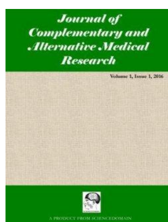

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Evaluation of Sub-acute and Sub Chronic Toxicity Profile of the Aqueous Leaf Crude Extract of Melanthera Scandens (Schumach & Thonn) in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Although *Melanthera scandens* is a plant widely used in traditional medicine for the management of seizures, stomach ulcers and sores, dysmenorrhea, diabetes and malaria, there was scanty information about its safety. There was, therefore, a need to evaluate the sub-acute and subchronic toxicity studies of this plant which would reflect on its safety.

Methodology: This was an experimental laboratory study. The research was conducted at Kampala International University-Western Campus at the Pharmacology laboratory from February to June 2017. The sub-acute toxicity was evaluated after administering daily oral doses of *M. scandens* crude extract (250, 500 and 1000 mg/kg) for 28 days and 90 days for subchronic study, after which the effect on haematological, biochemical and histopathological parameters were assessed in male and female Wistar rats (five of each sex).

Results: Sub-acute toxicity results revealed that there was a significant decrease in the AST between the male Wistar rats that received 250 mg/kg (P= .005) and those that received 500 mg/kg (P= .05) as compared with the control group. Subchronic studies showed a significant increase in ALP (P= .05) at 1000 mg/kg compared with 500 mg/kg. Terminal necropsy did not reveal any treatment-related histopathological findings. There were also no toxicologically significant treatment-related effects on haematological parameters. The sub-acute toxicity results suggest that doses of 250mg/kg and 500mg/kg are safe and could be hepatoprotective due to reduced levels of AST and ALP, while the subchronic toxicity study results suggest that doses greater than 1000 mg/kg could be toxic to the plasma membrane, liver cells or endoplasmic reticulum due to increased ALP levels at this dose.

Conclusion: The *M. scandens* crude extract did not cause significant toxicity on haematological and histopathological indices, after sub-acute and subchronic administration in Wistar rats.

Keywords: Sub-acute; Subchronic toxicity; aqueous leaf extract; Melanthera scandens (Schumach and Thonn); wistar rats.

1. INTRODUCTION

Plants are an important source of drugs worldwide [1]. The herbal and natural products of folk medicine have been used by men since the beginning of the human race [2]. Plants continue to be a major source of medicine, as they have been throughout human history [3]. Eighty per cent (80%) of the world's population use plant as their primary source of medication [4]. However, the traditional medicinal usage of plants is not always a guarantee of the plant's safety. In accordance with Ashafa et al. [5], it is plausible to assume that a history of a plant usage does not prove its safety.

Plants such as *M. scandens* (Schumach&Thonn) have been used by many African communities for medicinal purposes [6]. For example; in Nigeria, the leaves *M. scandens* are used to treat dysmenorrhea, diabetes and malaria [6]. It is also used by the Bete people of Issia district of Cote d'Ivoire to treat malaria [7]. Scientific evidence to validate the claimed efficacy of *M. scandens*, have been documented. The plant (*M. scandens*) has been scientifically proven to have anticonvulsant and anxiolytic properties [8], antioxidant properties [9], antiplasmodial activities [7], antidiabetic and hypolipidemic activities [10]. Furthermore, studies to determine anti-inflammatory and analgesic properties [11], antiplasmodial and antiulcer properties [12] have been carried out. These efficacy studies, therefore, justify the medicinal use of this plant to treat the various ailments in the traditional African community.

In Bushenyi, Western Uganda, the infusion of the leaves of *M. Scandens* is used in the management of seizures in addition to being used to clean teeth [8]. The leaves are used traditionally to treat various ailments such as stomach ulcer and sores in Gosomtwi-Atwimakwanwoma district of Ghana [13]. There is no pharmacological efficacy studies have been conducted on seizures as well as acute toxicity. However acute toxicity studies do not reflect on the safety for the treatment of seizures which is a long term process.

Regarding safety, acute toxicity studies have been conducted to assess the safety of the leaf extract of M. Scandens: an LD50 of 370.00± 23.33 mg/kg following intraperitoneal administration [12]. However, Twinomujuni et al. [8] reported an LD50 for the ethanolic leaf extract estimated at 6708 mg/kg; while the aqueous extract was found safe up to 9000 mg/kg following oral administration. Based on the report by Twinomujuni et al. [8] it may be concluded

that the aqueous extract of *M. scandens* form is safe for short term use. However, there is a scarcity of studies that have been done regarding the long term effects of *M. scandens* and yet seizures treatment is a long term process. There is, therefore, an important need to investigate the long term toxic effects of the plant, which would reflect on the long term use of this plant in the treatment of seizures. This study was therefore carried out to assess the long term toxicity profile of *M. scandens* aqueous leaf crude extract in Wistar rats, using the haematological, biochemical and histopathological parameters as indices of toxicity.

2. MATERIALS AND METHODS

2.1 Study Design and Setting

The experimental study design was used and research was conducted at Kampala International University-Western Campus at the Pharmacology laboratory from February to June 2017. Sub-acute toxicity study was performed according to the standard protocol based on OECD 407 guidelines for 28-day repeated oral toxicity study in rodents [14] while the subchronic study was based on OECD guidelines for testing chemicals, health effects test guidelines, for repeated dose 90-day oral toxicity study in rodents [15]. The study was conducted in compliance with the OECD principles complying with Good Laboratory Practices [16]. The following parameters were evaluated WBC count, WBC differential counts including; neutrophils [NO], lymphocytes (LY), monocytes (MO), eosinophil (EO) and basophils (BA) were evaluated. Red blood cells (RBC) total count, haematocrit (HCT), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count (PCH). The biochemical parameters indicative of liver and kidney function that were evaluated include AST, ALP, ALT, urea and creatinine. Histopathological studies of liver and kidney were also conducted at Makere University College of Veterinary medicine, Animal Resources and Biosecurity.

2.2 Plant Collection and Identification

The leaves of M. scandens were collected from a bush in Nyakabirizi, Bushenyi district in the morning so as to preserve the active constituents. This area was chosen because of the ethnobotanical information obtained from the traditional healer (Miss. Mbabazi Josephine) in this area who also collects from this location, absence of pollution in the area and due to the abundant plant species in the area. The plant was identified and authenticated by a taxonomist Dr Eunice Olet, at Mbarara University of Science and Technology and a voucher specimen was kept at the university herbarium.

2.3 Storage, Drying and Pulverization

The collected plant samples were stored and dried in a shade to avoid direct sunshine that could degrade some of the compounds in the plants. The dried samples were ground using a metallic mortar and pestle according to standard procedures described by Cseke et al. [17] and the powder was obtained by sieving. The powder was weighed using an analytical scale and packed in clean labelled air tied bottles until extraction.

2.4 Extraction and Determination of Percentage Yield

Extraction was done according to Sutapa et al. [18], with modifications in which the shade dried leaves were ground. A mass of 300 g of the powder was soaked in two litres of distilled water at room temperature, with intermittent shaking for 24 hours, filtered using Whatmann filter paper No 1 to obtain water extract. The filtrate was then concentrated under reduced pressure using a rotary evaporator at 40°C. The concentrated extract was then dried in a hot air oven at 40-600C to obtain the dried crude water extract. The modifications included the use of cold water with constant stirring instead of hot methanol under reflux and extraction for 24h in this study instead of 12h by Sutapa et al. [18]. The modifications were chosen because the local population in Bushenyi mainly uses water infusions of M. scandens and not methanol [8].

The percentage yield (%) of the extract was calculated using the following formula;

$$\text{Percentage yield (\% age yield)} = \frac{\text{Weight of concentrated extract}}{\text{Weight of the plant powder}} X\,100$$

The dried crude extract was stored in clean labelled bottles in a refrigerator at 4°C for further analysis.

2.5 Preparation of Stock Solution and Experimental Animals

Preparation of the stock solution was done by dissolving two grams of the extract in 10 ml of

distilled water to give a stock solution of concentration 200 mg/ml. This stock solution was prepared at the time of administration to the rats. The volumes of the extract to be administered to the animals were calculated using the formula by Gosh [19] as shown below;

Volume was given to each animal (ml) =

$$\frac{\text{Body weight of the animal (kg) X Dose (mg/kg)}}{\text{Concentration of the extract (mg/ml)}}$$

Male and female Wistar rats 7-8 weeks old, weighing about (150-220) g were used for this experiment. Selected females were nulliparous and non-pregnant. They were obtained from pharmacology animal house at Kampala International University-Western Campus. The animals were housed in standard cages and maintained on a standard feeds and water ad libitum, with 12 hours of the day and 12 hours of the night. The animals were allowed to acclimatize for a minimum of seven days before the initiation of experiments. Procedures involving animals were conducted in accordance with the Guidelines for the Humane Care and Use of Laboratory Animals published by National Institutes of Health, United States [16,20] guidelines. Animals were randomly assigned to the control and treatment groups. Each animal was assigned a unique identification number. Cages were arranged in such a way that possible effects due to cage placement are minimized.

Rats received three different oral doses of the extract per group as follows;

Group I: 250 mg/kg single dose for 28 days.

Group II: 500 mg/kg single dose daily for 28 days.

Group III: 1000 mg/kg single dose daily for 28 days. Group IV: 1 ml of distilled water single dose for 28 days. Group V: 250 mg/kg single dose daily for 90 days. Group VI: 500 mg/kg single dose daily for 90 days. Group VII: 1000 mg/kg single dose daily for 90 days. Group VIII: 1ml of distilled water single dose daily for 90 days. Each group consisted of ten rats, five of each sex. Groups I, II, III and IV were used for sub-acute toxicity studies, while groups V, VI, VII and VIII were used for subchronic toxicity studies. The doses were chosen based on the study by Twinomujuni et al. [8] which showed the three doses (250, 500 and 1000 mg/kg) to be efficacious for the treatment of seizures in a rat model.

2.6 Clinical Observations and Weekly Body Weights Monitoring

As in the study conducted by Chintan et al. [21], all animals were observed twice daily for morbidity and mortality. Clinical examinations included any abnormal physical and behavioural changes. The observations included changes in the skin, fur, eyes, mucous membrane, and autonomic activity like lacrimation, piloerection, pupil size and unusual breathing pattern. All animals were weighed at least once a week.

2.7 Haematological Evaluation

A method described by Prasanth et al. [22] was used. In this method, the animals fasted overnight at the end of the study period (28-day period for sub-acute and 90-day period for sub-chronic), the animals were fasted overnight and then anaesthetized with sodium pentobarbitone (60mg/kg) the following morning and six millilitres of blood were then collected through cardiac puncture into heparinised (3 ml) and non heparinised tubes (3 ml). Heparinised blood samples collected were analyzed using a fully automated haematology analyser WBC count, WBC differential counts including; neutrophils [NO], lymphocytes (LY), monocytes (MO), eosinophil (EO) and basophils (BA) were evaluated. Red blood cells (RBC) total count, haematocrit (HCT), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count (PCH) were done. These were carried out according to a routine method described by Dacie [23]. The haematological evaluation was conducted at the clinical chemistry laboratory of Kampala International University-Teaching Hospital.

2.8 Biochemical Evaluation

A method described by Prasanth et al. [22] was used. The non-heparinsed blood was allowed to coagulate and then centrifuged at 3000 rpm for 5 minutes to obtain serum. The serum was separated from non-heparinised blood and the serum biochemical parameters including creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), were analysed by using the semi-automatic biochemical analyser.

2.9 Histopathological Studies

Method of euthanasia: Euthanasia was conducted according to the American Veterinary

Medical Association (AVMA) guidelines [24]. According to these guidelines, euthanasia methods should result in rapid loss of consciousness, followed by cardiac or respiratory arrest and the subsequent loss of brain function. In addition, animal handling and the eutha¬nasia technique should minimize distress, pain and anxiety experienced by the animal prior to the loss of consciousness. In this study, animals were anaesthetized with sodium pentobarbitone (60mg/kg), after which they were dissected and blood collected by cardiac puncture. After the experiment, ani¬mal remains were handled appropriately and in accordance with AVMA guidelines [22] which recommend incineration of all animal remains involving use of pentobarbitone as an anaesthetic agent because it poses a danger to wildlife and fish. According to AVMA guidelines [22], a combination of criteria is most reliable in confirming the death, including lack of pulse, breathing, corneal reflex and response to a firm toe pinch, inabil¬ity to hear respiratory sounds and heartbeat by use of a stethoscope, greying of the mucous membranes, and rigour mortis. None of these signs alone, except rigour mortis, confirms the death.

Necropsy procedure to remove the liver and kidney: Necropsy was done according to a method described by Jean-Charles [25]. The major steps were; examination of the live animal, euthanasia, exsanguination, opening of the abdominal cavity, the opening of the thoracic cavity, and examination of muscles and skeleton.

Removal, examination and sampling of the liver: This was done in accordance with a method described by Jean-Charles [25]. The liver has four lobes: medial lobe, right medial lobe, right lateral lobe, caudate lobe, plus a papillary process. The liver was removed gently with the forceps. The xiphoid process was firmly grasped with the forceps. The diaphragm was punctured with scissors and trimmed completely away from the ribs. Using the diaphragm as a handle, the liver was pulled out of the abdominal cavity. It was then separated from the diaphragm by cutting the falciform and coronary ligaments that attach it to the diaphragm and all remnants of the diaphragm removed. A piece was taken from the left lateral lobe (transverse) and the right medial lobe (transverse). The liver samples were immersed in the 10% buffered formalin labelled sample bottles.

Removal, examination, and sampling of the kidneys: This was also done according to a method by Jean-Charles [25] as described below:

The kidney and adrenals were removed by grasping the caudal part of the ureter with forceps near its opening and keeping the adrenals attached to the kidneys. The adrenals were then separated from the kidneys. The kidneys were then examined and all freed of all remnants of connective and adipose tissues. A longitudinal section from the left kidney and a transverse section from the right kidney were then taken and fixed in 10% buffered formalin labelled sample bottles.

Tissue studies on liver and kidney: The tissues were prepared according to the method described by Wallace et al. [26] and histopathological studies by a method described by Prasanth et al. [22]. At the end of the study period, animals in test and control groups were sacrificed under 60mg/kg of pentobarbitone, the livers and kidneys were carefully removed. These organs were processed, sectioned and stained according to standard laboratory methods. They were fixed in 10% formalin- saline and 3-4 mm thick tissue were cut from each organ for processing. The cut tissues were transferred to the automatic tissue processor where they were further fixed in 10% buffered formalin- saline for two hours and dehydrated for two hours in each ascending grades of alcohol (70%, 90%, and 100% v/v). The dehydrated tissues were then cleared in xylene for two hours and the tissues impregnated in molten paraffin wax for another two hours and left to cool. The sections were then trimmed and re-sectioned on the microtome at 5 microns (µ). The sections were then floated out in a warm water bath, then attached to slides, and dried on a hot plate and stained with haematoxylin and eosin (H&E). The slides were viewed on a microscope with the assistance of a registered and practising veterinary pathologist from Makerere University who was not aware of the biochemical data to enable interpretation of morphological changes in the tissues. All gross lesions were examined and described by the pathologist. The examination on the liver included focal necrosis, hypertrophy of kuppfer cells, fatty degeneration, and sinusoidal congestion in the liver, inflammation of portal tract and degeneration of hepatocytes. The kidney was examined for necrosis of tubular epithelium, normal glomeruli and other parameters as required by standard guidelines in toxicity testing such as the OECD guidelines [15].

2.10 Data Analysis

Data was entered in MS Excel Version 2010. This was cleaned and imported into Graph Pad Prism version 6 in which all analysis was conducted. Information was expressed as mean ± SEM (Standard Error of Mean). ANOVA (Analysis of Variance) was conducted and a Tukey's post hoc test was conducted to show sources of variation. Significance was measured at P=.05. Histopathological data were qualitatively described and presented in photomicrographs.

2.11 Ethical Considerations

International guidelines for the handling of laboratory animals were followed and the animals were sacrificed under general anaesthesia. The guidelines for handling animals include using mature healthy inbred animals, feeding on standard pellets, giving them water ad libitum, giving them twelve hours of light and twelve hours of darkness, maintaining them in clean cages with standard humidity between 50% to 60% and temperature between 20 to 250C, acclimatizing the animals for at least one week, randomizing the animals during grouping and labeling them without causing pain, administering the extract using gastrointestinal tubes of suitable size, administering volumes of the extract of not more than 2 ml/100g at any time and using a suitable number of animals to generate results that are reproducible and valid. The benefit of using animals is that they have many digestive and metabolic similarities to human beings, and thus increase the confidence in the prediction of safety in humans. The research was approved by the Kampala International University Institutional Research and Ethics Committee (IREC).

3. RESULTS AND DISCUSSION

The percentage yield of the crude extract was 11.5%. This yield is relatively low since from 300g only 11.5% was got, however, the plant remains a potential source of drugs and thus this yield encourages more cultivation of the plant to improve on its availability.

3.1 Preliminary Results of Changes in Body Weights

The results in body weight did not reveal any significant increases or declines in body weight in all groups for both sub-acute and subchronic treatment groups. However, female rats treated with 500 mg/kg/day for 90days of the extract showed a slight increase in body weight from 9th to12th week which could be due to hormonal changes that occur in the female animals after puberty [27]. If it were due to the extract the weight changes would be across all groups which could suggest that the extract stimulates appetite but such cannot be concluded from this study. During this period the activity of the female animals in this group was found to be normal. These were preliminary results that were taken at weekly intervals. The results generally suggest that administration of the extract up to doses of 1000 mg/kg had no adverse effects on clinical observations and body weights (Figs. 1 and 2). This was in line with findings of Chintan et al. [21] where Punica granatum fruit crude extract administered orally for 90 days did not show any adverse effects on body weights of both male and female Wistar rats.

3.2 Hematological Parameters after 28 and 90 Days following Oral Administration of *M. scandens* Crude Extract

All rats in the 28-day group (sub-acute toxicity) and 90-day groups (subchronic toxicity) survived throughout the experiment for both control and treatment groups receiving daily oral doses of *M. scandens* aqueous crude extract (Tables 1 and 2). The signs of toxicity observed during the study period were not serious and included paw licking and excitation.

The results for sub-acute toxicity study (28-day group) which received doses of 250 mg/kg, 500 mg/kg and 1000mg/kg of for the treatment group did not reveal any significant changes in haematological parameters when compared with the control group (*P* = .05) for both males and females. There was also no significant difference between the doses with *P*=.1 (Table 1).

The results for subchronic toxicity (90-day treatment groups) revealed that daily administration of the *M. scandens* crude extract did not show any significant differences (*P=.04*) among all treatment groups as compared to the control and in between the treatment groups (Table 2) for the selected haematological indices in Wistar rats. Multiple comparisons between the 28-day groups (sub-acute toxicity) and 90-day groups (subchronic toxicity) did not show any significant differences at (*P*=.3) among all groups (Table 2).

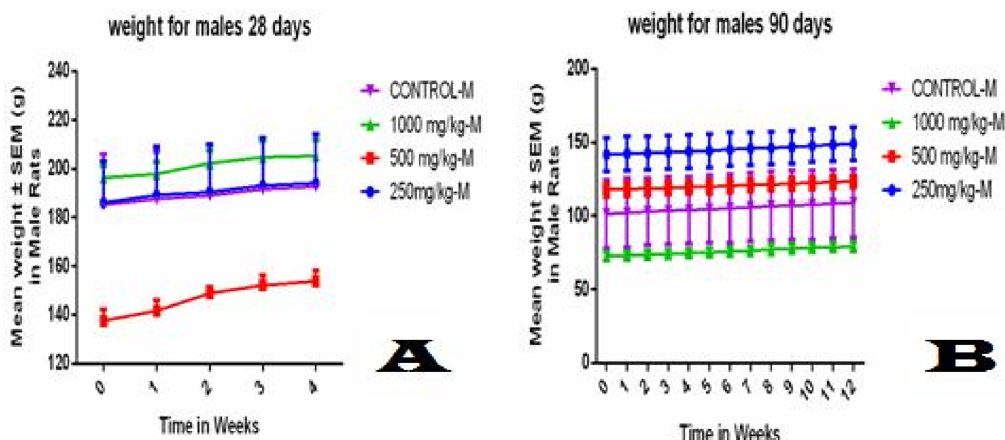

Fig. 1. (A) Weight for males 28 days, (B) Weight for

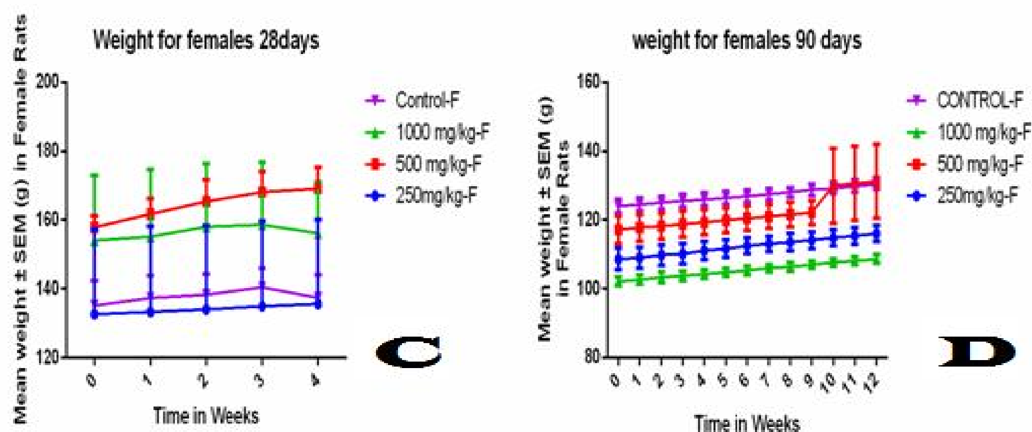

Fig. 2. (C) Weight for females 28 days, (D) Weight for males 90

Assessment of haematological parameters can be used to determine the extent of the deleterious effect of a foreign compound including plant extracts on the blood [28]. It can also be used to explain the blood-related functions of a chemical compound/plant extract. Such laboratory investigations have been reported to be highly sensitive, accurate, and reliable and it remains the bedrock of ethical and rational research, disease diagnosis, prevention and treatment [12, 29]. The normal range of these parameters can be altered by the ingestion of some toxic plants [30]. The extract of M. scandens at all doses did not significantly alter the haematological parameters in both male and female Wistar rats following oral sub-acute and subchronic administration.

There was no significant change in haematological parameters like haemoglobin, hematocrit, RBC, platelets, reticulocytes, monocytes, basophils, MCV, MCH, MCHC, and PCV in the extract treated animals. Haematological changes such as anaemia are often accompanied by bone marrow toxicity [31]. According to Onyeyilli et al. [32], anaemia that results after administration of an agent can be a result of lysis of blood cells. However, no such anaemia was observed after sub-acute and subchronic treatment with the M. scandens extract suggesting that there is no lysis of blood cells.

The non-significant effect on the RBC between the treatment and control group and between treatment groups observed in the study may be an indication that the balance between the rate of production and destruction of the red blood cells (erythropoiesis) was not affected by the administration of the *M. scandens* leaf extract.

This may imply that the extract does not contain chemical substances that can either stimulate or inhibit the formation and secretion of erythropoietin from the kidney, which is the precursor for RBC formation.

Since MCHC, MCH and MCV relate to the individual blood cells while the HB, RBC and PCV are linked to the population of red blood cells, the non-significant effect of the extract on these indices may imply that neither the incorporation of haemoglobin into red blood cells nor the morphology and osmotic fragility of the red blood cells was altered. Therefore, it is unlikely that the extract will affect the oxygen-carrying capacity of each RBC and the total population. In addition, since the anaemia diagnostic indices of MCV, MCH and MCHC were not altered, it indicates that the extract had no effect on the average size of RBC as well as the concentration of haemoglobin per RBC.

The non-significant difference in the white blood cells, monocytes, lymphocytes, neutrophils and eosinophils between the treatment and control groups and between the treatment groups implies that the extract does not contain chemicals that enhance the differentiation of the haematopoietic stem cells of the bone marrow into these parameters. Secondly, the extract does not affect the rate of removal of these parameters from circulation.

The non-significant change in the platelets implies that the extract does not contain chemical substances that stimulate or inhibit the release of thrombopoietin which is the main regulatory protein for synthesis of platelets. Secondly, it may be due to the absence of significant tissue damage that could trigger platelets release so as to mediate blood clotting formation of a meshwork of fibrous. According to Hamid et al. [31] analysis of blood parameters with respect to animal studies have high relevance and predictive value for humans and therefore this could indicate safe usage of *M. scandens* in humans.

The results of this study are in agreement with a study by Ighodaro et al. [27], for the sub-acute toxicity of Voacanga Africana which is also used in the treatment of seizures and other ailments. Also, a study by Chintan et al. [21] revealed that subchronic administration of pomegranate (*Punica granatum* L.) extract did not show any treatment-related adverse effects on haematological parameters in both male and Wistar rats after 90-days. Gandhare et al. [33] also showed that the sub-acute oral administration of Ceiba pentandra leaf extract, a plant used in the treatment of many conditions including diabetes did not show any toxic effects on haematological parameters.

3.3 Biochemical Parameters after 28 and 90 Days Following Oral Administration of *M. scandens* Crude Extract

The results of biochemical analysis revealed that after 28 days (sub-acute toxicity) of single daily administration of the *m. Scandens* crude extract there was a significant decrease in the ast between the male wistar that received 250 mg/kg (*p= .005*) and the males that received 500 mg/kg (*p =.05*) as compared with the control group (Table 3). The rest of the parameters for liver function tests such as alp and alt did not show any significant differences between the treatment groups and the control as well as in between dosages of the treatment groups. There were no significant changes in the results for the concentration of selected kidney function tests (urea and creatinine) for the sub-acute toxicity study group between treatment and control groups as well as between the different dosages of treatment groups.

Results for the subchronic toxicity study (after 90 days) showed a significant decrease in the levels of alp for the female rats that received 500mg/kg as compared to the control group (*p=.05)*. A significant increase in alkaline phosphatase (alp) activity was noted in male rats treated with 1000 mg/kg of the extract compared with the 500 mgkg for the 90-day treatment group (*p.05*). The concentrations of the other serum liver diagnostic enzymes (alt and ast) did not show any significant differences as well as concentrations for urea and creatinine (Table 4).

According to wurochekke et al. [34], significant kidney damage reduces the glomerular filtration rate and this leads to the retention of urea and creatinine in the blood. Although urea levels can be affected by many other factors such as dehydration, anti-diuretic drugs and diet, creatinine is more specific to the kidney. This is because it is only kidney dysfunction that significantly increases serum creatinine [35]. Thus significant changes in serum urea and creatinine are due to functional damage to the kidney.

Table 1. Mean and standard deviation values for haematological parameters after 28 days of the extract administration

Haematology indices	N	Dose (mg/kg)							
		250		500		1000		Control (1ml DW)	
		Mean ± SEM on Day 28							
		Male*	Female*	Male*	Female*	Male*	Female*	Male*	Female*
WBC 10^9/l	5	11.3±3.70	5.40±1.60	9.85±0.55	15.75±7.85	7.8±4.60	3.2±0.00	7.7±1.60	3.9±1.50
LYM (%)	5	9.10±3.00	3.15±3.15	7.65±0.15	13.6±7.00	5.8±3.40	1.8±0.00	5.85±0.55	3.05±1.35
NEU (%)	5	2.20±0.70	2.25±1.55	2.2±0.40	2.15±0.85	2.0±1.20	1.45±0.05	1.87±1.04	0.80±0.20
RBC ($x10^6$/µl)	5	8.93±0.16	8.30±0.30	9.2±0.90	7.23±0.22	9.23±0.17	7.90±0.01	8.05±0.11	9.75±1.23
HGB (g/dl)	5	15.95±0.35	14.8±0.80	16.65±1.05	12.75±1.15	16.65±0.05	14.55±0.45	15.45±0.55	17.55±1.65
HCT (%)	5	52.8±1.10	49.35±1.75	54.85±3.85	50.2±7.10	54.35±0.85	48.5±3.50	50.65±1.55	57.35±5.85
MCV (fl)	5	59.15±0.15	59.5±0.00	59.75±1.65	69.25±7.75	58.95±0.15	57.75±0.75	62.9±1.10	59.0±1.40
MCH (pg)	5	17.85±0.05	17.85±0.35	18.15±0.65	17.7±2.10	18.05±0.35	17.95±0.05	19.2±0.40	18.1±0.60
MCHC (g/dl)	5	30.2±0.00	29.95±0.55	30.4±0.20	26.25±6.05	30.65±0.55	31.4±0.10	30.5±0.20	30.65±0.25
PLT ($x10^5$/µl)	5	720.5±38.50	900.5±209.5	829±49.00	1411.5±468.50	653.5±208.5	747±47.00	588±257.0	967.5±152.5

*KEY: WBC = White Blood Cells; LYM = Lymphocytes; NEU = Neutrophils; RBC = Red blood cells; HGB = Hemoglobin; HCT = Hematocrit; MCV =Mean Corpuscular Volume; MCH = Mean Corpuscular hemoglobin; MCHC = Mean Corpuscular Hemoglobin Concentration; PLT = Platelets. N = Sample size for each sex. DW: distilled water, NB: There was no statistical significance between the treatment groups and the control (ANOVA, p=.1 between control and treatments), * Significance difference (p=.05) between male and female*

Table 2. Mean and standard deviation values for haematological parameters after 90 days of daily dosing of the extract

Haematology indices	N	Dose (mg/kg)							
		250		500		1000		Control(1ml DW)	
		Mean ± SEM on Day 90							
		Male*	Female*	Male*	Female*	Male*	Female*	Male*	Female*
WBC (10^9/l)	5	7.80±0.40	3.21±0.29	9.63±0.33	6.09±1.74	3.24±1.38	3.77±1.91	7.67±4.06	4.99±0.76
LYM (%)	5	6.04±0.07	1.86±0.34	6.51±1.00	4.22±1.67	1.58±0.35	4.79±4.09	5.91±3.70	3.25±0.65
NEU (%)	5	1.52±0.02	1.03±0.03	2.86±1.06	1.46±0.03	1.30±0.82	1.43±0.39	0.89±0.08	1.38±0.02
RBC ($x10^6$/µl)	5	9.53±0.44	8.29±0.31	9.09±1.01	8.54±0.17	9.02±0.24	9.31±0.81	8.53±0.17	8.05±0.05
HGB (g/dl)	5	17.0±0.70	14.4±0.40	16.05±1.65	15.85±0.25	15.0±0.30	16.20±0.80	15.65±0.35	14.20±0.10
HCT (%)	5	56.05±2.15	50.35±0.75	52.55±6.15	51.30±0.8	51.55±2.65	55.30±4.00	54.55±3.05	47.5±0.80
MCV (fl)	5	58.85±0.45	60.85±1.35	57.75±0.35	60.1±0.20	57.10±1.40	59.55±0.85	63.90±2.30	59.05±1.35
MCH (pg)	5	17.85±0.05	18.35±0.15	17.65±0.15	18.55±0.05	16.6±0.10	17.45±0.65	18.35±0.05	17.65±0.25

Haematology indices	N	Dose (mg/kg)							
		250		500		1000		Control(1ml DW)	
		Mean ± SEM on Day 90							
		Male*	Female*	Male*	Female*	Male*	Female*	Male*	Female*
MCHC (g/dl)	5	30.30±0.10	30.15±0.35	30.6±0.40	30.90±0.00	29.15±0.95	29.35±0.65	28.75±0.95	29.9±0.30
PLT ($x10^5/\mu l$)	5	850.0±60.0	885.5±24.5	888.5±10.50	744.0±0.09	887.0±74.0	774.50±117.5	679.0±128.00	710.5±193.5

*KEY: WBC = White Blood Cells; LYM = Lymphocytes; NEU = Neutrophils; RBC = Red blood cells; HGB = Hemoglobin; HCT = Hematocrit; MCV =Mean Corpuscular Volume; MCH = Mean Corpuscular hemoglobin; MCHC = Mean Corpuscular Hemoglobin Concentration; PLT = Platelets. N = Sample size for each sex. DW: distilled water, NB: There was no statistical significance between the treatment groups and the control (ANOVA, P=.3), * Significance difference (p=.0.4) between male and female*

Table 3. Biochemical parameters in experimental groups after 28 days

Biochemical parameters	N	Dosage mg/kg						Control (1ml DW)	
		250		500		100			
		Male	Female	Male	Female	Male	Female	Male	Female
		Mean ± SEM (mg/dl) at Day 28							
ALT (U/l)	5	178.35±6.65	112.75±30.95	185.95±54.65	131±24.7	113.15±5.75	147.1±6	255.75±72.15	121.9±16.7
AST (U/l)	5	246.2±27.60**	244.3±60.6	282.75±13.55*	431.15±223.75	404.05±31.15	519.25±120.15	570.9±163.9	381±62.3
ALP (U/l)	5	271.2±195.8	133.45±45.85	205.55±26.65	299±2	130.35±16.05	232.95±157.75	230.15±81.55	226.9±49.2
Urea (mg/dl)	5	27.45±7.05	25.35±4.95	22.5±2.70	20.95±2.45	20.5±2.70	21.25±3.95	23.6±1.90	25.35±10.55
Creatinine (mg/dl)	5	1.52±0.15	1.525±0.03	1.53±0.18	1.22±0.23	1.51±0.15	1.055±0.03	1.44±0.15	1.685±0.22

*KEY: ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; ALP = Alkaline Phosphatase; N = number of samples per sex. DW: distilled water, Multiple comparisons were conducted against control *P =.05;**P =.005*

Table 4. Biochemical parameters in experimental groups after 90 days

Biochemical parameters	N	Dose mg/kg						Control (1ml DW)	
		250		500		1000			
		Male	Female	Male	Female	Male	Female	Male	Female
		Mean ± SEM (mg/dl) at Day 90							
ALT (U/l)	5	142±27	120.5±18.5	259±125	118.5±6.5	215±121	109±11	208.5±15.5	160.5±33.5
AST (U/l)	5	338.5±166.5	245±28	355.5±13.5	298±9	361±148	233.5±49.5	370.5±62.5	307.5±90.5
ALP (U/l)	5	538±53	502±140	544±126	293.5±11.5[b]*	562.5±68.5	711.5±248.5[a]	699.5±28.5	605±12
Urea (mg/dl)	5	50.1±1.3	54.6±2.9	63.85±5.25	54.5±5.10	55.25±10.65	61.7±3.0	63.1±3.9	59.6±4.5
Creatinine (mg/dl)	5	0.625±0.06	0.5±0.07	0.56±0.09	0.54±0.01	0.635±0.05	0.595±0.05	0.69±0.01	0.60±0.05

*KEY: ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; ALP = Alkaline Phosphatase; N = number of samples per sex. DW: distilled water, Multiple comparisons were conducted against control *P=.05;**P=.005.a, b means significant differences in dosages*

The non-significant changes in urea and creatinine levels between the treatment and the control groups observed in this study imply that the aqueous leaf extract of m. Scandens did not cause significant damage to the kidney to affect its functioning. Thus the glomerular filtration rate (gfr) that contributes to urea and creatinine excretion out and retention in blood was not affected. This is further supported by histopathology results that revealed no significant damage to the kidney. These results are in line with those of what other researchers have conducted on plant extracts such as protus et al. [36] who revealed that sub-acute and subchronic administration of the aqueous leaf extract of carica papaya, a plant used in treatment of diabetes, malaria and anaemia did not show any signs of sub-acute or chronic toxicity on the kidney up to oral doses of 1000 mg/kg.

Significant liver damage leads to loss of the integrity of cellular membranes resulting in leakage of serum diagnostic enzymes in the blood. Thus a significant increase in the level of serum diagnostic enzymes after extract administration indicates liver damage. According to wurochekke et al. [34], an increase in the levels of ast after administration of the plant extract could probably be due to liver damage, cardiac infarction or muscle injury. This is because ast is known to be a non-specific indicator for liver damage since it is found in the liver, heart, kidney and other skeletal muscles. When the integrity of these organs are damaged, then ast enzymes leak and increase in the bloodstream and as a result a rise in the serum. Alt is the most sensitive biomarker diagnostic parameter for detecting liver injury. This is because it is localized within the cells of the liver. When the integrity of the liver cellular membrane is damaged, alt leaks into the bloodstream leading to its rise in the serum [37]. Alp is an enzyme biomarker important in detecting the health status of the plasma membrane and endoplasmic reticulum. Significant increase in the levels of alp may suggest that the plasma membrane, liver cells or the endoplasmic reticulum could have been compromised by the plant extract leading to alp extrusion and therefore its elevation into the serum.

The results of the biochemical analysis revealed that after 28 days (sub-acute toxicity) of single daily administration of the extract of *m. Scandens* there was a significant decrease in the ast between the male wistar that received 250mg/kg and the males that received 500mg/kg as compared with the control group. According to chintan et al. [21], an increase in the activities of alt and ast represents liver damage, a decrease in the activities of these enzymes is not considered of any toxicological significance. In accordance to lin et al. [38], a significant decrease in ast and alt is due to hepatoprotective effects. However in this study, only the ast serum levels were significantly reduced and not the alt thus this decrease could probably be due to hepatoprotective effects of the *m. Scandens* extract that further needs to be investigated at doses of 250mg/kg and 500mg/kg, or this decrease could be due to biological variation in response.

Results for the subchronic toxicity study (after 90 days) showed a significant decrease in the levels of alp for the female rats that received 500mg/kg as compared to the control group. According to saidu et al. [39], liver, bone, placenta and intestine are clinically important sources of the plasma activity of alp. This shows that alp is a nonspecific biomarker and its increase in serum should be supported by other parameters. A significant decrease in alp could also suggest inhibition or inactivation of the enzyme by the extract [40]. The significant decrease in alp at 500mg/kg versus the control, therefore, shows no adverse effect of the plant at this dose. Panunto et al. [41] also reported a decrease in alp after 270 days of evaluation of toxicity of terminalia chebula, and these changes were considered minor and not related to the toxicity of the plant.

Results for the subchronic toxicity study (after 90 days) also revealed a significant increase in alkaline phosphatase (ALP) activity. This was noted in male rats treated with 1000mg/kg of the extract compared with the 500mgkg for the 90-day treatment group (P=.05). The concentrations of the other serum liver diagnostic enzymes (ALT and AST) did not show any significant differences. Significant increase in the levels of ALP may suggest that the plasma membrane, liver cells or the endoplasmic reticulum could have been compromised by the plant extract leading to ALP extrusion and therefore its elevation into the serum. However, the ALP increase may not be related to the toxicity of the plant on the liver and may be due to biological variability in response since this increment was also not statistically significant as compared with the control group. At the same time AST is considered a more specific biomarker for liver toxicity since it's localized in the cells of the liver

and since it did not increase together with ALP, this result is not conclusive of liver damage. Furthermore, this increase in ALP may not be attributed to damage to the liver because the histopathological investigations did not reveal any significant lesions on the liver at a dose of 1000mg/kg for 90 days. There was no visible effect of the extract on the liver cells because the normal hepatic architecture showing central vein and portal tract interphase with hepatic plates separated by sinusoids was maintained. These results are in agreement with other studies such as by Chintan et al. [21] which showed an increase in ALP but that was not attributed to liver damage.

3.4 Histopathological Examination

Liver and Kidney 28 days: Necropsy at the end of study 28 did not reveal any gross pathological abnormalities in rats at doses up to 1000mg/kg following oral administration of *M. scandens* extract in both male and female Wistar rats. There was no visible effect of the extract on the liver cells because the normal hepatic architecture showing central vein and portal tract interphase with hepatic plates separated by sinusoids were maintained Fig. 3A.

Histopathological examination of the kidneys in the control and the *M. scandens* extract fed groups showed no differences, indicating that sub-acute administration of the extract of roots at these levels to the rats did not result in any adverse toxicological effect on the kidney. This is illustrated in Fig. 3B. These results suggest that sub-acute and subchronic administration of *M. scandens* extract at dose levels up to 1000 mg/kg/day to rats for 90 days has no adverse macroscopic or microscopic effects on liver and kidney.

Liver and Kidney 90 days: Necropsy at the end of the 90 day study period did not reveal any gross pathological abnormalities in male and female rats at doses up to 1000mg/kg following oral administration of *M. scandens*. There were no treatment-related macroscopic findings at the scheduled necropsy following administration of the extract to rats Fig. 4A.

Histopathological examination of the kidneys in the control and the *M. scandal* extract treated groups showed no differences, indicating that feeding the rats with the extract up to 1000 mg/kg for 90 days did not result in any adverse toxicological effect on the kidney as shown in Fig. 4B.

Necropsy at the end of the study did not reveal any gross pathological abnormalities in rats at doses up to 1000 mg/kg following oral administration of *M. scandens* extract in both male and female Wistar rats. There were no treatment-related macroscopic findings at the scheduled necropsy following administration of the extract to rats. All findings observed were consistent with normal background lesions in clinically normal rats of the age and strain used in this study and was considered spontaneous and/or incidental in nature and unrelated to the treatment. These results suggest that administration of *M. scandens* extract at dose levels up to 1000 mg/kg/day to rats for 90 days has no adverse macroscopic or microscopic effects on liver and kidney. The results are further supported by the non-significant changes in biochemical parameters, except for the 1000mg/kg group that showed significantly raised levels of ALP as compared to that of 500mg/kg after 90 days post oral administration of the *M. scandens* extract which has already been discussed. Histopathological studies showed the appearance of the normal architecture of the vital organs of the treated rats and did not induce any toxic effects at different doses. In histopathological studies, the liver of treated animals showed normal histological feature at 250, 500 and 1000 mg/kg. No degeneration of hepatocyte, focal steatosis, congestion of central vein or inflammation of the portal tract when compared with control animals was observed. The kidney of treated rats showed normal glomeruli and there was no necrosis of tubular epithelium in the kidney. Gross examination of liver and kidney on histology did not reveal any abnormalities. Thus, it was concluded that *M. scandens* did not produce any toxic effect in male and female Wistar rats on liver and kidney.

Observations from this study are in agreement with other sub-acute and subchronic studies conducted on herbal plant extracts widely used in traditional medicine such as, a study by Chintan et al. [21] where the administration of herbal extract did not show any adverse effects on Liver and kidney after 90 days and a study by Gandhare et al. [33], histopathological examination of the liver and kidneys did not result in any adverse toxicological effect on these organs after 28 days of extract administration compared with the control.

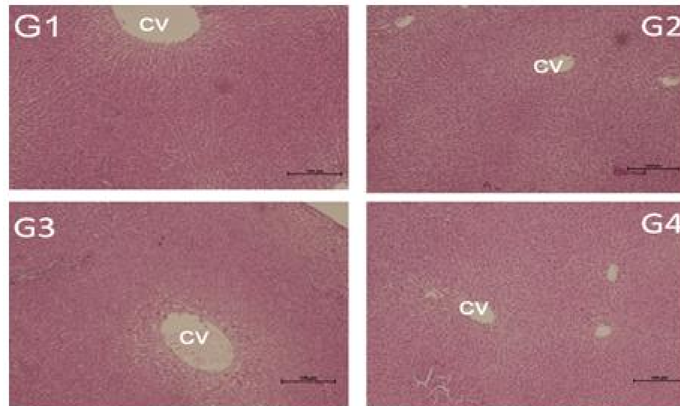

Fig. 3A. Photomicrograph of liver tissues after 28 days
G1 = 250 mg/kg; G2 = 500 mg/kg; G3 = 1000 mg/kg; G4 = Control; CV = Central vein. No significant lesions seen in all experimental groups

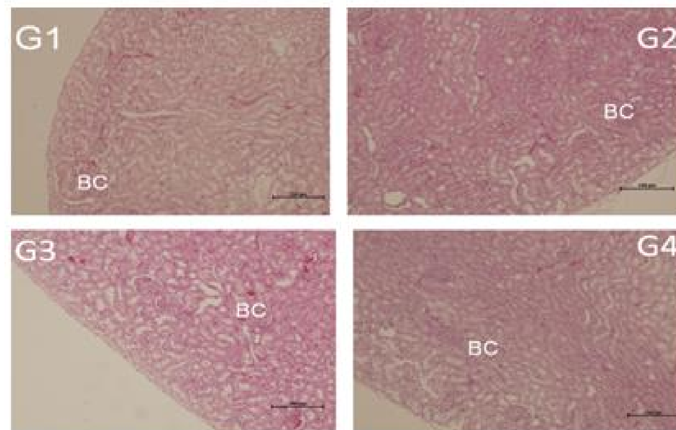

Fig. 3B. Photomicrograph showing liver tissues after 28 days
G1 = 250 mg/kg; G2 = 500 mg/kg; G3 = 1000 mg/kg; G4 = Control; H = Hepatocytes; CV = central vein. No significant lesions seen in all experimental groups. Red blood cells in central vein

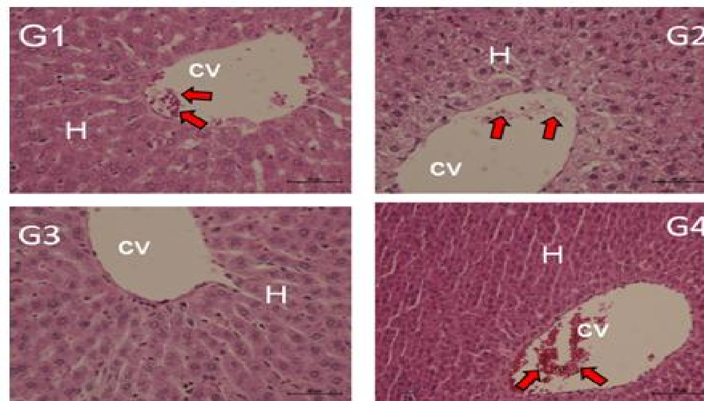

Fig. 4A. Photomicrograph showing liver tissues after 90 days
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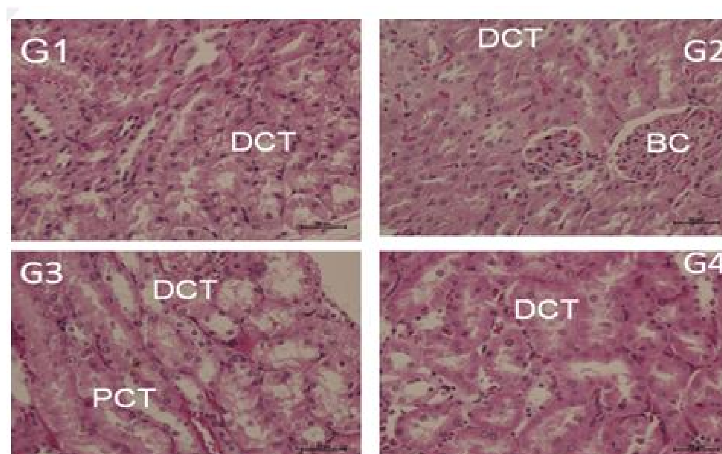

Fig. 4B. 1 Photomicrograph showing Kidney after 90 days

G1 = 250 mg/kg; G2 = 500 mg/kg; G3 = 1000 mg/kg; G4 = Control; BC = Bowman's capsule; DCT = Distal convoluted tube; PCT = Proximal convoluted tubule. No significant lesions seen in all experimental groups

4. CONCLUSION

The findings of this study conclude that the aqueous leaf crude extract of *M. scandens* is safe on haematological indices after sub-chronic and sub-acute administration at doses up to 1000mg/kg in Wistar rats. Furthermore, the extract was safe on the liver and kidney functioning as it did not significantly increase serum biochemical markers indicative of liver and kidney function after sub-acute and subchronic administration. However, a dose of 1000mg/kg should be monitored and doses beyond this are not recommended based on the results as it caused an increase in ALP after 90 days of administration of the extract and this should be studied further especially over long periods of time. The results also suggested that sub-acute and subchronic administration of *M. scandens* extract at dose levels up to 1000 mg/kg/day to rats has no adverse macroscopic or microscopic effects on liver and kidney. The *M. scandens* crude extract did not cause significant toxicity on haematological and histopathological indices, after sub-acute and subchronic administration in Wistar rats.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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