inhibiting mitosis (Azeez et al. 2020), Hela cell line by preventing growth (Sivajothi, et al.2015) and on SKBR3 human breast adenocarcinoma cell line by preventing proliferation (Goyal and Sharma 2014). Indeed, the plant has proved to be cytotoxic. This observation is suggestive of its potential use in treating cancer cells. However, care should be taken when using the plant for medicinal purposes as it damages healthy cells as well.

The plant leaf extract caused severe damage to spermatogonia, manifested by absence of spermatids and spermatozoa (ceased spermatogenesis) in males. Also it caused damage to ovary stroma which is the source of follicles and follicular cells in females. These findings indicate that the plant extract causes infertility in mammals. Antifertility activity of the plant extract has also been clearly demonstrated using anti-implantation and abortifacient rat models (Savadi et al. 2009, Villa et al. 2016) in which failure in implantation and abortions were observed. In a different experiment in which *in-vitro* sperm motility studies were performed using different concentrations of the plant extract,

the results showed detrimental effects of the plant extract to rat spermatozoa (Savadi et al. 2009). The plant extract revealed progressive lowering of the epididymal sperm count, percentage sperm viability and the blood levels of gonadotropins and testosterone. The percentage of abnormal sperm morphology and altered sperm motility also progressively increased with time of treatment (Savadi et al. 2009).

Damaged cells were also seen in the tunica mucosa within epithelial cell lining and in the lamina, propria of the glandular and non-glandular parts of the stomach, hepatocytes, cells of the collecting ducts in medullary rays of the kidney, pneumocytes type I, II and alveolar macrophages of the lungs and various cells in the spleen. The specific compound which caused the damage could not be identified as it was out of scope of this study. However, *H. indicum* is reported to contain pyrrolizidine alkaloids which are hepatotoxic (Fayed 2021, Oluwatoyin et al. 2011). Pyrrolizidine alkaloids are also shown to cause sticky chromosomes and DNA fragmentation in Brine Shrimps nauplii (Azeez et al. 2020). DNA fragmentation has been clearly demonstrated in this study by immunohistochemistry for ssDNA, a characteristic of degenerating cells by apoptosis. In addition, herbal medicinal plants such as *Saussurea lapp* (Costus root) used for the treatments of cancers in East Asia are reported to exert their apoptotic effect by inducing expression of p53 and its downstream effector p21, both increasing the expression of bax and cleavage of active caspase-3 protein, resulting into apoptosis (Ko et al. 2004, Rasbridge et al. 1994). The mechanism of apoptosis to various tissues seen in this study is not well understood. However, it may be associated with the presence of multiple unknown biological compounds accumulated in *H. indicum* from soil, and hence necessity to perform experiments for soil analysis from where the plant samples were collected and probably compare with that of the Asian soil where the plant leaves are reported to have health benefits (Dash, Abdullah 2013, Yeo et al. 2011, Machan et al. 2006).

Variation in the apoptotic index in tissues was not significant among the three doses of 5, 10 and 15mg/kg body weight. All tissues were more or less equally damaged by the plant leaf extract at all the treatment doses, perhaps due to thin dose margin. Generally, cytotoxic effect was different amongst organs in which the highest apoptotic index (apoptotic cell count) was observed in the liver which receives blood from intestines through hepartic portal vein and does the detoxification role, followed by the spleen and kidney which also receive high amount of blood. Damage was moderate in the gonads, testis and ovaries as well as the stomach and lungs.

Conclusion

This study demonstrates that the methanolic leaf extract of *H. indicum* induces cell death in various body organs. Caution should be taken in consuming the leaf of *H. indicum* for traditional medicinal use. This study also opens opportunities for further studies on the isolation of the active compounds of the plants indigenous to Tanzania and analysis of soil where the plant grows.

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Declaration of interest

The authors declare that they have no competing or incompatible interests

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