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Acute and Subacute Toxicological Assessment of the Leaf Aqueous Extract of *Eremomastax speciosa* (Acanthaceae) in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Authors GTS, ABD and PVT designed the study and wrote the protocol. Authors GTS, APA and CM managed the biochemical analysis, author GEEO conducted the analysis of histological sections, authors GTS and PVT did the literature search and statistical analysis, author GTS wrote the first draft, authors GEE, ABD and PVT supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was carried out to investigate the possible toxic effects of the leaf aqueous extract from *Eremomastax speciosa* and also to evaluate the acceptable safety level of this extract in Wistar rats.
Place and Duration of Study: Department of Animal Biology and Physiology, Faculty of Sciences of the University of Yaounde I, between September and December 2013.
Methodology: The acute assay used 9 female rats distributed into 3 groups of 3 rats each. A control group received distilled water and the two test groups received by oral route a unique dose of the extract at 2000 mg/kg with 48 hours interval. In the subacute assay, 60 rats of both sexes were distributed into 6 groups of 10 rats each (5 males and 5 females) and received the extract by oral route for 28 days consecutively. The tests groups received the extract at doses of 400, 800 and 1600 mg/kg. The controls and satellite test groups received, respectively, distilled water and extract at the dose of 1600 mg/kg. Anthropometric, hematological and biochemical parameters were measured and histological sections of liver, kidneys and lungs were realized.
Results: The results showed no signs of toxicity such as general behavior change, mortality or change in gross appearance of internal organs even at high dose (2 g/kg). In subacute toxicity assay few modifications were observed in hematological and biochemical parameters. Histopathology showed the presence of disturbances at the dose of 800 and 1600 mg/kg particularly in the females.
Conclusion: The aqueous extract of the leaves of *E. speciosa* could be moderately toxic at high doses and adequate caution should be exercised in its use in ethnomedicine.

Keywords: E. speciosa; acute toxicity; subacute toxicity; Wistar rats.

1. INTRODUCTION

The use of traditional and complementary medicine is becoming widely popular both in the developing as well as in the developed nations. The increasing cost, non-availability of modern drugs and limited access to adequate health care have compelled about 80% of the world population to use traditional pharmacopoeia for primary health care [1]. Since the mid-nineteenth century, serious efforts have been made to isolate and purify the active principles of these remedies. Thus a large variety of biologically-active compounds have been obtained and their structures determined e.g. morphine from the opium plant, cocaine from coca leaves, and quinine from the bark of the cinchona tree [2]. Some of the most common practices involve the use of crude plant extracts which may contain a broad diversity of molecules with often unknown biological effects [3]. Besides this, the fact that a drug has a natural origin does not assure its innocuousness. Therefore, a pre-clinical toxicity study is indispensible to validate their safe medicinal use [4].

Eremomastax speciosa (Hochst.) Cufod. (Acanthaceae) is a plant widely distributed in tropical Africa and is one of the two species of the genus *Eremomastax* (syn. *Paulo wilhelmia* (Lindau); and *Ruellia* (S. Moore)). It is a robust, polymorphous shrub that grows to 2 m high and has a characteristic quadrangular stem and violate underside of the leaves which has earned for it the local name *Pang nyemshe*, meaning 'red on one side' in the Bamileke region of Cameroon [5]. This plant is commonly used in Cameroonian ethnomedicine for the treatment of various stomach complaints and information from tradipractitioners suggested that it possesses antiulcer effects. The antidiarrhoeic activity of *E. speciosa* leaf aqueous extract has been reported [6]. The leaf extract is used for the treatment of male infertility among the *Ifa Nkari* people of Akwa Ibom State, Nigeria (where it is known commonly as "golden seal"; "African blood tonic plant"; local name, *Edem Ididout, Ndana-edem*) [7]. Its widely-claimed anti-anemic activity has been experimentally demonstrated by authors [8] who also showed anti-microbial actions against pure clinical cultures of *Staphylococcus aureus*, *Eschericha coli*, *Candida albicans* and *Aspergillus niger.* The Douala people of Cameroon employ *E. speciosa* variously for malaria, kidney pain, scabies, anemia, diabetes, and nerve pain [9]. *E. speciosa* has been cited for its local use in the treatment of female infertility in the west region of Cameroon [10], as well as for its use in the treatment of irregular menstruation by the Aguambu-Bamumbu peoples of the Lebialem highlands in the South West Region of Cameroon [11]. The plant has also been cited [12] for the treatment of

appendicitis, menstrual pains, gonorrhea, burns, as an anti-poison, and to increase and purify blood in the mount Cameroon region. Phytochemical screening of the water extract of *E. speciosa* revealed the presence of tannins, alkaloids, resins, flavonoids, anthocyanins, phenols, quinones, oils, sterols, triterpenoids, glycosides and proteins [13].

In spite of the wide ethnotherapeutic applications of the plant, there is no literature information related to the safety limits of *Eremomastax speciosa* aqueous extract in traditional medicine. Thus, in the present study, we evaluated the oral acute and subacute toxicity of the aqueous extract of the leaves of *E. speciosa* in rats.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

The leaves of *E. speciosa* plant were collected in May 2013 in Yaoundé (Centre Region of Cameroon) and identified botanically by Paul Mezili of the Cameroon National Herbarium (by comparison with existing voucher specimen No. HNC/136984).

2.1.2 Animal

Adult female Wistar rats (140-150 g) were used for acute toxicity and young Wistar rats of both genders (85-90 g) were used for subacute toxicity. The female rats were nulliparous and non-pregnant. The animals were raised in the Animal house of the Animal Physiology Laboratory, Faculty of Science, University of Yaounde I. They were fed with a standard laboratory diet and tap water *ad libitum*. Each cage contained 3 to 5 rats of the same sex with a bedding of wood shavings. Natural day/night cycles were provided. Environmental conditions (a temperature of 26ºC±2ºC and a relative humidity of 60%±10%) were maintained. Prior authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethics Committee (Reg. No. FWA-IRB00001954). The use, handling and care of animals were done in adherence to the European Convention (Strasbourg, 18.III.1986) for the protection of vertebrate animals used for experimental and other purposes (ETS-123), with particular attention to Part III, articles 7, 8 and 9.

2.2 Methods

2.2.1 Preparation of plant extract

The leaves were chopped and quickly dried in the shade to avoid them getting moldy and then ground in a mechanical grinder to obtain a fine powder. Eight hundred grams (800 g) of powder were extracted by infusion in 5 liters of boiled distilled water for 15 minutes. After filtration through Whatman filter paper No. 3, the filtrate was evaporated at 40ºC using a Raven convection air oven (Jencons-PLS, UK). The brownish solid obtained (150.8 g (18.85% yield)) was stored at 4ºC for subsequent experiments.

2.2.2 Acute toxicity

Acute toxicity assay was carried out according to the Organization of Economic Co-operation and Development (OECD) guideline No. 423 for testing of chemicals [14]. The overnight fasted (water *ad libitum*) female rats were divided into 3 groups of 3 animals each. Animals in the control group received distilled water. The first test group received, by oral route, a unique dose of the extract at 2000 mg/kg and the second test group (the confirmation group), received the same dose of extract 48 hours later. Neither food nor water was given up to 4 h after extract administration and the animals were observed closely during this time for any toxicity manifestations. Body weight change, signs of toxicity, behavior and mortality were observed for the initial 24 hours after extract administration and once daily for 14 days.

2.2.3 Subacute toxicity

The repeated doses (28 days) procedure for oral toxicity study was carried out in rats according to the OECD test guideline No. 407 [15]. Sixty rats of both sexes were distributed into 6 groups of 10 rats each (5 males and 5 females) as follows: Group I (Control; distilled water), Groups II, III and IV (extract: 400, 800 and 1600 mg/kg, respectively). They received vehicle or extract daily for 28 days. The remaining 20 rats were distributed into 2 satellite groups (distilled water, 1600 mg/kg extract) that were equally treated for 28 days, but were allowed an extra 2 week period of observation after treatment withdrawal. Body weight, food and water intake were measured daily. The animals were observed daily for any death and abnormal clinical signs during the entire study period. At the end of the treatment period, 40 animals (Control I and Groups III, IV & V) were fasted overnight (water

ad libitum). On day 29, the animals were weighed, and sacrificed (one at a time) using an overdose of ether. Each rat was opened up surgically and blood samples were drawn by cardiac puncture. Blood was collected into tubes with and without ethylene diamine tetra acetic acid (EDTA) for hematological and biochemical analysis, respectively. The internal organs such as liver, kidneys, stomach, spleen, lungs, heart and testis/ovaries were excised, prepared for gross pathology and weighed (paired organs were weighed together) to determine relative organs weights. Liver, kidney and lung tissue samples were rinsed in 0.9% saline and preserved in 10% neutral buffered formaldehyde solution for histopathological examination. Fourteen days later, the 20 remaining animals (Control II & Group VI) were sacrificed and submitted to the same procedures as above in order to observe reversibility, persistence or delayed occurrence of toxic effects.

2.2.3.1 Hematological parameters

Blood samples were collected into sample tubes containing EDTA. The tubes were shaken gently to mix up the blood with EDTA and prevent clotting. Red blood cell count (RBC), white blood cell count (WBC), differential leukocyte count (lymphocyte, monocyte, granulocyte), platelets, hemoglobin (HGB), hematocrit (HCT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) were determined using an automatic analyzer (Hospitex Diagnostics Hema Screen 18).

2.2.3.2 Biochemical parameters

Blood samples collected without anticoagulant were immediately centrifuged at 3400 rpm for 10 minutes to obtain serum for analysis of biochemical parameters. The serum was carefully aspirated with a Pasteur pipette into sample bottles for the various biochemical assays. Serum contents of alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), triglyceride, cholesterol (Chol) (total, HDL and LDL), creatinine, urea, total bilirubin and total protein were evaluated using standard analytical kits obtained from Fortress Diagnostics Ltd, UK. Atherogenic index was calculated as described by authors [16].

2.2.3.3 Histopathological examination

Tissue samples of liver, kidneys and lungs preserved in 10% neutral buffered formaldehyde solution were dehydrated using upgraded ethanol series and embedded in paraffin blocks. Ultra-thin sections (5 µm) were de-waxed by xylene, rehydrated through a degraded ethanol series and stained with hematoxylin and eosin (H&E). A pathologist (Author GEE) performed the histopathological examination with an optical microscope and microphotographs of the sections were recorded.

2.2.4 Statistical analysis

Statistical analysis was done by one-way analysis of variance (ANOVA) followed by the Dunnett's test for multiple comparisons and p values less than 0.05 were considered as significant. The results are expressed as mean ± standard error of mean (SEM).

3. RESULTS

3.1 Acute Toxicity

The limit dose of 2000 mg/kg did not cause death or any toxic signs in treated female rats. All six rats were normal throughout the study and survived until the end of the 14-day experimental period. No behavioral changes such as tremor, convulsion, self-mutilation, salivation, lethargy or sleep were observed during the first four hours of *E. speciosa* extract administration. No changes in feces and body coat condition and reactivity to noise and touch were observed. The eating habits (26.13-45.63 g/animal/day) and drinking habits (13.00-18.66 ml/animal/day) of all the animals (Tables 1 & 2) remained normal, and there were no significant differences in body and organ weights of rats treated with the extract compared with the controls. Macroscopic examination did not reveal any changes in organ condition.

3.2 Subacute Toxicity

No deaths, unusual changes in behavior or in locomotor activity were recorded and no signs of intoxication were observed during the 28-day treatment period. Significant differences (in males) were found in growth rate between the satellite control group and the animals treated with the highest dose of the extract (Table 3). The relative organ weights showed no significant differences between treated and control groups (Table 4). The macroscopic observation of the target organs (liver, lung, heart, spleen, kidney and sex organs) of the treated animals did not show significant changes in color and texture when compared with the controls.

Hematological parameters; RBC, HGB, MCHC, MCH and MCV in both control and experimental rats, were not significantly different. However, in male rats, platelet counts significantly decreased from 810±143 10^3/µL in the controls to 632±45 10^3/µL at the dose of 800 mg/kg. WBC counts of the extract-treated groups increased in the females (9.57±0.25 vs 24.02±6.78 10^3/µL) and decreased in the males (14.50±0.98 vs 7.90±2.06 10^3/µL) compared with the controls (Table 5).

The results of biochemical parameters in rats treated with various doses of the aqueous extract of *E. speciosa* for 28 days are shown in Table 6. In the extract-treated male rats, none of the parameters was significantly different compared with the controls. But in female rats, ASAT significantly increased from 112.70±37.32 u/l in the controls to 211.84±48.48 U/L at the doses of 800 and 1600 mg/kg while total bilirubin values increased from 2.08±0.49 mg/dl to 5.21±1.10 mg/dl for the same extract doses compared with the 28 day controls.

Histology of the liver sections of control rats showed normal hepatic architecture and normal liver lobular structure with portal triad, prominent nucleus and well-preserved cytoplasm. Hepatic cell damage was not perceptible for the lower dose (400 mg/kg) but at the higher doses (800 and 1600 mg/kg), the liver sections showed histological disturbances characterized by localized inflammation, cell degeneration, vascular congestion and biliary stasis (Figs. 1 & 2).

Histopathological examination of kidney sections showed generalized tubular epithelial cell necrosis associated with reduction of glomerular space and scattered inflammation at the dose of 1600 mg/kg. At the lower doses, cellular architecture was not different when compared with controls (Figs. 1 & 2).

Histology of the lung sections of control rats and of all the extract-treated groups revealed a normal architecture with bronchioles, thin-walled alveoli and alveolar sacs. However, at the dose of 1600 mg/kg, histopathological examination showed the presence of very developed alveoli in both sexes (Figs. 1 & 2).

4. DISCUSSION

The results of the acute toxicity study indicated that the water extract of *E. speciosa* administered by oral route at the dose of 2000 mg/kg did not produce death or any sign of toxicity in the rats indicating that the LD50 of the extract is greater than 2000 mg/kg of body weight. No signs of writhing and body weakness were observed following acute intake as reported by authors [8] who administered the extract of *E. speciosa* by intraperitoneal route. There were no significant differences in organ and body weights in the extract-treated groups compared with the control. In line with the chemical labeling and classification of acute systemic toxicity, the water extract of *E. speciosa* can be assigned to the lowest toxicity class (class 5; no label; unclassified) [14].

Acute toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses. Hence multiple dose studies are almost always essential in evaluating the safety profile of phytomedicines. Thus, subacute toxicity study was carried out for evaluation of long-term effects of the water extract of *E. speciosa*. The study revealed that no adverse clinical sign or toxicity sign or death was observed throughout the treatment duration of 28 days in the rats. This is in line with the acute toxicity studies where experimental animals treated orally with the extract doses up to 2000 mg/kg body weight showed neither toxicity sign nor death in mice. This may be an indication that long-term oral administration of the extract within this dose range may be safe.

Table 1. Effects of the extract on the food intake (g/animal/day) in acute assay

	Day 1	Day 2-5	Day 6-9	Day 10-13	Day 14	Day 1-14
Control	36.30	41.29	42.86	42.60	38.26	40.26
Extract	31.30	31.76	39.10	39.35	33.73	35.05

Table 2. Effects of the extract on the water intake (ml/animal/day) in acute assay

	Day 1	Day 2-5	Day 6-9	Day 10-13	Day 14	Day 1-14
Control	17.33	14.25	14.67	15.83	13.00	15.01
Extract	16.66	17.25	16.00	17.50	18.00	17.08

Table 3. Effects of the extract on the body weight (g) in subacute assay

	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
Male							
Control (dH_2O)	84.4±3.6	103.6±4.8	128.4±5.9	143.1±10.2	157.2±6.9	-	-
Extract (400 mg/kg)	78.6±3.3	96.4±4.3	112.6±6.3	130.8±4.6	150.7±6.4	-	-
Extract (800 mg/kg)	81.2±4.2	104.4±4.7	129.5±8.2	146.0±7.3	160.9±7.9	-	-
Extract (1600 mg/kg)	83.9±4.8	99.7±3.9	121.9±7.9	141.4±9.2	157.1±6.4	-	-
Satellite (dH_2O)	85.1±3.8	103.9±4.2	118.7±6.9	128.3±8.4	148.8±4.7	164.6±10.4	166.8±10.1
Sat (Extract 1600 mg/kg)	89.7±5.9	111.1±4.6	138.3±5.7*	155.2±8.3*	174.5±6.5*	193.4±8.7*	180.2±7.9*
Female							
Control (dH_2O)	80.3±3.9	99.4±5.4	118.8±8.23	133.2±6.3	142.3±8.4	-	-
Extract (400 mg/kg)	80.7±4.8	99.3±6.4	116.2±8.2	128.0±5.7	137.1±7.9	-	-
Extract (800 mg/kg)	82.2±5.2	99.2±4.6	116.0±7.3	127.4±7.4	139.0±6.8	-	-
Extract (1600 mg/kg)	82.8±4.2	99.7±4.6	115.8±6.6	127.1±3.6	138.1±10.5	-	-
Satellite (dH_2O)	83.1±4.8	101.8±8.6	117.4±5.8	124.9±7.3	129.8±6.5	134.4±6.5	148.6±9.9
Sat (Extract 1600 mg/kg)	81.0±4.3	101.5±6.9	106.4±7.4	117.9±6.5	126.7±8.1	129.9±6.4	140.3±7.5

*Values are expressed as mean ± S.E.M. (n=5), *p<0.05; statistically significant compared to control II. Sat=Satellite*

Table 4. Effects of the extract on the relative organ weights (g/100 g of b.w.) in subacute assay

	Heart	Lungs	Kidneys	Liver	Stomach	Spleen	Testis/Ovaries
Male							
Control (dH_2O)	0.336±0.02	0.811±0.04	0.678±0.05	3.593±0.07	0.728±0.06	0.285±0.02	1.203±0.09
Extract (400 mg/kg)	0.317±0.02	0.619±0.04	0.663±0.06	3.465±0.07	0.727±0.04	0.27±0.03	1.218±0.08
Extract (800 mg/kg)	0.311±0.03	0.625±0.03	0.636±0.05	3.442±0.05	0.685±0.06	0.283±0.03	1.222±0.03
Extract (1600 mg/kg)	0.308±0.02	0.623±0.03	0.615±0.04	3.323±0.06	0.637±0.05	0.317±0.02	1.377±0.06
Satellite (dH_2O)	0.367±0.04	0.601±0.03	0.654±0.04	3.642±0.08	0.794±0.07	0.262±0.03	1.26±0.08
Sat (Extract 1600 mg/kg)	0.296±0.01	0.719±0.04	0.591±0.04	3.231±0.07	0.701±0.08	0.266±0.03	1.329±0.07
Female							
Control (dH_2O)	0.362±0.03	0.735±0.08	0.712±0.07	3.96±0.11	0.666±0.08	0.338±0.03	0.06±0.06
Extract (400 mg/kg)	0.333±0.03	0.668±0.06	0.691±0.07	3.797±0.13	0.677±0.06	0.306±0.02	0.072±0.005
Extract (800 mg/kg)	0.368±0.04	0.847±0.09	0.671±0.03	3.493±0.13	0.727±0.8	0.264±0.01	0.075±0.007
Extract (1600 mg/kg)	0.354±0.03	0.723±0.07	0.698±0.04	3.34±0.10	0.778±0.09	0.284±0.03	0.071±0.007
Satellite (dH_2O)	0.382±0.02	0.684±0.06	0.682±0.05	3.796±0.10	0.675±0.07	0.304±0.02	0.071±0.005
Sat (Extract 1600 mg/kg)	0.348±0.02	0.771±0.08	0.681±0.08	3.042±0.06	0.786±0.06	0.315±0.04	0.081±0.008

Values are expressed as mean ± S.E.M. (n=5) Sat=Satellite

Table 5. Effects of the extract on hematological parameters in subacute toxicity

	Control (dH_2O)	Extract (400 mg/kg)	Extract (800 mg/kg)	Extract (1600 mg/kg)	Satellite (dH_2O)	Satellite (extract 1600 mg/kg)
Male						
RBC (10^6/µl)	6.40±0.33	7.30±0.12	6.15±0.84	7.12±0.24	6.60±1.37	6.56±0.32
WBC (10^3/µl)	14.50±0.98	10.47±1.98	9.82±2.17	7.90±2.06*	14.27±0.66	15.52±0.69
Lymphocyte (%)	53.55±4.54	69.20±4.04*	65.60±2.10	68.73±2.57	64.12±6.13	72.10±1.02
Monocyte (%)	15.20±0.52	18.40±2.27	19.77±1.08	19.02±1.19	18.97±2.60	13.52±1.73
Granulocyte (%)	31.25±5.07	12.40±1.78**	14.60±1.91**	11.32±0.90**	16.90±3.70	14.37±2.51
Platelets (10^3/µl)	810±143	632±45	561±120*	739±119	611±86	521±162
HGB (g/dl)	10.92±0.69	11.82±0.43	10.85±0.67	11.47±0.47	15.92±3.59	11.60±1.44
HCT (%)	34.30±1.79	38.47±0.40	34.32±2.34	37.92±1.29	36.85±7.26	33.25±1.79
MCHC (g/dl)	31.77±0.56	30.72±0.80	31.82±2.06	30.30±0.75	35.70±5.96	33.10±4.36
MCH (pg)	17.05±0.69	16.20±0.43	18.70±2.79	16.12±0.47	21.82±1.89	21.55±2.15
MCV (fl)	53.50±1.25	52.75±0.47	58.00±5.74	53.00±0.40	55.50±5.90	50.75±1.65
Female						
RBC (10^6/µl)	6.57±0.43	6.65±0.34	7.27±0.39	6.72±0.15	7.18±0.26	6.09±0.27
WBC (10^3/µl)	9.57±0.25	24.02±6.78**	14.35±2.49	23.80±5.72**	8.92±0.62	18.50±2.33#
Lymphocyte (%)	67.97±3.03	67.12±2.42	64.32±0.89	60.55±6.86	77.00±2.43	65.20±8.69
Monocyte (%)	17.25±0.98	15.40±0.34	16.90±1.17	15.32±1.17	13.47±0.89	12.20±1.36
Granulocyte (%)	14.77±2.18	17.42±2.22	18.75±2.05	24.07±1.18*	10.80±1.74	18.25±5.14#
Platelets (10^3/µl)	661±36	498±81	599±103	641±42	656±30	590±50
HGB (g/dl)	10.75±0.73	11.00±0.68	11.42±0.39	11.10±0.31	12.10±0.51	11.72±0.21
HCT (%)	34.20±2.71	33.82±1.65	38.32±2.66	35.07±0.54	35.15±1.48	31.15±0.75
MCHC (g/dl)	31.55±1.11	32.35±0.57	30.10±1.53	31.65±0.49	34.42±0.37	37.65±1.14
MCH (pg)	16.37±0.71	16.25±0.54	15.77±0.65	16.50±0.23	16.85±0.38	19.32±0.70
MCV (fl)	51.75±1.03	51.00±1.00	52.25±1.10	52.00±0.91	48.75±0.75	51.25±1.43

*Values are expressed as mean ± S.E.M. (n=5), *p<0.05; **p<0.01: statistically significant compared to control I. #p<0.05: statistically significant compared to control II. dH_2O=distilled water*

Table 6. Effects of the extract on biochemical parameters in subacute toxicity

	Control (dH_2O)	Extract (400 mg/kg)	Extract (800 mg/kg)	Extract (1600 mg/kg)	Satellite (dH_2O)	Satellite (extract 1600 mg/kg)
Male						
ALAT (U/l)	63.60±9.54	58.65±12.15	47.65±7.66	52.25±14.29	53.72±12.4	67.45±9.71
ASAT (U/l)	117.33±24.84	143.54±36.43	129.21±23.89	133.70±37.90	116.52±18.76	155.23±21.02
Total Chol (mg/dl)	117.76±10.42	129.00±18.21	118.40±6.59	135.52±8.83	115.25±4.99	132.68±15.02
Triglyceride (mg/dl)	92.81±20.78	116.01±48.13	114.55±25.82	84.07±15.74	83.99±9.02	123.00±33.04
HDL-Chol (mg/dl)	66.36±2.78	76.96±11.31	52.11±6.85	72.41±15.41	73.03±9.05	85.45±13.91
LDL-Chol (mg/dl)	32.83±6.43	28.83±5.85	43.38±7.99	46.30±7.32	25.42±4.23	22.63±4.05
Atherogenic index	1.77±0.21	1.68±0.13	2.27±0.44	1.87±0.35	1.58±0.27	1.55±0.31
Creatinine (mg/dl)	0.71±0.13	0.62±0.10	0.52±0.07	0.57±0.08	0.52±0.05	0.47±0.08
Urea (mg/dl)	51.60±6.40	47.25±17.09	67.40±7.01	38.50±2.50	49.00±12.97	43.00±6.68
Total bilirubin (mg/dl)	2.57±0.41	2.85±0.89	3.08±0.36	3.18±0.26	2.66±0.50	3.84±0.50
Total protein (mg/dl)	7.08±1.12	9.34±2.05	7.07±1.08	5.85±1.33	6.57±1.12	8.18±2.03
Female						
ALAT (U/l)	69,95±12.71	69.12±8.02	58.52±13.68	61.00±10.66	63.67±7.21	50.50±16.18
ASAT (U/l)	112.70±37.32	134.49±24.64	209.50±29.11*	211.84±48.48*	137.00±23.62	193.25±37.07
Total Chol (mg/dl)	111.00±15.28	122.56±10.82	138.56±6.73	126.00±4.94	119.31±10.89	131.60±5.43
Triglyceride (mg/dl)	102.90±76.64	113.80±30.68	95.52±25.71	127.18±61.37	71.34±9.37	100.96±25.00
HDL-Chol (mg/dl)	66.96±10.65	79.8±16.08	85.15±12.08	81.26±18.21	84.84±8.02	76.96±7.64
LDL-Chol (mg/dl)	23.46±4.22	20.72±3.87	34.31±6.35	19.30±3.45	20.20±3.98	34.45±5.18
Atherogenic index	1.66±0.32	1.55±0.27	1.63±0.30	1.55±0.28	1.41±0.22	1.71±0.34
Creatinine (mg/dl)	0.48±0.07	0.59±0.11	0.44±0.06	0.34±0.05	0.58±0.08	0.39±0.07
Urea (mg/dl)	47.00±13.52	61.50±11.38	60.00±10.67	62.50±9.67	55.25±14.33	44.00±14.60
Total bilirubin (mg/dl)	2.08±0.49	4.27±0.96*	5.21±1.10*	4.84±1.79*	2.76±0.07	3.49±1.03
Total protein (mg/dl)	8.12±1.78	8.15±1.55	8.40±2.07	8.45±1.98	7.43±1.32	8.51±1.77

*Values are expressed as mean ± S.E.M. (n=5), *p<0.05: statistically significant compared to control 1. dH_2O=distilled water*

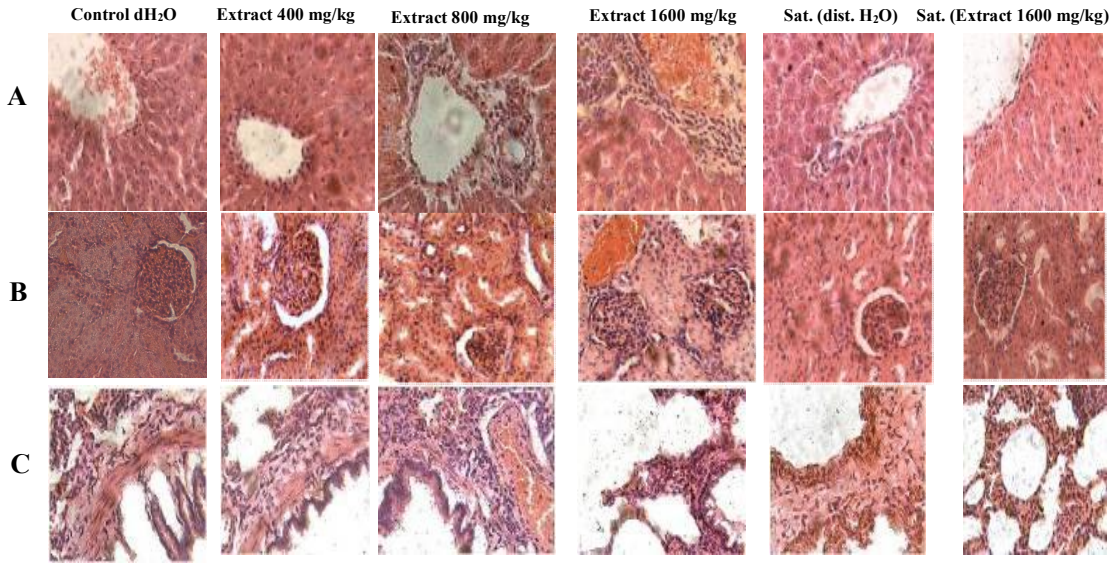

Fig. 1. Effects of the extract on histology of liver (A), kidneys (B) and lungs (C) in female rats (H&E × 400). Sat=Satellite

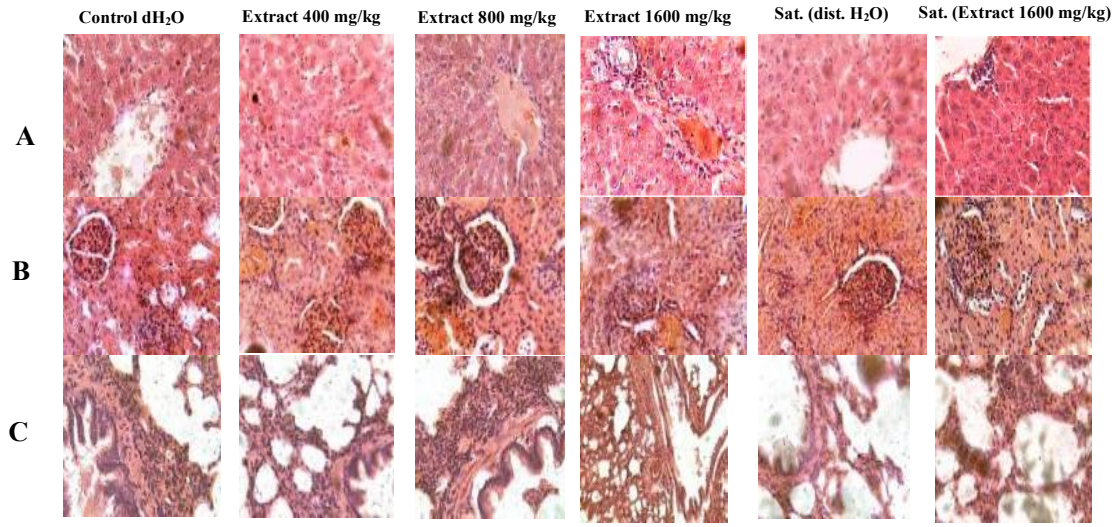

Fig. 2. Effects of the extract on histology of liver (A), kidneys (B) and lungs (C) in male rats (H&E × 400) Sat=Satellite

Changes in body weight (especially weight loss) have been used as an indicator of adverse effects of drugs and chemicals [17]. Significant differences in body weight gain were noted only in the male satellite controls (1600 mg/kg extract) which gained more body weight from weeks 2 to 6 compared with the untreated (distilled water) satellite controls. Since a similar result was not obtained for the extract-treated male rats (1600 mg/kg) that were sacrificed on day 28, it cannot be attributed to an effect of the extract. Changes in organ weights are also indices of toxicity in animals which are readily determined in long-term toxicity tests. There is a possibility that herbal products, when ingested into the body may be toxic to important organs such as the kidneys, liver, spleen, stomach, and lungs because of their diverse roles in the human body. Macroscopic observation of the major organs showed no abnormalities in morphology, consistency and appearance in the rats treated for 28 days with the extract.

The hematopoietic system is one of the most sensitive targets of toxic compounds and provides important indices of physiological and pathological status in man and animals [18]. Blood constitutes the main medium of transport for many drugs and xenobiotics in the body. For this reason blood components such as red blood cells, white blood cells and blood platelets are often exposed to significant concentrations of toxic compounds. Blood parameter analysis in rodents can provide a high predictive index (up to 91% concordance) for risk of toxicity in humans [19]. Our results showed no significant difference in hematological parameters between controls and treated groups except for the sex-related differential variation of white blood cell count which could be attributed to the presence of compounds in the extract which interact with sexual hormones to maximize their effects on immune response [20]. Indeed some flavonoids such as quercetin can induce opposing actions on white blood cell counts depending on their concentrations and/or different conditions [21]. By the way, our results did not show any concordance with those obtained by authors [8] who demonstrated the anti-anemic actions of *E. speciosa*. This could be due to differences in phytochemical composition between the two extracts. The significant decrease of platelet counts in male rats at the dose of 800 mg/kg may lead to clinical implications such as high risk of hemorrhage due to thrombocytopenia. Clinical symptoms of thrombocytopenia can include prolonged bleeding from cuts, mouse or nose bleeds, blood in urine or stools, unusually heavy menstrual flows, and fatigue. Factors that cause thrombocytopenia include low platelet production by the bone marrow, rapid destruction of platelets by the body (due to autoimmune diseases, medicines or infections), and high platelet retention by enlarged spleen (as in severe liver disease such as cirrhosis). Some medicines such as diuretics, ibuprofen, aspirin and chloramphenicol can slow the production of platelets [22]. The significant reductions in blood platelet counts and platelet aggregation reported in rats given, respectively, aqueous extracts of *Ocimum gratissimum* [23] and *Ocimum basilicum* [24] were attributed to the presence of saponins and cardiac glycosides. The presence of glycosides (but not saponins), has been reported in the aqueous extract of *E. speciosa* [13], and this may be responsible for the decrease in blood platelets observed in this study. The decrease of WBC counts and its differentials in the males such as basophils, monocytes, eosinophils, lymphocytes and neutrophils could be linked to suppression of leucocytosis in the bone marrow which may induce poor defensive mechanisms against infection; consequentially, they might have effects on the immune system and phagocytic activity of the animals [25,26]. On the other hand, increases in WBC counts can boost the immune system and defensive mechanisms against infection.

Serum biochemical analyses were carried out to evaluate the effect of the extract on hepatic and renal functions and also on lipid profile. Many xenobiotics are capable of causing some degree of liver injury [27]. The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation and its anatomical and physiological structure. Generally, analysis of the activities of some basic liver enzymes (such as ALAT and ASAT) in the plasma or serum can be used to indirectly assess the integrity of tissues after being exposed to certain pharmacological agents [28]. Necrosis or membrane damage releases the enzymes into circulation; therefore, they can be measured in the serum. Usually, about 80% of ASAT is found in the mitochondria whereas ALAT is a purely cytosolic enzyme. Therefore, ASAT appears in higher concentrations in a number of tissues (liver, kidneys, heart and pancreas) and is released slowly in comparison to ALAT. But since ALAT is localized primarily in the cytosol of hepatocytes, this enzyme is considered a more sensitive marker of liver inflammation or damage than ASAT and within limits can provide a quantitative assessment of the degree of damage sustained by the liver [29]. The significant increase of ASAT levels in the female rats at the doses of 800 and 1600 mg/kg could therefore be an indication of hepatocellular changes and/or histological changes in organs such as kidneys, heart and pancreas induced by the extract. Bilirubin is formed by the breakdown of hemoglobin in the liver, spleen and bone marrow. Levels of serum bilirubin reflect the liver's ability to take up, process, and secrete bilirubin into the bile. An increase in tissue or serum bilirubin concentrations occurs as a result of increased breakdown of RBC (hemolysis) or liver damage e.g., hepatitis or bile duct obstruction [30]. The high levels of serum bilirubin concentration observed in the female rats at all doses of the extract used in this study are indicative of possible adverse effects of the extract on hemoglobin metabolism pathways or bile duct obstruction. Clinically, this could lead to the complications such as the development of

gallstones either in the gall bladder or in bile ducts. The observed increases in bilirubin levels with little or no increase in ALAT levels in this study are clinical indications of cholestasis [31]. In fact, histology of the liver revealed the presence of inflammatory cells, vascular congestion and biliary stasis at the doses of 800 and 1600 mg/kg of extract. However, ASAT and total bilirubin levels in the satellite control animals left in observation for 14 days tended to revert back towards initial control values. This suggests that any hepatocellular damage induced by *E. speciosa* could be temporary and reversible after medium term intake.

Estimation of total protein is one of the most widely used means of measuring hepatocellular injury. Total protein measurements can reflect nutritional status and may be used to screen for and help diagnose kidney disease, liver disease, and many other conditions. Low total protein levels can suggest a liver disorder, a kidney disorder, or a disorder in which protein is not digested or absorbed properly. High total protein levels may be seen with chronic inflammation or liver infections. Total cholesterol test is used to estimate risk of developing a disease (specifically heart disease) and some liver dysfunctions. Increase in the total protein and cholesterol as well would have indicated hepatocyte damage [32].There were no significant changes in any liver function parameters (such as total cholesterol, total protein) and in serum lipid profile (triglyceride, HDL, LDL cholesterol) as compared to the control groups. All these results suggest the absence of major hepatotoxicity and cardiovascular risk factors induced by *E. speciosa*.

The kidneys are highly susceptible to toxicants for two reasons; a high volume of blood flows through it and its ability to filter large amounts of toxins which can concentrate in the kidney tubules. It can result in systemic toxicity causing decreased ability to excrete body wastes, inability to maintain body fluid and electrolyte balance and decreased synthesis of essential hormones. Blood urea nitrogen is derived in the liver protein/amino acid from dietary or tissue sources and is normally excreted in the urine. In renal disease, serum urea accumulates because the rate of serum urea production exceeds the rate of clearance [33]. Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatine breakdown. The plasma creatinine concentrations in normal individuals are usually affected by a number of factors such as the muscle mass, high protein diet and catabolic state, thus serum urea concentration is often considered the more reliable renal function predictor than serum creatinine [29]. There were no significant changes in the levels of serum creatinine and urea in the treated groups compared with the controls. However, histopathological examination of kidney sections showed that high doses of extract (1600 mg/kg) produced generalized tubular epithelial cell necrosis associated with reduction of glomerular space and scattered inflammation. In toxic injury, renal epithelial cell necrosis is accompanied by massive tissue damage leading to rapid collapse of internal homeostasis of the cell characterized by cell swelling, loss of plasma membrane integrity, major changes to organelles, and swelling of the nucleus. The affected cells rupture, spilling cellular contents into the surrounding tissue space evoking an inflammatory response [34]. Glomeruli play a variety of important functions that are critical in maintaining homeostasis. These include plasma ultrafiltration, regulation of blood pressure and tubular metabolism, and removal of macromolecules from circulation. Many drugs can induce glomerular damage by a variety of pathogenic mechanisms which in turn, can lead to a loss of nephron function and significantly affect blood flow to other nephron segments [35]. Rats given high doses of Cimetidine (an H2 antagonist of histamine), developed glomerular lesions associated with proteinuria within 2 to 4 weeks of iv injection, the magnitude of proteinuria being proportional to the administered dose. *E. speciosa* extract has been shown to possess gastric antisecretory activity that is achieved through a mechanism of action which may involve the histaminergic pathway similar to cimetidine [13]. Clinically, the drug and chemically induced lesions may lead to irreversible nephron loss, and in an attempt to compensate for this, the remaining nephrons may undergo cellular and functional hypertrophy as well as increased glomerular filtration and glomerular hypertension. These events can set up a cycle of sclerosis, progressive nephron loss, and renal fibrosis which may lead to renal dysfunction and failure [35].

Histological sections of lungs of the rats treated at the dose of 1600 mg/kg revealed the presence of very developed pulmonary alveoli which could be an indication of emphysema or diffuse alveolar damage. Diffuse alveolar damage can lead to various complications in the body including hypoxia (low oxygen tension),

hypercapnia (increased carbon dioxide build-up), pulmonary hypertension (increased pulmonary artery pressure due to compression of the lung vasculature by the collapsed parenchyma) and cardio respiratory arrest. Diffuse alveolar damage can be arrested and stabilized but it is irreversible [36].

5. CONCLUSION

The water extract of the aerial parts of *E. speciosa* can be considered safe, as it did not cause either lethality or adverse changes in general behavior of rats in acute toxicity study up to the dose of 2000 mg/kg. The high safety margin of the extract through oral route justifies its widespread use by traditional healers. However, since the extract had effects on selected organ histology and on some hematological and biochemical parameters, caution should be exercised in its use especially at high doses. Further investigations (chronic, reproductive, developmental and genetic toxicity studies) need to be done for the complete elucidation of the safety profile of *E. speciosa*.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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