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Original Research Article

Acute Toxicological Evaluations of the Methanol Leaf Extract of *Justicia flava* (Vahl) Acanthaceae in Mouse Models

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ABSTRACT

Justicia flava is used traditionally for several ailments including preterm births prevention in Africa. The study's objective was to evaluate the acute toxicity profile of *J. flava* (MJF) (family: Acanthaceae) in mice.

The acute toxicity was performed with a limit dose of 5 g/kg of the extract. A 24 h and 14 d single dose study was performed. Control and treated groups of 5 animals each were used. The control animals received 10% tween 80 while the treated group received 5 g/kg of the freshly-prepared extract. The animals were weighed and parameters such as mortality, behavioural and weight changes, injury, signs of illness were observed daily. At the end of each study, haematological analysis, and histopathological examination of the uterus, kidney, liver and heart tissues were performed.

There were no significant differences ($p > 0.05$) observed in the organs, and body weights in both 24 h and 14 d study. Platelets count was significantly increased ($p < 0.05$) after 24 h while lymphocyte counts were significantly decreased ($p < 0.05$) after 14 d. There were no gross lesions on the tissues after 24 h but mild congestion of the blood vessels was observed on the kidney and liver tissues with mild vascular stenosis observed on the heart. No mortality was however recorded. This study therefore, suggests that short-term oral administration of high dose MJF may not result in death but may cause mild haemorrhage in some organs. Lower doses of the plant extract are therefore suggested for use.

Keywords: *Justicia flava*, Toxicity, Weight, Histology, Haematology, Acanthaceae.

Introduction

The plant *Justicia flava* is a medicinal plant utilized for the management of several conditions in Africa.[1,2] It is used in traditional medicine for skin infections and disorders.[3] In Southern Nigeria, traditional healers use the leaf of *J. flava* for the prevention of preterm birth (personal communication with traditional healers in Edo State Nigeria). There have also been some scientific reports supporting the biological effects of the plant. The methanol leaf extracts of *J. flava* have been reported to improve angiogenesis, collagenation, and reepithelialization in wound tissues.[4] The leaves of *J. flava* have also been reported to exhibit radical scavenging effect as well as inhibition of lipoxygenase activity.[2] The alpha-amylase inhibitory activity of the leaves of *J. flava* has also been reported [1] and has been proposed to have antiviral properties as well.[5] Despite the plant being used for several health conditions, there have so far been no report on the toxicity profile of *J. flava*.

Justicia flava (Forssk.) Vahl of the family Acanthaceae is found growing in disturbed habitat, on a wide range of soil types and in full sun or semi-shady areas.[6] It is widespread in tropical and southern Africa[6]. It is called "Afema" in local Asante-Twi language in Ghana and 'Ighereje'in Urhobo language of Nigeria. It is an abundant perennial herb also referred to as yellow *Justicia* of the Acanthaceae family because of its distinct yellow flowers. It is an erect plant of about 450 mm high.

Toxicity profile of plants provides necessary information to guide natural product's use. This study, therefore, examines the acute toxicological profile of the leaves of *J. flava* using mouse models.

Materials and Methods

Plant material

The matured leaves of *J. flava* were collected from Ugonoba village, Uhunmwonde Local Government Area Edo State, Nigeria and were identified by Dr. H.A. Akinnibosun of Plant Biology and Biotechnology Department, Faculty of Life Sciences, University of Benin, Benin City, Edo State Nigeria. The plant was provided a herbarium number UBHj 386 and a voucher specimen has been deposited at the Department of Plant Biology and Biotechnology and the Department of Pharmacognosy, University of Benin, Nigeria.

Preparation of plant material

The leaves were shade-dried for two weeks and pulverized to powder with the use of a bench top milling machine. Five hundred grams of the powdered leaves were subjected to exhaustive extraction by maceration in 2000 mL of methanol for 72 h with constant stirring. The macerate was concentrated under reduced pressure using a rotavapor (BUCHI Labortechnik AG, Flawil Switzerland) set at 60°C and dried to a constant weight in an oven set at 40°C. The dried extract gave a yield of

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20.14% (w/w) and was stored in an air-tight container in a refrigerator at about 4°C until required.

Animals

Nulliparous and non-pregnant female mice weighing between 17 and 25 g were used. The animals were obtained from the Animal Unit of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Edo state, Nigeria. The animals were housed in plastic cages at an environmentally controlled room temperature of approximately 27 ± 5°C and lighting conditions. Ethical consent was obtained prior to start of the experiments from the Faculty of Pharmacy Ethics Committee, University of Benin, Nigeria (EC/FP/016/07). The animals were handled as much as possible according to standards of the Public Health Service policy on humane care and use of Laboratory Animals.[7,8] The animals were maintained on pelleted standard diet of animal pellets and clean tap water. However, food was withdrawn 4 h before the start of the experiment.

Twenty four (24) hour single dose administration

A modified acute toxicity method was employed.[9,10] Female mice were assigned to two groups of 5 mice per group. The treatment group received the methanol leaf extract of *J. flava* (MJF) at a dose of 5 g/kg p.o. resolubilized in 10% tween 80,[11] while the control group received 10% tween 80 (10 mL/kg p.o.). Administration was done with the aid of a feeding syringe. Following administration of the substances, the animals were observed for toxic manifestations for the next 4 h and subsequently were observed intermittently for signs of morbidity and mortality. Body weights were taken before extract administration and before euthanasia.[12] After 24 h, the animals were exposed to chloroform anaesthesia via inhalation for euthanasia.[10]

Fourteen (14) days single dose administration

Mice were also assigned to two groups of 5 mice per group and MJF (5 g/kg p.o.) was administered while the control group received 10% tween 80 (10 mL/kg p.o.) via the oral route with the aid of a feeding syringe as above. Following extract and tween 80 administration on day 1, the animals were observed for toxic manifestations and maintained for 13 days thereafter, with regular observations[9,10] after the single dose administration.

Observation and examination methods

Toxic manifestations like abnormal motor activity, alteration in water or food intake, writhing, straub reaction, sedation, diarrhoea, piloerection, opisthotonus, exophthalmos and tremors were observed for.[13]

Body weight measurement

Body weights of the animals were recorded for the 14 d group on Day 0 (before administration), and on day 15 before sacrifice.

On day of sacrifice, the animals were anaesthetized by chloroform inhalation and while under anaesthesia blood and tissue samples were collected.

Blood sample collection and analysis

Blood samples (about 0.2 - 0.4 mL) were collected via cardiac puncture using hypodermic needle and syringe and transferred into tubes containing Na^+ EDTA (1.5 mg) for haematological analyses, which was determined using automated SYSMEX KX-21N haematology analyser (Japan). Haematological study included red blood cell (RBC) count, haemoglobin (Hb) concentration, haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets (Plt), white blood cell (WBC) count and leucocyte parameter count.

Organ collection and analysis

After blood sample collection, the animals were exsanguinated and dissection performed to harvest key organs which included the kidney, liver, heart and the uterus. These organs were isolated, weighed and kept in 10% neutral buffer formalin, but the uterus was kept separately in Bouin's fluid and were all submitted for histopathology.[14] The organs were subsequently cut into short segments using the paraffin technique as described.[14] Briefly, sections of 5 µm thicknesses were cut and stained using routine haematoxylin and eosin method. All organs were observed and measured on haematoxylin and eosin stained slides, and 3 randomly chosen areas of the sections were measured per slide. The fixed tissue sections were processed for histopathological examination. The tissue sections were washed in tap water for 30 min, and later dehydrated in graded changes of equal volumes of chloroform; xylene mixture and cleared in two changes of pure xylene. The sections were impregnated in two changes of molten paraffin wax at 60 °C to remove the clearing agents, and embedded in the molten paraffin enblocked in a mould. The blocks were allowed to solidify. Solid blocks of tissues in paraffin wax were sectioned to the required thickness of 15 µm, using microtome (Behr Manning Troy, N.Y). The embedded specimens were cut into thin paraffin ribbons and smeared on the slide and stained with haematoxylin (Sigma, U.S.A) and eosin (Sigma, USA) following a standard staining procedure.[15] The prepared slides and processed specimens on the slides were examined with an Olympus optical microscope (Germany). Photomicrographs of the tissues were captured with a digital camera, 14 mega pixels attached to the microscope and connected to a computer by a USB cord.

Animals were handled carefully during collection of the various parameters to avoid unnecessary stress to the animals.

Statistical Analysis

All data are represented as the mean ± standard error of mean (S.E.M.). The one-factor analysis of variance (one factor ANOVA) with Dunnett's post hoc testing for group differences was employed. Differences were considered to be significant when p-values were less than 0.05.

Results and Discussion

Herbal medicines are largely considered safe and effective since they arise from natural products. This may not always be the case since in some cases slight side effects have gone unnoticed.[16] Assumptions such as these, influence the indiscriminate use of herbal medicines particularly in the rural populace leading to usage without proper dosage monitoring and awareness of the adverse effects that may result.[16] Scientific knowledge of possible toxicity of herbal medicines is imperative in order to appreciate that certain forms of regulations must be put in place to ensure they are used safely.

In the 24 h study group in this study, no adverse reactions or mortality were observed. No mortality or morbid effects were also observed for the 14 d single dose study. General behaviour appeared unchanged throughout the time period. In drug screening, LD_{50} which is the dose that causes death in 50% of the population is often determined.[17] It serves as an initial assessment of toxic manifestations likely to arise from the test compound and can also provide a therapeutic index showing the margin of safety of the test compound.[17] The methanol leaf extract of *J. flava* showed no deaths after 24 h and after observation for 14 d, suggesting that the LD_{50} of the extract goes beyond 5 g/kg. This also suggests that therapeutic doses for *J. flava* can be used at doses between 0 – 5 g/kg where necessary. There were also no signs of abnormal or morbid behavioural changes during the course of this study which also suggests the relative safety of *J. flava* leaf extract. This is so because when experimental models survive high doses such as 5 g/kg no further acute testing is required as the test compound is considered safe.[7,11,18]

There were no differences in the body weights of animals in the 14 d study (Figure 1). The body weight changes serve as sensitive indications of general health status of animals[19–21] and also suggests that there was no interference with normal metabolism which would also have affected food and water intake.[19–21] Lack of change in body weight in this study therefore suggests that the extract neither interfered with the body's metabolism nor with food and water intake on a short term.

No significant changes were observed in the blood analysis of the animals in the 24 h period except for a significant increase ($p < 0.05$) in the platelet count of animals treated with MJF (Table 1). Similarly, no significant changes were observed in blood analysis of the parameters

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Table 1: Haematological indices in 24 h toxicity group.

Haematological parameters	Groups	
	Control	**MJF (5 g/kg)**
WBC[a]	4.16 ± 0.16	4.20 ± 0.26
HGB[b]	14.62 ± 0.24	15.42 ± 0.46
MCV[c]	53.20 ± 3.30	43.80 ± 2.28
MCH[d]	34.40 ± 1.20	36.20 ± 1.59
MCHC[e]	32.00 ± 1.41	31.80 ± 0.92
PLT[f]	133.60 ± 3.36	144.20 ± 2.91*
LYM (x 10^{-3})[g]	64.60 ± 7.20	68.00 ± 9.68
PCV (%)[h]	44.20 ± 0.66	46.40 ± 1.29
NEUT[i]	29.60 ± 5.54	36.25 ± 5.48

a = white blood cell (x 10^3 mm^{-3}); **b** = haemoglobin concentration (g/dL); **c** = mean corpuscular volume (fl); **d** = mean corpuscular haemoglobin (pg); **e** = mean corpuscular haemoglobin concentration (g/dL); **f** = platelet (x 10^3 mm^{-3}); **g** = lymphocytes (x 10^3 mm^{-3}); **h** = platelet count (x 10^3 mm^{-3}); **i** = neutrophil count (cells/µL). * = $p < 0.05$ compared to control only; n = 5 animals.

Table 2: Haematological indices in 14 d toxicity group.

Haematological parameters	Groups	
	Control	**MJF (5 g/kg)**
WBC[a]	29.10 ± 13.15	15.66 ± 1.39
HGB[b]	13.70 ± 0.37	14.78 ± 0.51
MCV[c]	44.88 ± 0.64	46.60 ± 1.53
MCH[d]	24.48 ± 0.61	23.42 ± 1.66
MCHC[e]	54.65 ± 1.80	50.08 ± 2.09
PLT[f]	295.80 ± 14.62	389.80 ± 51.82
LYM (x 10^{-3})[g]	67.50 ± 3.51	58.32 ± 1.83*
PCV (%)[h]	41.18 ± 1.12	44.36 ± 1.56
NEUT[i]	23.65 ± 2.78	29.96 ± 1.44

a = white blood cell (x 10^3 mm^{-3}); **b** = haemoglobin concentration (g/dL); **c** = mean corpuscular volume (fl); **d** = mean corpuscular haemoglobin (pg); **e** = mean corpuscular haemoglobin concentration (g/dL); **f** = platelet (x 10^3 mm^{-3}); **g** = lymphocytes (x 10^3 mm^{-3}); **h** = platelet count (x 10^3 mm^{-3}); **i** = neutrophil count (cells/µL). *$p<0.05$ compared to control; n= 5 animals.

studied in the 14 d period (Table 2). There was however, a significant decrease ($p < 0.05$) in the lymphocyte value in animals treated with MJF in the 14 d study (Table 2). The haematological parameters can be used to assess the effect of the extract on blood functions. The haemopoietin system is a sensitive target of compounds with toxic actions and it alsoprovides an important barometer for assessing the pathological and physiological consequences of toxicant exposure.[11,22] On administration of the extract, there were no significant differences in the red blood cell (RBC) indices in both the 24 h and 14 d study groups suggesting that MJF does not significantly alter the morphology, erythropoiesis, or osmotic fragility of RBCs.[23] The white blood cells (WBCs) respond to tissue injury, infectious agents, or any inflammation and are usually the first line of cellular defence as well as being some of the biomarkers of inflammation.[24] MJF administration resulted in no significant changes in either the 24 h or 14 d study group suggesting that the extract does not exert significant challenge on the immune system of the animals in a short term. However, while no significant alterations occurred with neutrophils and monocytes, lymphocytes were significantly decreased after 14 d of MJF single dose administration. The regulation of systemic immune responses is dependent on lymphocytes. Lymphocytes affect the immunologic repertoire by directing immune effector cells to area of antigens are present.[25] Therefore, a drop in lymphocyte count may signal a positive effect due possibly to the extract's ability to reduce the body's load of antigens which augment the immune system. An increase in platelet count was observed after 24 h administration which seemed to level out during the 14 d observation period. Platelets have several functions in the body which range from cellular mediator of thrombosis, to being immune cells that usher in and increase several vascular inflammatory conditions.[26] Platelets are therefore linked to the pathogenesis of inflammatory diseases. In some contexts, platelet immune functions are protective, whereas in others, platelets contribute to adverse inflammatory outcomes.[24] It is unclear whether the increase in platelets seen after 24 h MJF is beneficial or adverse at this stage.

For the organ weights, there were no statistically significant differences between the control and treated groups of the organs collected, however some differences were observed. In the 24 h study, the uteri of the treated group were observed to weigh more than the control groups with a 14.29% difference (Figure 2A). The kidneys of the treated group were also observed to increase in weight by 74.07% (Figure 2B). For the liver and the heart there were only slight increases in weight of 14.61 and 5.08% increase respectively in the MJF treated group compared to the control (Figure 2C and D). For the 14 d study, the organ weights showed no significant changes between the MJF-treated groups and the control (Figure 3). There were however slight differences. The uteri of the treated groups were observed to weigh more than the control groups with a 12.59% increase (Figure 3A). The kidneys of the treated group were also observed to increase in weight by 14.50% (Figure 3B). For the liver and the heart there were also slight increases in weight of 14.37 and 13.60% increase respectively in the MJF treated group compared to the control (Figure 3 C and D). The lack of significant changes in the organs suggest that administration of MJF at on a short term may not alter normal growth and development. The procedure of weighing organs in toxicity testing arose from their sensitivity to toxic agents and is sometimes correlated with histopathological changes.[16] No significant changes were observed in the absolute organ weight in both the 24 h and 14 d single dose study.

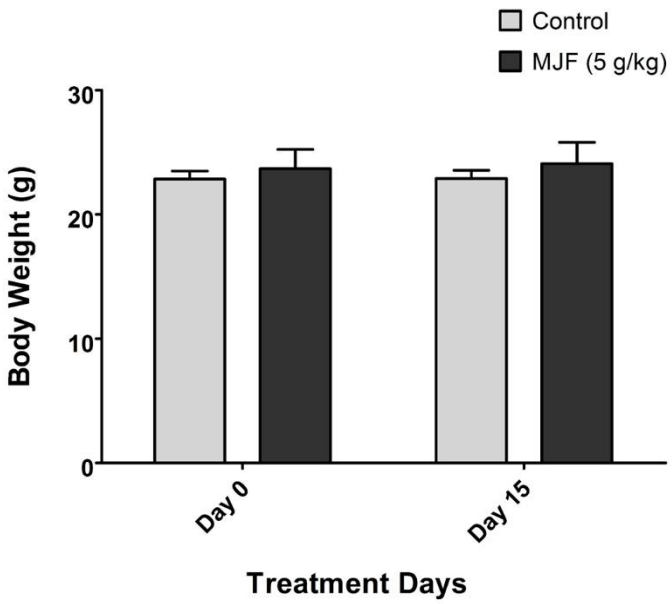

Figure 1: Bar graph showing the body weight changes in the 14 d toxicity study group. No significant changes in body weights were observed after treatment with MJF (5 g/kg). MJF = methanol leaf extract of *Justicia flava*; n = 5 animals.

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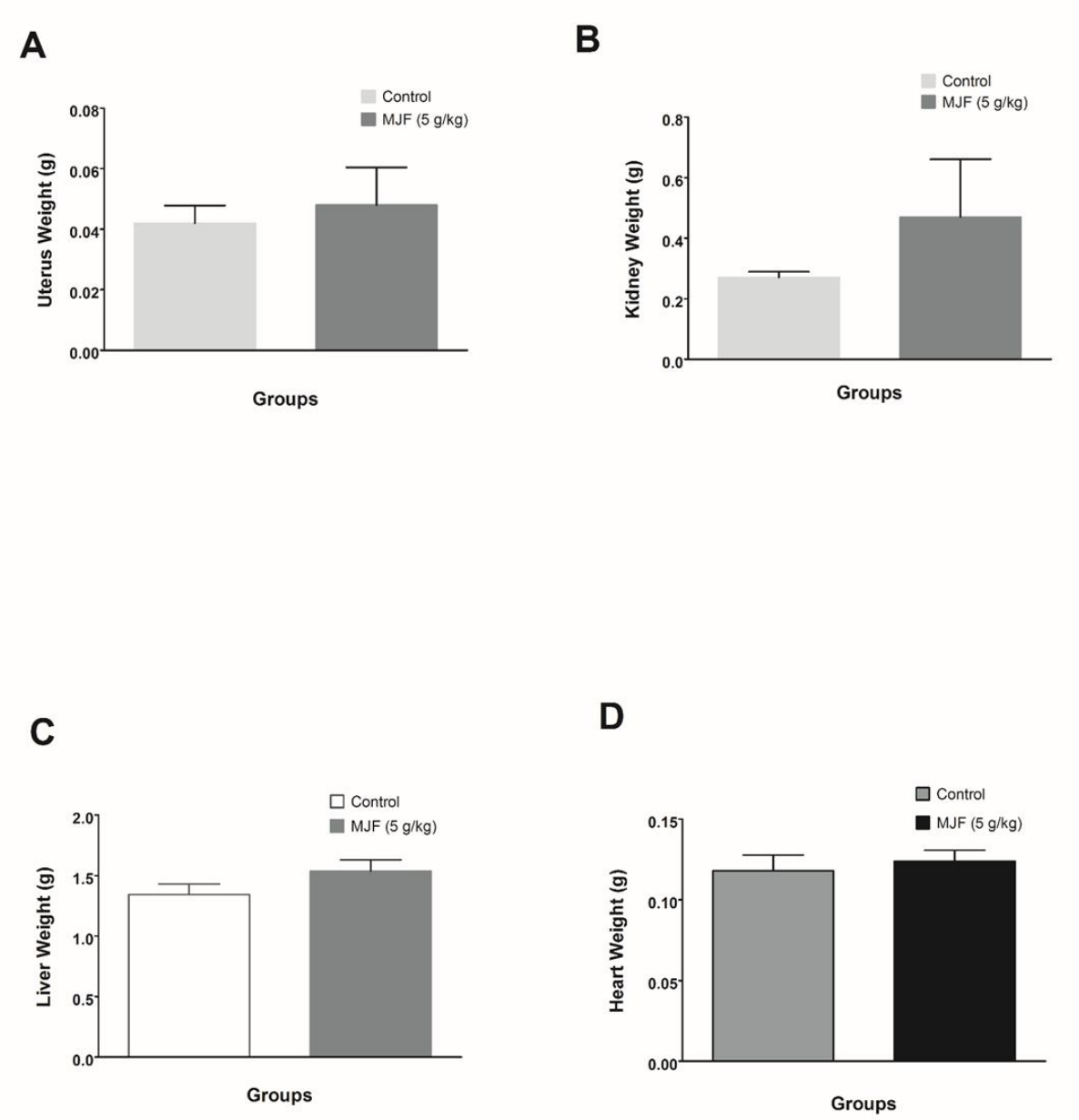

Figure 2: Bar graphs showing the weights of organs isolated from 24 h experimental toxicity group. The weights of the uterus (A), kidney (B), liver (C) and the heart (D) showed no significant changes. Though mild increases where observed after MJF treatment. MJF = methanol leaf extract of *Justicia flava*; Control = 10% tween 80; n = 5 animals.

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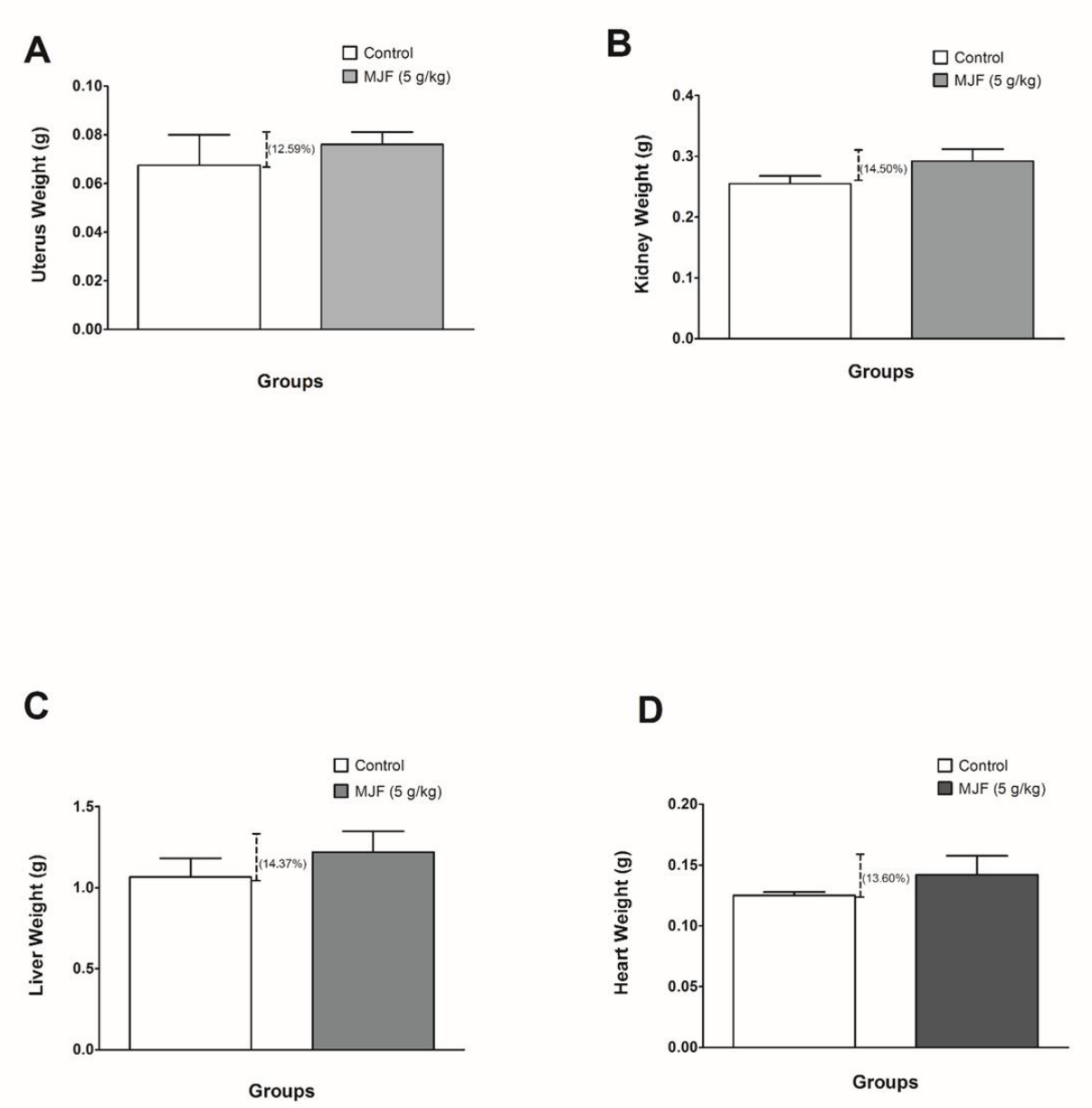

Figure 3: Bar graphs showing the weights of organs isolated from 14 d experimental toxicity groups. The weights of the uterus (A), kidney (B), liver (C) and the heart (D) showed no significant changes. Though mild increases where observed after MJF treatment. MJF = methanol leaf extract of *Justicia flava*; Control = 10% tween 80; n = 5 animals.

However slight increases in the absolute weights were observed but these changes were not statistically significant. Whether these will be significant in longer term exposures remains to be determined.

Histological analysis revealed no changes in tissue architecture for all tissues examined in the 24 h study (Figure 4). Histomorphological investigation after 14 d, showed no change in the uterine architecture (Figure 5A and B). However some mild changes were observed in the other organs. The kidneys of animals treated with MJF showed mild congestion in the renal corpuscles compared to the controls (Figure 5 C and D). The liver tissues from animals treated with MJF showed some visible congested centrioles which appeared surrounded by inflammatory cells compared with the control tissues (Figure 5 E and F).

Similarly, the heart or cardiac tissues from animals treated with MJF showed mild vascular stenosis and mild congestion of the interstitial spaces compared to thee control tissues (Figure 5 G and H). The mild congestion sites in the liver and kidney which may be a sign of mild hemorrhage[27] and mild stenosis in the heart tissues on 14 d observation. The mild haemorrhage observed in the 14 d exposed groups may be associated with the increase in platelets observed at 24 h. These may have occurred due to the high dose used, suggesting a preference for lower doses. The uterine tissue however was unaffected. The overall tissue architecture in all organs remained normal.

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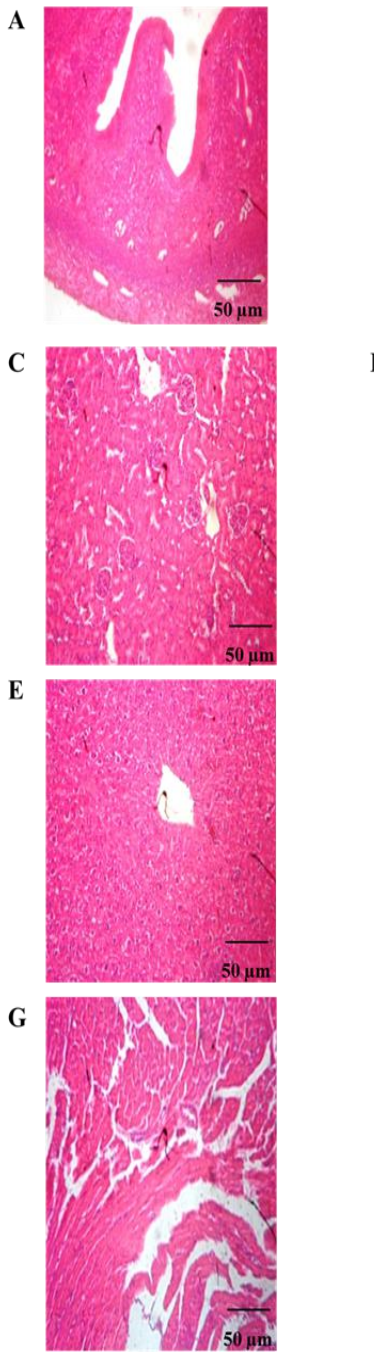

Figure 4: Representative photomicrographs H&E (X 100) of tissues from mouse models after 24 h treatment with MJF (5 g/kg) compared with controls (10% tween 80) administration. Tissues from controls are represented in the left panel while tissues from the treated group are represented in the right panel. The uterus (A and B), the kidney (C and D), the liver (E and F) and the heart (G and H) showed no changes in tissue architecture 24 h after MJF administration. MJF = methanol leaf extract of *Justicia flava*; n = 5 animals.

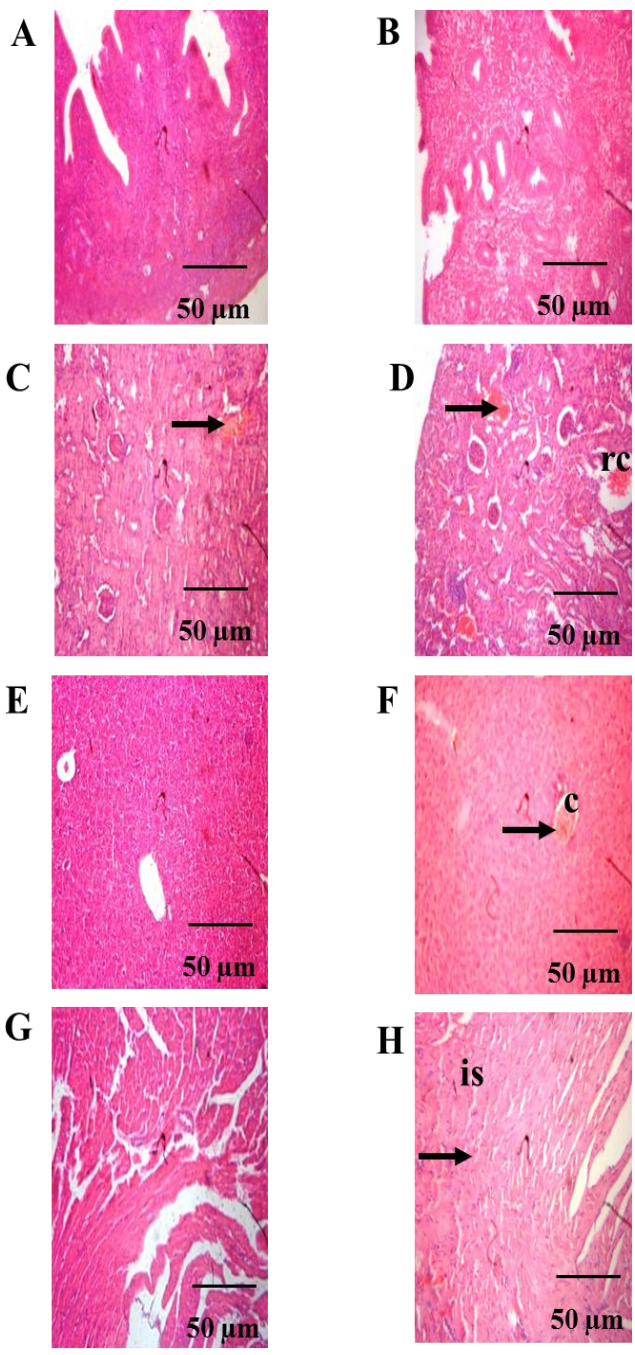

Figure 5: Representative photomicrographs H&E (X 100) of tissues from mouse models after 14 d single dose treatment with MJF (5 g/kg) compared with controls (10% tween 80) administration. Tissues from controls are represented in the left panel while tissues from the treated group are represented in the right panel. The uterus (A and B) showed no changes in tissue architecture after MJF administration. However mild inflammatory changes were observed in the kidneys (C and D), liver (E and F) and the heart (G and H) shown with block arrows. MJF = methanol leaf extract of *Justicia flava*; n = 5 animals.

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Conclusion

This study has shown that short term administration of high dose MJF is relatively safe with no death and no adverse effects on most haematological parameters. However, clearance of a high dose may pose problems even on short term administration and it is therefore advised that lower doses of MJF may be safer. Studies on the toxicity profile of lower doses are recommended in order to build a complete pathological profile for MJF. At this stage, it also appears that MJF at a high dose shows no untoward effect on the uterus and therefore may be safe for managing reproductive conditions.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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