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Research Paper

ACUTE AND CHRONIC TOXICITY STUDIES OF THE AQUEOUS EXTRACT OF MIST DIODIA, ITS CONTRIBUTING PLANTS: *Diodia scandens* AND *Aframomum melegueta* IN MALE AND FEMALE SPRAGUE-DAWLEY RATS

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Abstract

Mist Diodia(MD), is a poly-herbal preparation for the management of hypertension in Ghana. Acute and chronic toxicity studies were carried out on it and also, on *Diodia scandens* (DS) and *Aframomum melegueta* (AM) its two component plants, to ascertain its safety. For acute toxicity study, both sexes of Sprague-Dawley rats (SDRs) were given a single oral dose (5000 mg/kg) of aqueous extract of MD, DS and AM and observed for mortality and physical signs of toxicity occurring within 24 hours and for additional 13 days. For chronic toxicity study, the therapeutic dose (X), 10X, 20X of MD, DS and AM, respectively were orally administered daily to both sexes of SDRs for a period of 6 months. The acute toxicity study estimated the LD50 to be above5000 mg/kg for all the test materials. The AST, ALP, ALT, LDH-1 enzyme activities as well concentrations of bilirubin, creatinine and urea measured for chronic toxicity study did not indicate significant difference ($p > 0.05$) between control and treatment groups for all the test materials. Urinalysis and histopathological examination of the heart, kidneys and liver also did show injury to these organs including the lungs and the spleen. The body weights, wet organ weights of the rats as well as their haematological profiles were not affected by the extracts at the end of the treatment period. Pentobarbital-induced sleeping times of the animals were not altered by the extracts. Thus, no toxicity both acute and chronic was associated with Mist Diodia and its components*: D. scandens* and *A. melegueta* in both sexes of SDRs.

Key words: *Mist Diodia, D. scandens, A. melegueta toxicity and SDRs*.

INTRODUCTION

Traditional medicines including herbal medicines, according to the World Health Organization (WHO), are being used by about 80% of populations of developing countries to treat various kinds of diseases [26]. Reasons for the increase in the usage of herbal medicine may include increased interest in preventive medicine, affordability, accessibility, lack of trust with some allopathic medicines and the notion that these herbal medicines may be safe and have minimal side effects.

At the Centre for Plant Medicine (CPMR), Mampong-Akuapem, Ghana, Mist Diodia (MD), prepared from the plants *Diodia scandens* (DS) and *Aframomum melegueta* (AM), has been developed as a herbal alternative for the management of hypertension. This is based on ethno-pharmacological evidence obtained from local herbalists. *D. scandens* belongs to the Rubiaceae plant family. It is a straggling, scrambling evergreen perennial or annual herb, with slender stems and yellowish-green leaves and found in tickets and secondary forests of West Africa. It is tasteless, odourless and whole parts of the plant is used in curing various ailments [10]. *D. Scandens* has been reported to possess antimicrobial properties for example; the leaves have been used to treat eczema in Nigeria [10]. Ethanolic extract of the plant possesses anti-thromboplastic activity in humans and slows down blood-clotting [22]. Petroleum ether extract of the leaves possesses anti-inflammatory effect in rats, increased the threshold of pain stimulus in mice and significantly protected rats from aspirin, indomethacin and reserpine induced ulcers [4]. The leaf extracts of the plant are used to stop bleeding, treat minor cuts, bruises, ear problems in Nigeria [11].

Phytochemical analyses of the leaf extract showed the absence of terpenoids, alkaloids and anthraquinones and the presence of tannins, saponins, flavonoids, steroids and cardiac glycosides [20].

A. melegueta, known commonly as Alligator pepper, Guinea Grains or Grains of Paradise, is found in most forest regions of West Africa. It has a reed-like structure and belongs to the plant family, Zingiberaceae (ginger family). It grows from a rhizome, reaching a height of about a meter with narrow and bamboo-like leaves with single pink flowers. It has reddish-brown ovoid capsules containing many reddish-brown spicy

seeds. Aqueous extract of the seeds of *A. melegueta* in rats acts as anti-diarrhoeal agent [27]. The plant is also known to be a stimulant and also possesses carminative and diuretic properties [22]. The seeds have antioxidant properties due to the presence of phenolic compounds [1]. In male Wistar albino rats, aqueous extracts of the dried seeds increased the secretions of epididymis and seminal vesicle [18].

Though the use of herbal medicines is becoming popular many of them have not been scientifically evaluated for their safety. Scientific evaluation of the safety of herbal medicines through pre-clinical studies is essential for their incorporation into healthcare systems globally. Therefore, in this study the safety of MD as an anti-hypertensive herbal preparation is evaluated by acute and chronic toxicity analyses in rats.

MATERIALS AND METHODS

Reagents and Chemicals

Kits for urinalyses and for the assay of aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), lactate dehydogenase-1 (LDH-1) enzymes activities and the estimation of creatinine and urea concentrations, were obtained from Cypress Diagnostics (Langdorp, Belgium). Reagent for drug interaction studies, histopathology and kits for myeloperoxidase enzyme activity were procured from Sigma-Aldrich (Milwaukee, USA).

Animals

All experiments done with animals were reviewed and approved by the Scientific and Ethics Committee of the CPMR, Mampong, Ghana (CPMR-Et/M.01/2015). Male and female Sprague-Dawley rats (SDRs) were procured from the Animal Experimentation Facility of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, Ghana and bred at the Animal Experimentation Unit of the CPMR, Mampong, Ghana. The animals were housed in aluminium cages with standard beddings of wood shavings. They were allowed to acclimatize for 14 days under standard environmental conditions of room temperature of 27 ^{o} C, relative humidity not less than 30% and not more than 70%. Further, the animals had 12 hours each of light and darkness and were provided with standard feed obtained from Ghana Agro Food

Company (GAFCO), Tema, Ghana. In addition, they were provided with and sterilized water *ad libitum*.

Plant Materials

D. scandens (leaves and stems) were obtained from the Akwapim Ridge, Eastern Region, Ghana, and *A. melegueta* seeds, from Praso, Central Region, Ghana, between the months of January – March. Both plant materials were authenticated by the staff of the Plant Development Department, CPMR, Mampong, Ghana and voucher specimen kept at the same Department (*A. Melegueta*; CPMR No 3710 and *D.scandens*; CPMR No 3711). Aerial parts of *D. scandens* and the seeds of *A. melegueta* were shade-dried for 4 weeks.

Preparation of Plant Material

The preparation of MD, DS) and AM were done in accordance with protocol provided for by the Production Department of the CPMR, Mampong, Ghana. The DS extract was prepared by boiling 2.5 kg of crushed shade-dried leaves and stems in 60 litres of water for 25 min. The resulting solution was allowed to cool and filtered with Whatman No. 1 filter paper. The filtered preparation was freeze-dried (Heto Power Dry LL 3000, Jouan, Nordic, Denmark). The seeds of AM were extracted by boiling 25 g of shade-dried seeds for 25 min in 60 litres of water and treated as described for DS. MD was prepared by boiling together the stated quantities of DS and AM in 60 litres of water for 25 min and treated as described previously. Prior to administration to experimental animals the freeze-dried plant preparations were reconstituted in sterilized water.

Acute Toxicity Test

This study was carried out based on OECD guidelines [3, 5], with some modifications. Adult Sprague Dawley rats (SDRs) (180 – 220 g) of both sexes were used for this study. Non-pregnant nulliparous female rats were selected for the study. For each sex a total of 20 rats were selected and were assigned to 4 groups of 5 rats per group and acclimatized for 14 days. Group 1 served as a control group and groups 2 – 4 were designated as the test groups and treated with MD, DS and AM. A quantity of 5000 mg freeze-dried sample of each plant preparation was weighed (Kern ALJ 310-4N, Kern & Sohn, Germany) and dissolved in sterilized water and the final volume made up to 10.0 ml. After initial physical examination, rats in the test groups were weighed and given a

single oral dose (10 ml/kg) of the test solution, after an over-night fast. The control group received only water. The rats were given feed 3 hours after treatment.

The rats in the various groupings were observed for mortality and physical signs of toxicity, 5, 15 and 30 min, after treatment with the plant preparations then hourly during the first 24 hr. Observation for mortality and physical signs of toxicity such as lack of alertness, presence of staggering gait and pilo-erection continued daily, for a total of 14 days. [3, 5].

Chronic Toxicity Test

Just as in acute toxicity studies, chronic toxicity studies were based on the OECD guidelines [3, 5], with slight modifications. SDRs of both sexes (180 – 220 g) were used for this study. For each plant preparation a total of 76 male rats and 76 female rats were selected. The rats were randomly assigned to 4 groups of 19 rats each. Group 1 served as controls and received water. Groups 2, 3 and 4 were the treatment groups and for MD received 30, 300, 600 mg/kg; 40, 400, 800 mg/kg for DS and 80, 800, 1600 mg/kg for AM, respectively representing the therapeutic dose (X), 10X and 20X of each plant preparation.

The treatment regimen was followed daily for a period of 6 months with periodic observation. The body weights of control and treated animals were determined at baseline (day 0) and at a 14-day interval during the study period. Wet weights of selected organs of control and treatment groups were determined at the baseline and at the 3rd and 6th months of treatment [3, 5, 2].

Urinalysis

On day 0, 3rd and 6th months of the study period urine samples of control and treated animals produced by involuntary discharge, were collected into clean ceramic containers in the morning and analysed with a urine test strip (Urine-10, Cypress Diagnostics, Belgium). The test results were compared with the colour chart on the bottle label to provide either qualitatively or semi-quantitatively results for the following urine parameters: glucose, bilirubin, ketones, specific gravity, blood, pH and urobilinogen,

Water/feed intakes

Five rats from each group were placed in metabolic cages and used to monitor feed and water intakes. The treated and untreated groups were provided daily with measured quantities of water and feed at a specific time in the morning. The quantities left in the cages the following morning at a particular time were measured and used to establish the quantity of water and feed consumed by each animal. This was done for a period of 6 months.

Blood sampling

Blood samples of each rat in the control and treatment groups were obtained by tail snip on day 0, 3rd and 6th months of the study period. Slides of thin blood smear were prepared on each occasion and the some of the blood drained into Eppendorf tubes without anti-coagulant, put on ice, allowed to clot and centrifuged (Denley BS 400, England) at 4,000 x g for 5 min. The serum formed was transferred into Eppendorf tubes and stored at -20 ^{o} C for biochemical analyses. Blood samples (0.5 ml) were separately collected into tubes coated with ethylenediaminetetraacetate (EDTA) and used for haematological analyses.

Serum Biochemical Analyses.

Activities of serum AST, ALT, ALP and LDH-1 enzymes and serum bilirubin (total and direct), creatinine and urea were determined spectrophotometrically (Photometer 4040, Robert Riele G & Cole-2000, Germany) with commercially available kits (Cypress Diagnostics, Langdorp, Belgium) with slight modifications in the scheduled of timing analysis [16].

Haematology

A volume (0.5 ml) of uncoagulated blood prepared earlier was analysed for white blood cells (WBCs), red blood cells (RBCs), haemoglobin (Hb) and haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), platelet (PLT) count, red cell distribution width (RDW) and mean platelet volume (MPV) were determined with the Sysmex KX-21 Automated Haema Screen (Europe), at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon, in

accordance with established protocol. The equipment is computerized and automatically generated the haematological data for each blood sample.

Pentobarbital-induced Sleeping Time

Each animal from the control and treatment groups underwent the pentobarbital-induced sleeping test. The animals were given intraperitoneal injection of pentobarbital in saline (40 mg/kg) at a volume of 10 ml/kg. The time the individual animals completely lost their reflexes was noted (sleep time) and the time they completely regained consciousness or righting reflex was recorded as awakening time. The difference between these two times gave the pentobarbital-induced sleeping time [25].

Histopathology

At the beginning of the study and at the 3rd and 6th months of treatment, two animals from the control and treated groups were killed by cervical dislocation and the heart, liver, kidneys, lungs and spleen excised and fixed in 10% buffered formalin solution (pH 7.4) and dehydrated with increasing concentration of alcohol. The tissues were cleared with xylene and impregnated with wax and 5 µm microtome sections were stained with haematoxylin and eosin and mounted on slides for light microscopic examination [24].

Cytochemistry

Slides of thin blood smear of each rat in the control and plant material-treated groups prepared before the start of treatment and on the 3rd and 6th months post-treatment were analysed for myeloperoxidase enzyme activity, following protocol contained in the Sigma-Aldrich Myeloperoxidase Protocol. Procedure Number 391.

Statistical Analysis

Statistical evaluation was performed using SPSS statistical software version 16.0 for Windows XP. All results were presented as means ± S.E.M. Data was analysed using one-way analyses of variance (ANOVA) to determine statistical differences between groups and when necessary Tukey post hoc to determine differences within groups. Significant difference was set at $P < 0.05$.

RESULTS

Acute Toxicity

Results for median lethal dose (LD_{50}) determination showed that none of the rats of both sexes treated with MD, DS and AM preparations at a dose of 5000 mg/kg, died during the twenty-four-hour observation period. Also, no observable signs of toxicity such as restlessness, staggering gait and pilo-erection for behavioural, neurological and autonomic response respectively, were detected in both control and treated rats of both sexes. No mortality occurred in both sexes during the period of observation of 14 days.

Chronic Toxicity

The results of biochemical parameters determination in male SDRs; for both controls and plant preparation-treated rats, at termination of treatment are presented in Figures 1 – 5. There were no significant ($p > 0.05$) differences in all parameters measured; AST, ALT and ALP (Fig 1), total bilirubin (TB) and direct bilirubin (DB) (Fig 2), LDH-1 (Fig 3), creatinine (Fig 4) and urea (Fig 5), between control values and plant preparation-treated groups, at termination of treatment. The baseline values of these biochemical parameters measured were not significantly ($p > 0.05$) different from controls at termination of treatment.

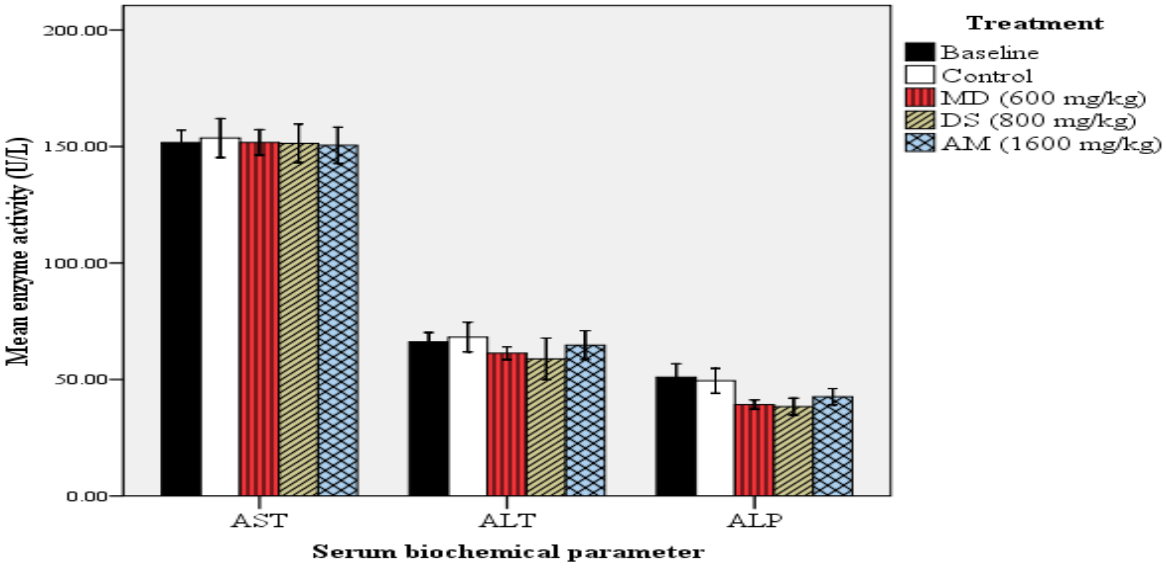


Figure 1: Effect of a 6-month oral treatment with aqueous preparations of Mist Diodia, (MD), *D. scandens* (DS), and *A. melegueta* (AM), on the activities of serum AST, ALT and ALP enzymes of male SDRs. Similar results were obtained for female SDRs, at the 3rd month of treatment and at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M for n = 10.

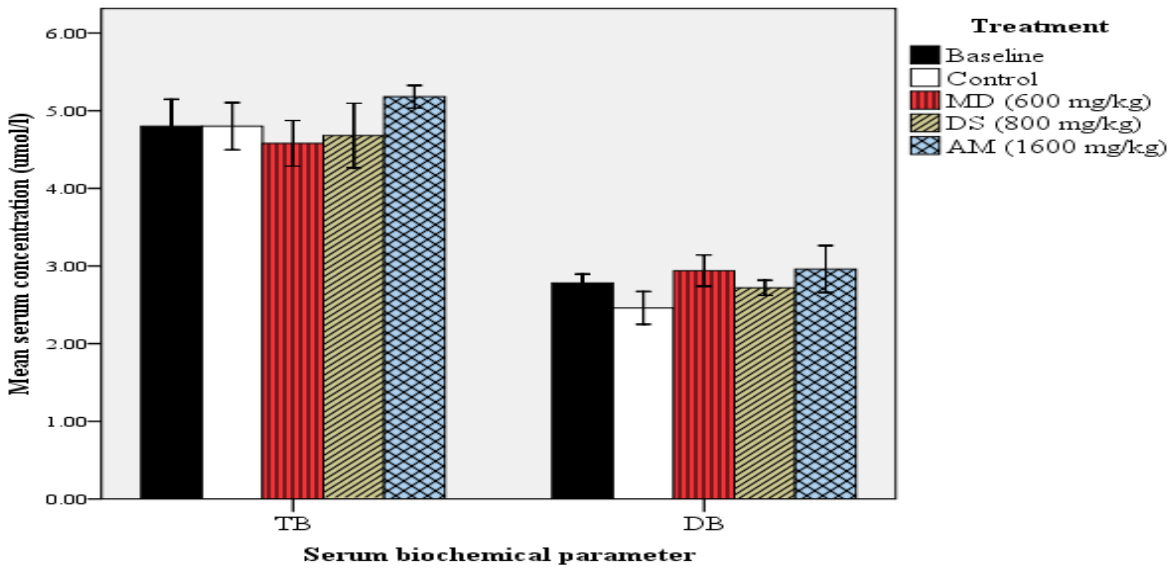


Figure 2: Effect of a 6-month treatment with aqueous preparations of Mist Diodia, (MD), *D. scandens* (DS) and *A. melegueta* (AM), on serum concentrations of total bilirubin (TB) and direct bilirubin (DB) of male SDRs. Similar results were obtained for female SDRs, at the 3rd month of treatment, at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M. for n = 10.

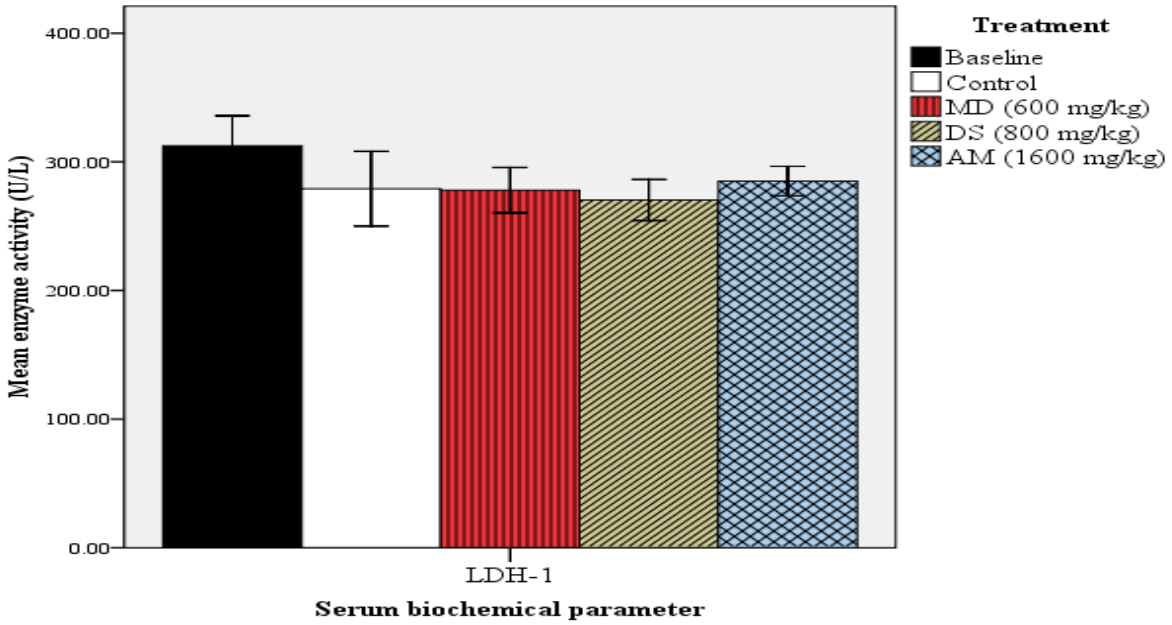


Figure 3: Effect of a 6-month treatment with aqueous preparations of Mist Diodia (MD), *D. scandens* (DS) and *A. melegueta* (AM), on activity of serum LDH-1 of male SDRs. Similar results were obtained for female SDRs, at the 3rd month of treatment, at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M. for n = 10.

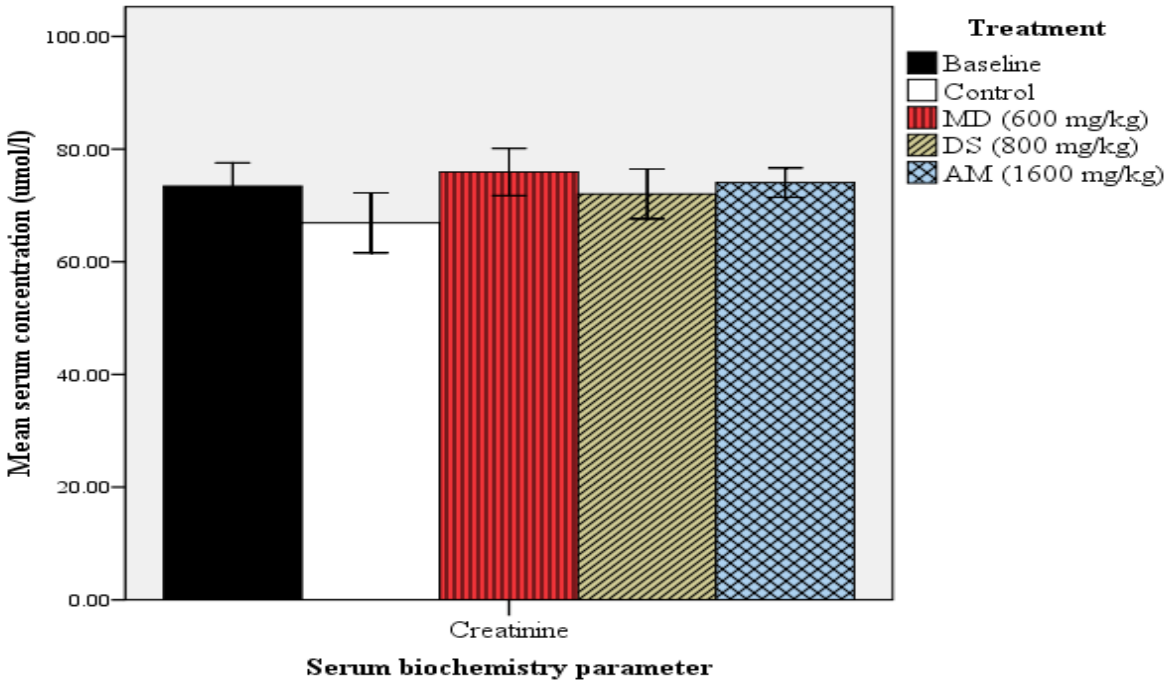


Figure 4: Effect of a 6-month oral treatment with aqueous preparations of Mist Diodia (MD), *D. scandens* (DS) and *A. melegueta* (AM), on serum concentration of creatinine of male SDRs. Similar results were obtained for female SDRs, at the 3rd month of treatment, at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M. for n = 10.

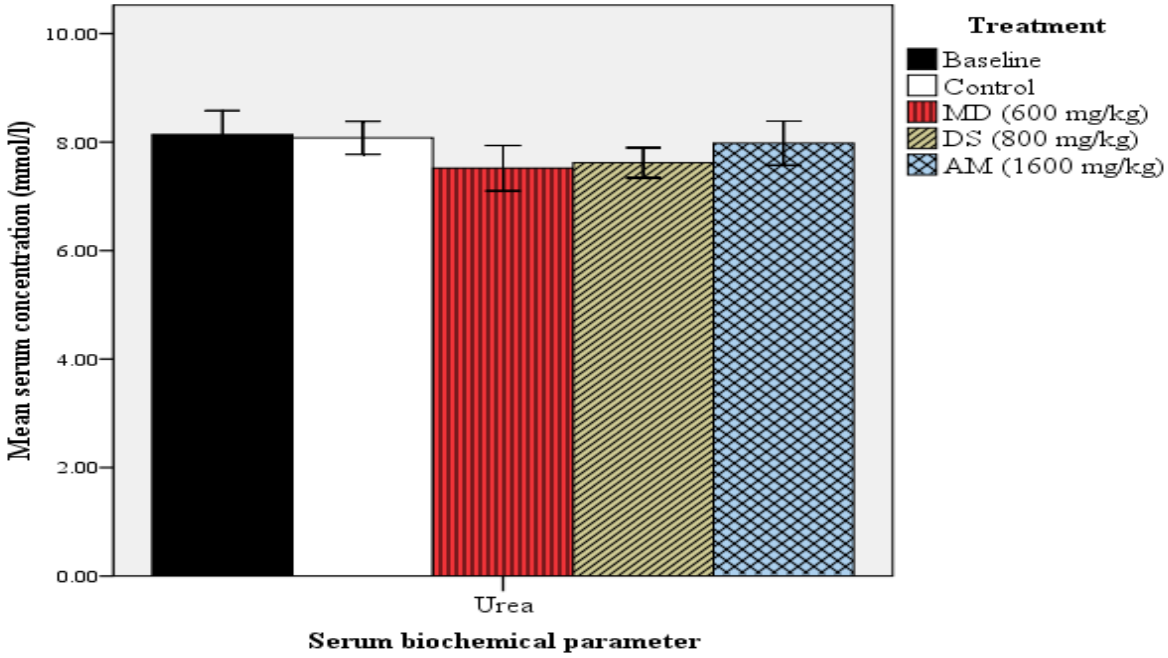


Figure 5: Effect of a 6-month oral treatment with aqueous preparations of Mist Diodia (MD), *D. scandens* (DS) and *A. melegueta* (AM), on serum urea of male SDRs. Similar results were obtained for female SDRs, at the 3rd month of

treatment, at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M. for n = 10.

Results of dipstick urinalysis of male and female SDRs treated with/without the plant preparations, MD, DS and AM at termination of treatment shown in Table 1, did not show significant difference ($p > 0.05$) between rat treatments groups in all parameters measured.

Table 1: **Urinalysis data of male and female SDRs at termination of treatment with plant preparations**

Parameter	**[a]Treatment**			
	Control	**MD**	**DS**	**AM**
Urobilinogen (µmol/l)	N (N)	N (N)	N (N)	N (N)
Glucose (mmol/l)	- (-)	- (-)	- (-)	- (-)
Bilirubin	- (-)	- (-)	- (-)	- (-)
Ketones (mmol/l)	- (-)	- (-)	- (-)	- (-)
Specific gravity	1.03 ± 0.002 (1.02 ± 0.001)	1.03 ± 0.002 (1.03 ± 0.001)	1.03 ± 0.002 (1.02 ± 0.001)	1.03 ± 0.001 (1.02 ± 0.001)
Blood (rbc/µl)	- (-)	- (-)	- (-)	- (-)
pH	6.90 ± 0.18 (7.80 ± 0.43)	7.00 ± 0.35 (7.85 ± 0.45)	6.90 ± 0.20 (7.80 ± 0.50)	7.00 ± 0.30 (7.80 ± 0.40)

[a] Rats were treated orally daily for 6 months with Mist Diodia (600 mg/kg), DS (800 mg/kg) and AM (1600 mg/kg). Values represent means ± S.E.M. for n = 10. Results in parenthesis represent those of female rats. Similar results were

obtained at the baseline, the 3rd month of treatment, at the therapeutic and medium doses of the plant preparations (Not shown).

(-): Absent; (N): Normal; (Tr): Trace.

Table 2 shows the pentobarbital-induced sleeping time after treating male and female SDRs chronically with higher dosages of MD, DS and AM, over a 6-month period. The results showed statistically insignificant difference ($p > 0.05$) between control and plant preparation-treated groups. Similar results were obtained at baseline and at the 3rd month of treatment (not shown).

Table 2: Pentobarbital-induced sleeping time in male and female SDRs after termination of treatment with plant preparations

Parameter	[a]Treatment			
	Control	**MD**	**DS**	**AM**
Sleeping Time (min)	77.00 ± 6.05 (88.10 ± 2.08)	78.40 ± 2.57 (83.80 ± 3.08)	78.00 ± 1.91 (82.10 ± 2.36)	76.23 ± 2.03 (86.10 ± 2.46)

[a] Rats were orally treated daily with Mist Diodia (600 mg/kg), *D. scandens* (800 mg/kg) and *A. melegueta* (1600 mg/kg). At termination of treatment (6 months), rats were given pentobarbital (40 mg/kg) and sleeping and awakening times recorded. Similar results were obtained at the baseline and at the 3rd month of treatment, at the therapeutic and medium doses of the plant preparations (not shown). Values are means ± S.E.M. for n = 10. Values in parenthesis represent results of female rats.

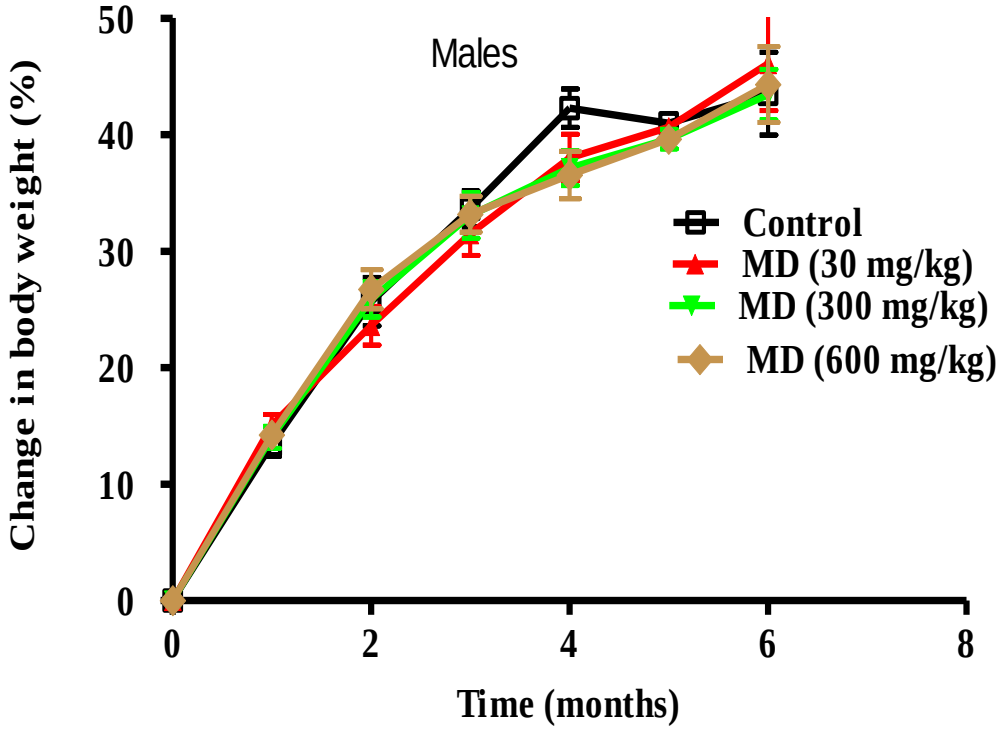
The haematological analyses for control and plant preparation-treated male and female SDRs at the 6th month of treatment did not show significant differences ($p > 0.05$) between control and the extract-treated animal groups, for all the parameters measured (Table 3). Similar results were obtained at the baseline and at the 3rd month of treatment (not shown).

Table 3: Haematological profiles of male and female rats after termination treatment with plant preparations

Parameter	[a]Treatment			
	Control	MD	DS	AM
WBC (x 10^3/ µl)	9.00 ± 0.85 (7.36 ± 0.29)	9.16 ± 0.14 (7.40 ± 0.25)	9.86 ± 0.65 (7.67 ± 0.22)	8.50 ± 0.93 (7.35 ± 0.26)
RBC (x 10^6/ µl)	7.88 ± 0.20 (7.19 ± 0.40)	8.28 ± 0.13 (7.72 ± 0.14)	8.06 ± 0.14 (7.71 ± 0.11)	8.01 ± 0.23 (7.63 ± 0.15)
HGB (g/ dl)	14.74 ± 0.36 (14.70 ± 0.48)	15.30 ± 0.26 (15.33 ± 0.20)	15.16 ± 0.20 (15.99 ± 0.30)	4.82 ± 0.50 (15.20 ± 0.25)
HCT (%)	44.84 ± 1.12 (44.22 ± 2.53)	47.06 ± 0.80 (48.46 ± 1.23)	46.70 ± 0.77 (49.15 ± 1.13)	47.24 ± 1.68 (46.98 ± 1.04)
MCV (fl)	56.96 ± 0.47 (61.50 ± 0.47)	56.88 ± 0.52 (62.72 ± 0.77)	57.94 ± 0.88 (63.74 ± 1.03)	58.94 ± 0.89 (61.61 ± 0.71)
MCH (pg)	18.72 ± 0.10 (18.81 ± 0.79)	18.46 ± 0.27 (19.87 ± 0.24)	18.80 ± 0.33 (20.74 ± 0.37)	18.48 ± 0.17 (19.97 ± 0.30)
MCHC (g/ dl)	32.88 ± 0.12 (30.59 ± 1.30)	32.52 ± 0.23 (31.74 ± 0.55)	32.46 ± 0.20 (32.61 ± 0.45)	32.34 ± 0.37 (32.42 ± 0.50)
PLT (x 10^3 µl)	941.80 ± 85.66 (794.80 ± 49.87)	975.00 ± 25.52 (845.90 ± 56.35)	929.20 ± 45.92 (743.60 ± 51.10)	932.00 ± 25.94 (744.60 ± 47.47)
RDW (fl)	30.04 ± 0.60 (33.03 ± 0.97)	28.84 ± 0.24 (35.73 ± 1.46)	30.56 ± 0.49 (33.34 ± 1.20)	32.10 ± 2.14 (34.54 ± 1.24)
MPV (fl)	6.14 ± 0.07 (6.53 ± 0.08)	6.22 ± 0.05 (6.60 ± 0.06)	6.16 ± 0.07 (6.39 ± 0.06)	6.36 ± 0.14 (6.52 ± 0.09)

[a]Rats were treated d orally daily for 6 months with MD (600 mg/kg), DS (800 mg/kg) and AM (1600 mg/kg). Values are means ± SEM for n = 10. Results in parenthesis are for female rats. WBC = white blood cells; RBC = red blood cells; HGB = haemoglobin; HCT = haematocrit; MCV = mean cell volume; MCH = mean cell haemoglobin; MCHC = mean cell haemoglobin concentration; PLT = platelet; RDW = red cell distribution width; MPV = mean platelet volume. Similar data were obtained for rats treated at the baseline, after 3-months of treatment, at the therapeutic and medium doses of the plant materials (not shown).

Figure 6 shows the effect of varying doses of MD on the body weights of male and female SDRs. There was a gradual increase in body weight up to the 6th month of treatment. However, compared to the controls the plant preparation-treated groups did not show significant ($p > 0.05$), changes in body weight over the treatment period. Similar results were obtained for the plant preparations DS and AM (not shown).



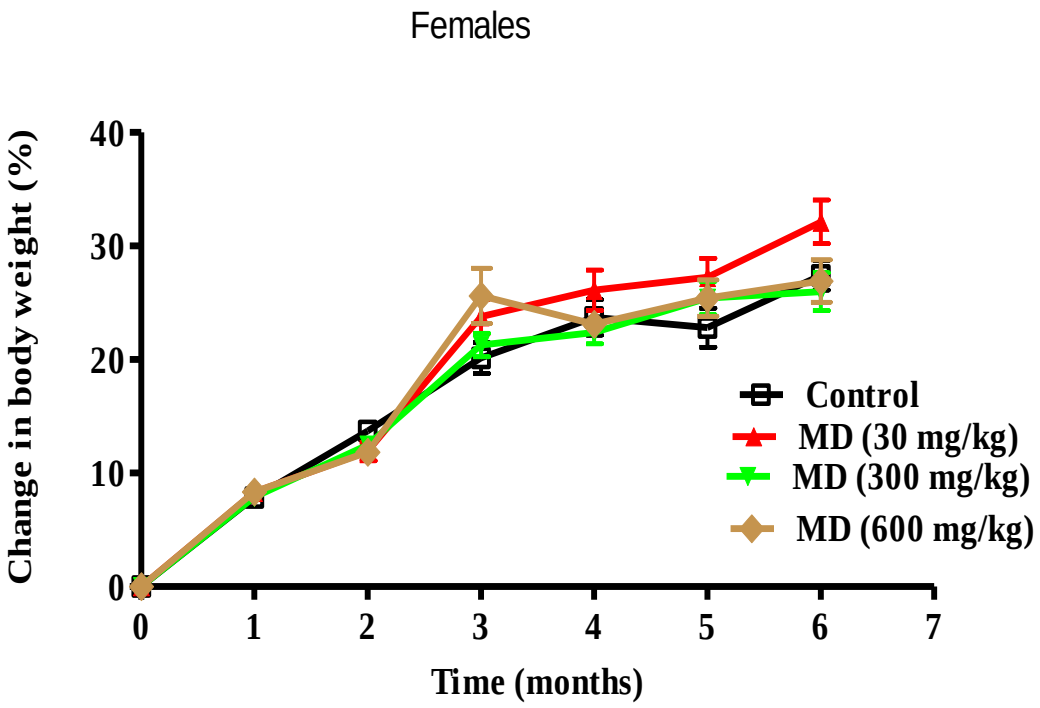


Figure 6: Effect of Mist Diodia (MD) on changes in on body weights of male and female SDRs. Changes in body weights expressed as a percentage of mean baseline values of 200.90 ± 3.77 and 213 ± 5.37 (g) for male and female SDRs, respectively were not statistically significant (P > 0.05). Each point represents mean ± S.E.M. for n = 10. Similar results were obtained for DS and AM (not shown).

The effects of various dosages of MD preparation on mean wet weights of selected organs (heart, kidneys, liver, lungs and spleen), expressed as a percentage of body weight in male SDRs, at termination of treatment are shown in Figure 7. There were no significant ($p > 0.05$) differences in mean organ wet weights between control and baseline values, at termination of treatment. Furthermore, at termination of treatment the plant preparations did not cause significant changes ($p > 0.05$) in these mean organ wet weights, compared with untreated controls. Similar results were obtained for female SDRs and for the plant preparations DS and AM (not shown).

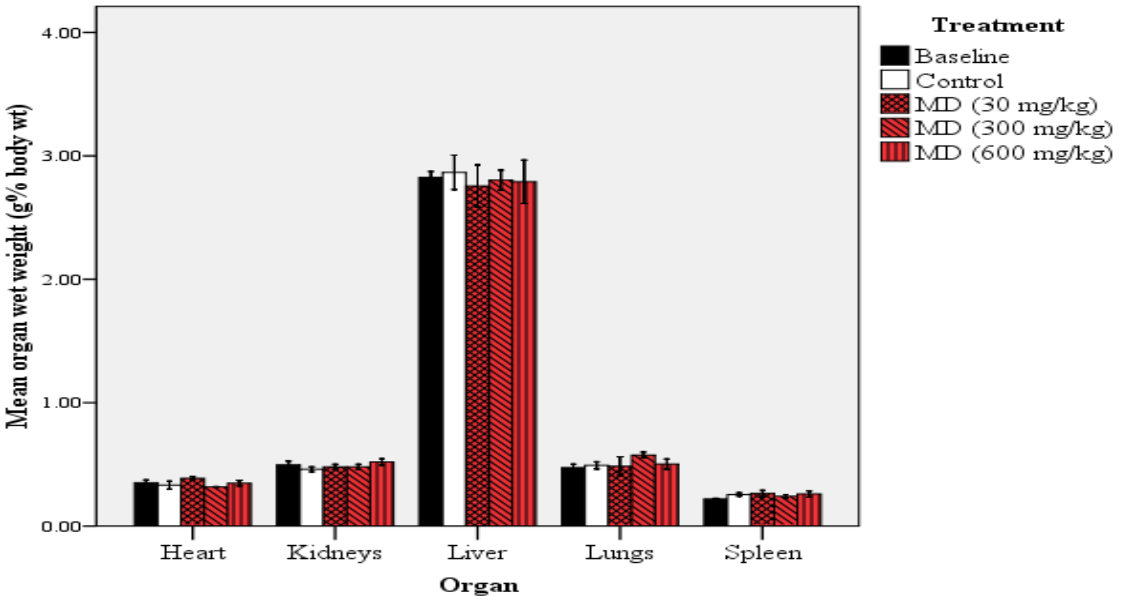


Figure 7: Effect of a 6-month oral treatment with Mist Diodia (MD) on organ wet weights of male SDRs. The organ wet weight was expressed as a percentage of total body weight. Values are means ± S.E.M. for n = 10. Similar results were obtained at the 3rd month, for female SDRs, DS and AM (not shown).

The daily intake of water by male SDRs treated with varying doses of MD is shown in Figure 8. Water intake appears to increase with increasing age in all animal treatment groups. There was no significant difference ($p > 0.05$) between control and plant preparation-treated groups, over the period of study. Similar results were obtained for female SDRs and for DS and AM (results not shown).

Similar to water intake, feed intake increased with age for the controls and the plant preparation-treated male SDRs (Fig 9). There were no significant changes in ($p > 0.05$) in feed intake in the treatment groups compared to the controls. Results for female SDRs, DS and AM-treated animals not shown, were similar to those obtained for MD.

Vol. 9(10), 2020 pp. 7988-8013

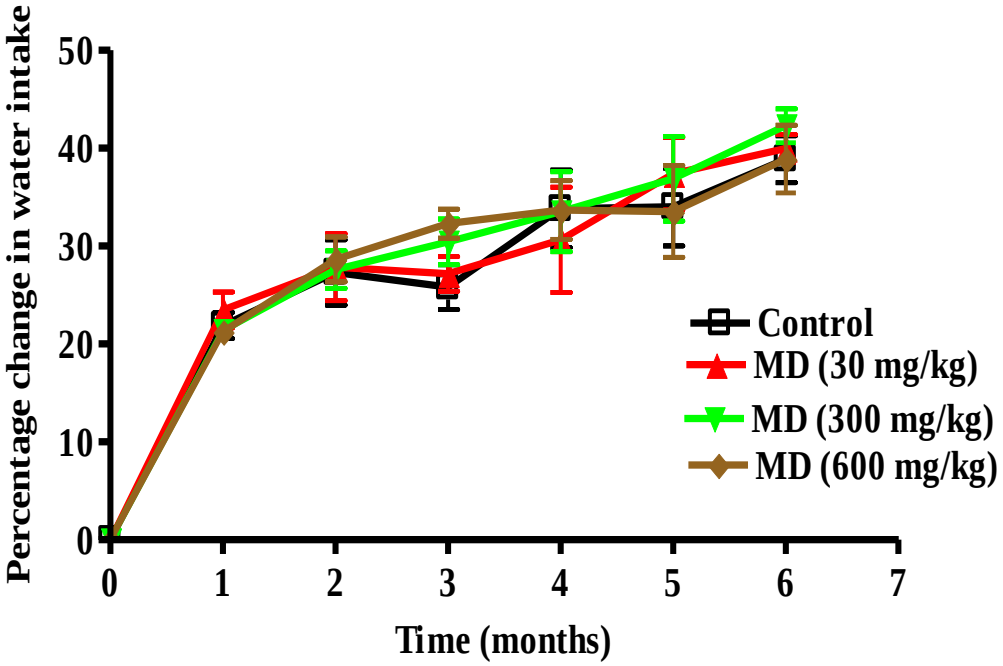


Figure 8: Water intake of male rats expressed as a percentage of baseline value of 20.40 ± 0.26 (ml) for male SDRs, after treatment with Mist Diodia (MD) for 6 months. Each point represents mean ± S.E.M. for n = 5. Similar results obtained for female SDRs, DS and AM (not shown).

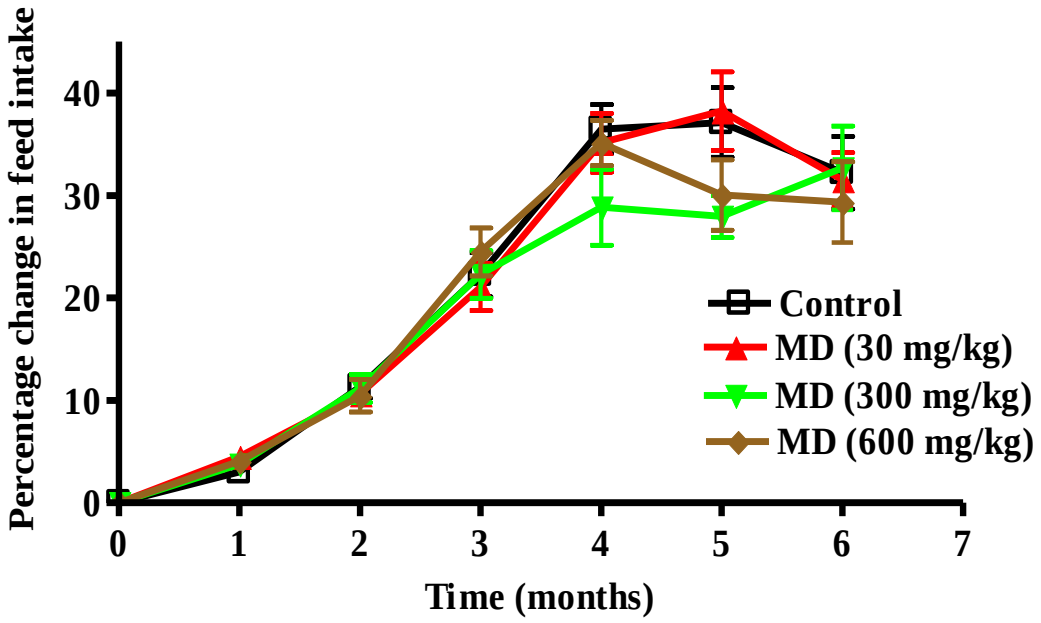


Figure 9: Feed intake of male rats after a 6-month daily oral treatment with Mist Diodia (MD). Feed intake was recorded at regular intervals and expressed as a percentage of baseline value of 19.35 ± 0.13 (g) for male SDRs. Each point represents mean ± S.E.M. for n = 5. Similar results were obtained for female SDRs, DS and AM plant preparations (not shown).

Histopathological examination of heart, kidneys and liver of male SDRs treated with high doses of MD, DS and AM did not show any significant morphological changes in these tissues, at the end of treatment (Figs 10, 11 & 12). Similar results were obtained at the baseline, 3rd month of treatment, for the liver, lungs and spleen and for female rats (results not shown). Also, results of immunohistochemical staining of the blood samples of experimental male rats treated with high doses of the plant preparation MD (Fig 13), did not show myeloperoxidase activity in both control and treatment groups, at the end of the experimental period of 6 months. Similar results were obtained for the plant preparations DS, AM and at the baseline, 3rd month of treatment and for female rats (results not shown).

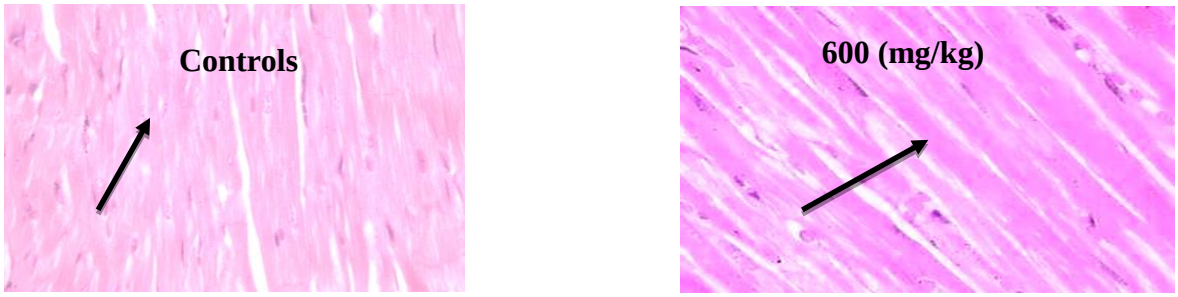


Figure 10: Micrographs of heart showing normal heart muscle fibres without necrosis and fibrous tissues in control, MD (600 mg/kg)-treated male SDRs, at termination of treatment (6 months). Similar results were obtained at baseline, 3- months post-treatment and for female SDRs, therapeutic and medium doses of MD. Results for DS and AM similar (not shown). Magnification: x200

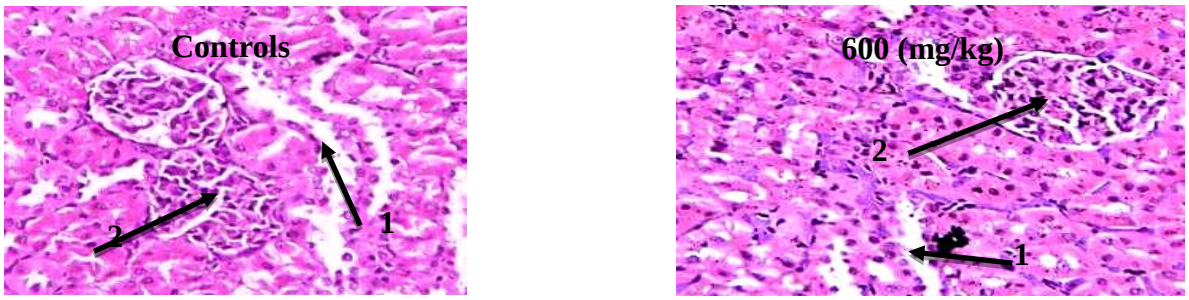


Figure 11: Micrographs of kidneys showing normal non-necrotic renal tubules (1) and glomerulus (2), which also did not show lesions or fat deposition, in control and MD (600 mg/kg)-treated male SDRs, at termination of treatment (6 months) Similar results were obtained at baseline, 3- months post-treatment,

female SDRs, therapeutic and medium doses of MD. Results for DS and AM were similar (not shown). Magnification: x200

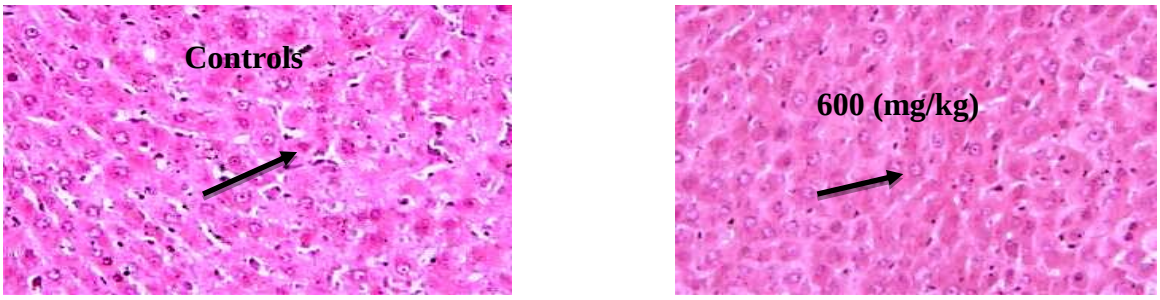


Figure 12: Micrographs of liver showing normal hepatocytes without fibrous tissues and fat deposition in control, MD (600 mg/kg)-treated male SDRs, at termination of treatment (6 months). Similar results were obtained at baseline, 3-month post-treatment, for female rats, therapeutic and medium doses. Results for DS and AM were similar (not shown). Magnification: x200

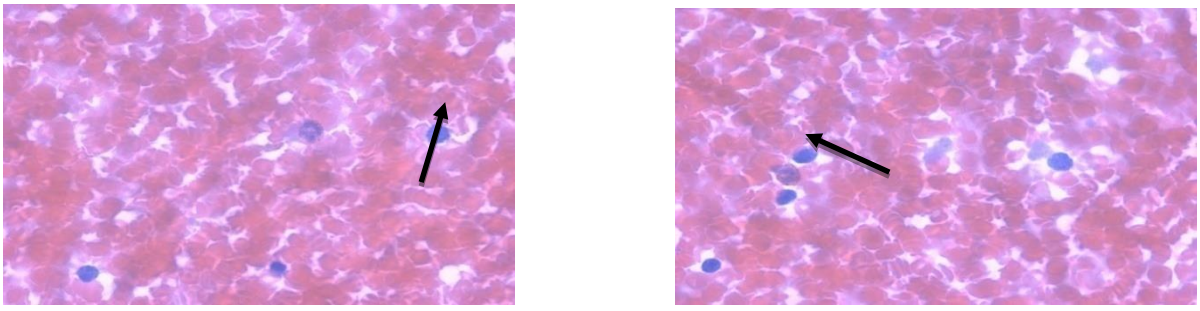


Figure 13: Micrographs of blood films showing normal granulated white blood cells in control and MD (600 mg/kg)-treated male SDRs at the termination of treatment (6 months). Similar results were obtained at baseline, 3- months post-treatment, for female SDRs, at the therapeutic and medium doses. Results for DS and AM were similar (not shown) Magnification: x68.

DISCUSSIONS

At a single dose of 5000 mg/kg, no deaths nor any physical signs of toxicity such as lack of alertness, staggering gait and pilo-erection were observed in both male and female SDRs treated with the plant preparations, during the first 24 hours and over the 14-day period of observation. This suggests that the preparations were fairly safe and did not affect the behavioural, neurologic and autonomic responses or the central nervous

system (CNS), in both sexes of SDRs [21, 19, 23] at the therapeutic doses of 30, 40 and 80 mg/kg for MD, DS and AM.

The 6-month chronic toxicity studies in both sexes of SDRs with high dosages of MD (600 mg/kg), DS (800 mg/kg) and AM (1600 mg/kg), did not also cause any physical signs of toxicity or mortality. Chronic toxicity studies shed light on possible risk involved in continuous exposure to drugs, since single dose exposure is not enough to show these risks. It is thus, carried out to determine any toxic effects induced through chronic exposure and to help determine No Observed Effect Level (NOEL).

One of the major functions of the liver in the body is the metabolism of substances. It is thus susceptible to chemically-induced tissue damage. When liver cells are damaged, they release intracellular enzymes such as, ALT, AST and ALP into the blood resulting in an elevation in their activities. Their elevations can thus, be used as an indices of liver damage [7]. Serum bilirubin concentration have been shown to increase in cases of liver injury [7]. The concentration of urobilinogen has been observed to be elevated in the urine in liver damage [12].

The serum activities of AST, ALT, ALP and concentrations of total bilirubin (TB) and direct bilirubin (DB) indicators for liver function and integrity assessed in the 6-month chronic toxicity studies, were normal (Figs 1 & 2), an indication that these plant preparations did not cause hepatic damage, at the dosages administered. This is supported by liver wet weight/body weight ratios which did not show any statistically significant differences ($p > 0.05$) between the control and treated groups (Fig 7) and by histopathological studies of the liver tissues which, in all cases showed normal hepatocytes and interstitial spaces. Urinalysis data did not also show any significant differences between control and test groups, with respect to urine urobilinogen and bilirubin. This further supports the absence of liver damage due to the administration of the plant preparations.

The kidneys are responsible for the maintenance of water and electrolyte balance in the body. Its damage results in poor glomerular filtration and tubular secretion of waste products causing elevations their concentrations in the blood. Thus, levels of urea and

creatinine in the blood can be used to assess kidney function [8]. The plant preparations did not cause significant elevations in the serum concentrations of creatinine and urea (Figs 4 & 5), the presence of which are indicative of kidney damage in both male and female SDRs at the dose administered over the period of study. These findings were further corroborated by the urinalysis data [3], kidney wet weights/body weight ratios (Fig 7) and histopathological examination of the kidney tissues of male and female SDRs all of which did not show significant differences between controls and test animals.

Cardiotoxicity, may be diagnosed biochemically [13] and by enzyme assays. Creatine kinase (CK-MB), AST, and lactate dehydrogenase-1 (LDH-1) especially are used to investigate cardiac function [6]. Histopathological techniques can also be used to diagnose this type of toxicity [13]. Absence of significant differences between test animals and controls in serum LDH-1 activities (Fig 3) together with the fact that there was no organ hypertrophy, oedema or atrophy (Fig 7) suggests no damage to heart muscle fibres.

The principal role of the lungs is to supply oxygen to the body and exhale carbon dioxide. Lung damage may lead to necrosis of the Clara cells and the lining of bronchiolar epithelium or alveolar cells change, leading to thickening of the alveolar septa. For this reason, histopathological examination of selected organs such as the lungs are carried out as part of toxicity studies in animals. Also, the wet organ weights of treated and un-treated animals can be used to determine organ-specific toxicity in animal studies [28]. The present study did not show lung damage after 6-months exposure to MD and its components, as evidenced by lack of new effect in the Clara cells of the bronchioles and alveolar cells. The wet weights of lungs of all the treatment groups were found to be normal (Fig 7),

Certain substances can affect the haematopoiesis adversely [9]. Some common haematological parameters often affected are haemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet (PLT), mean platelet volume (MPV), white blood cells (WBCs) and red blood cells (RBCs). Results of the

haematological assessment in both male and female SDRs treated with the three plant preparations did not show any statistically significant differences between the control and treatment groups, at termination of chronic toxicity testing. This indicates the absence of anaemia or disturbances in the haemopoietic system in both male and female animals.

Through a process called culling the spleen removes abnormally shaped blood cells from circulation. Thus, when the spleen is damaged abnormally shaped blood cells and some inclusion bodies will be found in circulation. However, when the spleen is enlarged by being filled with large numbers of red blood cells, as seen in some cases of hypertension, there can an anaemia and an increase in WBCs and platelets counts. Treatment with the plant preparations did not cause damage to spleen of test animals, as evidenced by the absence of morphological changes in the spleen, when compared to the controls. This is supported by the lack of difference in the size and weight of the spleen of the plant preparation-treated animals, when compared to controls (Fig 7).

The liver plays an important role in the detoxification of xenobiotics. It does this by the metabolism of xenobiotics, mainly by isozymes of the cytochrome P450 (CYP450) system. Some other substances can cause the induction or inhibition of isozymes of the CYP450 system, which can result in either beneficial or harmful drug interactions [17]. Pentobarbital is a hypnotic drug whose metabolism is catalysed by the cytochrome P450 isozymes, so its metabolism by the liver is assessed in drug interaction studies, by determining the effect on pentobarbital–induced sleeping time [25]. The fact that pentobarbital-induced sleeping time for all the treatment groups was similar to the values obtained for the controls, is an indication that the plant preparation neither induced nor inhibited isozymes of the CYP450 system.

There were progressive increases in body weights (Fig 6) of both sexes of SDRs over the period of chronic treatment with the varying doses of MD, DS and AM, which corresponded well with feed intakes (Fig 9). The absence of any significant differences in these determinations between the control and test groups, suggest that the plant preparations did not cause anorexia or changes in the metabolism of carbohydrates or

fats in the animals. Loss in body weights have been linked to anorexia [14], changes in body weights to altered carbohydrates and fats metabolism [29].

Water is the medium for the transport of nutrients, enzyme–catalysed reactions and the transfer of chemical energy. The degree of water intake increased in of both sexes of SDRs over the period of chronic treatment with the varying doses of MD, DS and AM. The lack of significant differences in these determinations between the control and treated groups, suggest that the plant preparations did not affect water intake in the animals.

Immunohistochemical staining for myeloperoxidase activity is used in the diagnosis of acute myeloid leukaemia to show that the leukemic cells were derived from the myeloid lineage [15]. Results from this study did not show positive staining for myeloperoxidase binding sites for both male and female SDRs. This suggests the absence of acute myeloid leukaemia in the test groups.

CONCLUSIONS

The acute toxicity studies involving a single dose administration of MD and its component, DS and AM, in male and female SDRs over a period of 14 days gave a lethal median dose (LD_{50}) for each plant preparation to be above 5000 mg/kg, suggesting that there might be no adverse side effects at the therapeutic dose.

Chronic toxicity studies on the plant preparations in both sexes of SDRs suggests that the preparations may not adversely affect the functions of organs like the liver, kidneys, spleen, lungs and heart and induce liver enzymes. They further suggest that they do not affect feeding and drinking habits and consequently the body weight. Exposure did not adversely affect haematopoiesis or cause acute myelolytic leukaemia. It can thus, be concluded that chronic administration of Mist Diodia (MD) preparation and its component extracts *D. scandens* (DS) and *A. melegueta* (AM) in SDRs, does not cause any deleterious effects.

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