Original Article

Antioxidant and Toxiciy Studies of Two Acaricidal Plants, *Cymbopogon citratus* and *Eucalyptus globulus*

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ABSTRACT

Background: The menace of acaricide resistance, which confronts animal owners with attendant losses, and the issue of safety with commercially available acaricides are enormous, hence the search for alternatives.

Objectives: This study evaluated the safety profile of essential oils (EOs) of two acaricidal plants with proven efficacy against ticks.

Methods: EOs from *Cymbopogon citratus* and *Eucalyptus globulus* were extracted by hydrodistillation, while acute, subacute oral, and dermal toxicities of the plants and their combination were evaluated in vivo using Wistar rats according to a procedure described by the Organization for Economic Cooperation and Development (OECD) guidelines. Thereafter, blood samples were collected for complete blood counts, serum biochemistry, antioxidant properties, and reproductive hormonal profiles, and tissues were harvested for histopathology. Data were analyzed using SPSS software, version 20, and analysis of variance (ANOVA) was conducted, with $P \leq 0.05$ considered statistically significant.

Results: The packed cell volume (PCV) for *C. citratus* (55±1.05%) and *E. globulus* (55.6±0.93%) was higher ($P<0.05$) than that of the control group (47.2±2.50%). Serum creatinine was significantly higher in all the test groups than in the control group ($P<0.05$). Progesterone, luteinizing hormone, and follicle-stimulating hormone levels were significantly

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lower, while estrogen levels were higher in the test groups compared to the control. Histopathology revealed normal hepatocytes, with mild distension of Bowman's capsule in the kidney at doses much higher than those required for acaricidal efficacy.

Conclusion: *C. citratus* and *E. globulus* EO combination has the potential to be developed into an environmentally friendly botanical acaricide.

Keywords: Antioxidant, Safety, Botanical, Acaricide

Introduction

Ticks are important vectors of bacterial, viral, rickettsial, and protozoan diseases of domestic animals and humans (Bartikova et al., 2016). The effects of ticks (and tick-borne diseases) are seen worldwide, especially in tropical and subtropical regions, because of their cosmopolitan distribution. Globally, an annual estimate of between 22 billion USD and 30 billion USD is expended on tick control, in an effort to mitigate the significant economic, veterinary, and public health implications (Lew-Tabor & Valle, 2016). Therefore, searching for effective tick control strategies is crucial.

Currently, in many parts of the world, the most adopted method for tick control is the topical application of synthetic acaricides on the animal or the environment (Agwunobi et al., 2021). The uncontrolled use of these acaricides has led to the evolution of tick populations that have become resistant, particularly those of one-host ticks, such as *Rhipicephalus microplus* and *Rhipicephalus decoloratus*, to these agents (Reck et al., 2014). Furthermore, these chemical acaricides are often characterized by several adverse effects following their use, either directly on the animal, the environment, or humans (Mazlan et al., 2017).

In Africa, acaricide resistance has made chemical tick control a less desirable option (Mvumi et al., 2021). The search for medicinal plants with acaricidal activity and their use for alternative control is strongly advocated (Fouche et al., 2016). In addition, these plants are locally available, affordable, and mostly environmentally friendly, and their use is compatible with traditional practices in Nigeria (Boate & Abalis, 2020).

The interest in using botanical acaricides is on the rise globally, and many plants have been screened so far, demonstrating acaricidal properties (Adenubi et al., 2021). Despite the vast African vegetation and the potential of medicinal plants, there is a dearth of botanical-based acaricidal products available on a commercial scale in the market, largely because their safety is still poorly understood.

Essential oils (EOs) are composed of several volatile compounds produced as secondary metabolites in medicinal plants, exhibiting demonstrated pesticidal, insecticidal, and fungicidal activities (Assadpour et al., 2023). This study, therefore, seeks to evaluate the safety profile of the EOs from two Nigerian plants (*Cymbopogon citratus*, *Eucalyptus globulus*), with documented ethnoveterinary use against ticks, as well as reported acaricidal efficacy (Adenubi et al., 2021; Danna et al., 2023).

Materials and Methods

Plant collection and identification

C. citratus and *E. globulus* were collected from their natural habitat in Wukari, Taraba State. The plants were identified and authenticated by Dr. B. Oche at the Nigeria Natural Medicine Development Agency (NNMDA), Lagos State. Voucher numbers: MNPH/2019/01321 (*C. citratus*), and MNPH/2019/01298b (*E. globulus)* were allocated and deposited at the NNMDA herbarium for reference purposes.

Extraction of EO

Five hundred grams of each plant sample (fresh *C. citratus* and *E. globolus* leaves) and about 4 L of distilled water were put into a round-bottom flask. This flask was attached to a Clevenger-type apparatus and heated for three hours (Elyemni et al., 2019). The volume of EO obtained for each plant sample was weighed, and the percentage yield was estimated as described by Ranitha et al. (2014) (Equation 1):

$$1.\ Yield\ of\ EO = \frac{Amount\ of\ EO(g) obtained}{Amount\ of\ plant\ raw\ materials(g)\ used} x100$$

In vivo toxicity studies

Study animals

Seventy-five apparently healthy Wistar rats of both sexes (160-180 g), obtained from the toxicological unit of NNMDA, Lagos State, were used for this study. Proper housing, adequate feed, and water were made available for the animals, and they were kept at the College of Veterinary Medicine Animal House, Federal University of Agriculture, Abeokuta. Before experimenting, the animals were acclimated for one week (Suriyavadhana & Pakutharivu, 2011).

Acute toxicity test

Acute toxicity tests, as outlined in Organization for Economic Cooperation and Development (OECD) guidelines 420 (OECD, 2001), were conducted to assess the safety of the EOs. The animals were deprived of food for 12 hours before the experiment. Twenty female Wistar rats were randomly assigned to 4 groups of 5 rats each. Group I- *C. citratus*, group II- *E. globulus*, group III- *C. citratus*+ *E. globulus* EO combination, and group IV- 2 mL of distilled water (control). One animal from each treatment group was administered 2000 mg/kg of the EO orally. After two days, the same dose was administered once orally to the 4 remaining animals in each group (Lulekal et al., 2019). The animals were observed closely at hourly intervals for the first six hours, and then at 12- and 24-h post-treatment (PT). The animals were observed for 14 days to detect any physical and behavioral changes.

Subacute toxicity test

Subacute toxicity tests were conducted in accordance with OECD guideline 420 (OECD, 2001). Thirty-five male Wistar rats were randomly divided into seven groups of five rats each. *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination were administered orally daily to the rats at the dose of 1000 mg/kg to three groups and 2000 mg/kg to another three groups for 28 days, while the control group received 2 mL of distilled water daily. This experiment was repeated using thirty-five female rats. Animals were observed for behavioral changes as signs of toxicity throughout the experimental period. The feed consumption of each group and their body weights were measured daily and weekly. At the termination of the experiment, 5 mL of blood was collected from the medial canthus of the eyes of the Wistar rats in all groups and transferred to heparinized tubes for hematological studies and non-heparinized centrifuge tubes for serum biochemistry and reproductive hormone profiles. The animals were thereafter sacrificed humanely using cervical decapitation. The abdominal cavity of each animal was excised, and a thorough examination of the viscera was conducted to observe any gross pathological lesions. The liver and kidneys were harvested, weighed, and fixed in formalin for histopathological examination.

Acute dermal toxicity test

The acute dermal toxicity of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination was conducted according to the OECD guidelines 420 (OECD, 2001), where the limit dose of 2000 mg/kg body weight was used. The subjects were deprived of food for 12 hours before commencing the experiment. Twenty female Wistar rats were randomly assigned to 4 groups of 5 rats each. Group I- *C. citratus*, group II- *E. globulus*, group III- *C. citratus* + *E. globulus* EO combination, and group IV- petroleum jelly (control). Twenty-four hours before the study, fur was shaved from the dorsal area of the animals' trunks using a sterilized razor blade (approximately 10% of the body surface area was shaved). Thereafter, 2000 mg/kg of each EO was applied uniformly on the shaved area on one animal from each group (Lulekal et al., 2019). Two days later, the same dose was administered topically to the four remaining animals in each group. Observations were made and recorded at 1, 2, and 4 hours, after which they were observed once a day for 14 days. Changes in skin and fur, mucous membranes, itching, erythema, or other signs of discomfort were also monitored. A portion of each animal's skin was excised and fixed in 10% formalin for histopathological investigation.

Hematological analyses

Hematological parameters, including Packed cell volume (PCV), red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), and differential WBC count (neutrophil, eosinophil, basophil, lymphocyte, and monocyte), were carried out as described by Bain et al. (2016). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated using the Equation 2, 3 and 4:

2. MCV (fL)=(PCV×10)/(RBC count)

3. MCH (pg)=(Hb×10)/(RBC count)

4. MCHC (g/dL)=(Hb×100)/PCV

Biochemical analyses

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, blood urea nitrogen (BUN), albumin, globulin and lipid profile [total cholesterol (TC), triglyceride (TAG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total bilirubin (TBL) and direct bilirubin (DBL)] were assayed by Randox assay kits (Randox® Diagnostics, UK), following procedures as recommended by the manufacturer.

Antioxidant assays

Thiobarbituric acid (TBA)/malondialdehyde (MDA)

MDA, identified as a product of lipid peroxidation, was assayed using the procedures described by Bhutia et al. (2011). A reaction with TBA to give a red color absorbing at 535 nm. The MDA concentration was calculated using the coefficient of extinction (1.56×10^5 m^{-1} cm^{-1}) as follows (Equation 5):

5. MDA concentration=

$$\frac{Absorbance\ of\ sample\ x\ Total\ volume\ x\ 1000}{Extinction\ coefficient\ x\ volume\ of\ sample\ x\ 1\ x\ mg\ of\ tissue\ in\ volume\ of\ sample}$$

Catalase (CAT)

CAT was determined spectrophotometrically as described by Hadwan (2018). Ten microliters of the sample were added to the test, and 10 µL of distilled water were added to the blank. Then, 40 µL of 0.66 M H_2O_2 was added to the test and standard. The mixture was incubated for 3 minutes, after which 200 µL of sulfuric acid (H_2SO_4) and 1 mL of potassium permanganate ($KMnO_4$) were added to all the test tubes. They were mixed by inversion, and the absorbance was recorded at 480 nm.

Superoxide dismutase (SOD)

The method described by Sadia and Irshadullah (2014) was used to determine SOD. Test tubes were labeled as "test" and "blank." Approximately 100 µL of buffer was transferred into the test solution tube, and 150 µL of buffer was transferred into the blank. Then, 830 µL of distilled water was pipetted into all test tubes, and 50 µL of the sample was added to the tube containing the test solution. The tubes were then incubated at room temperature for 10 minutes, and 20 µL of pyrogallol was added. Immediately after, the tubes were mixed by inversion and the differences in absorbance at 340 nm/min were measured thus (Equation 6):

6. Units $=\frac{(\%\ inhibition)}{100-(\%\ inhibition}$

%inhibition=

$$\frac{(\Delta absorbance/min\ of\ blank-\ \Delta absorbance/min\ of\ sample)}{(\Delta absorbance/min\ of\ blank)\times100}$$

Glutathione peroxidase (GPx)

GPx was determined according to the method of Cichoski et al. (2012). The reagent consists of 10 mM sodium azide (NaN_3), 4 mM reduced glutathione (GSH), 2.5 mM H_2O_2, 10% TCA, 0.3 M dihydrogen phosphate (H_2PO_4), 0.4 g/L dithiobis-2-dinitrobenzoic acid (DTNB), and phosphate buffer (pH 7.4). To the blank test tube, 500 µL of buffer, 100 µL of NaN_3, 200 µL of GSH, 100 µL of H_2O_2, and 500 µL of distilled water were added. All were added to the sample test tubes, except for distilled water, in place of which 100 µL of the samples was used. The entire reaction mixture was incubated at 37 ˚C for 3 minutes, after which 500 µL of TCA was added. It was then centrifuged at 3000 rpm for 5 minutes. To 1 mL of each supernatant, 2 mL of H_2PO_4 and 1 mL of DTNB were added. The absorbance was read at 412 nm against the blank. The GPx activity was calculated by plotting the standard curve and expressed in units per milligram of protein.

Hormonal profile assays

Serum levels of testosterone, progesterone, estrogen, follicle stimulating hormone (FSH), and luteinizing hormone (LH), were assayed using their respective enzyme-linked immunosorbent assay (ELISA) kits (Fortress Diagnostic Limited®, Antrim Technology Park, Antrrim, BT41 1Qs, United Kingdom) and read with the ELISA machine (Biotek ELX800®, USA, SN213376) following the manufacturers' instructions.

Histopathology

The liver, lung, kidney, and heart tissues were fixed in 10% neutral buffered formalin (containing 10% formalin in 0.08 M sodium phosphate at pH 7.4) and then dehydrated in graded alcohol. They were embedded in paraffin wax at 60 ˚C. These tissues were sectioned using a microtome at a thickness of 5 µm, and each section was floated in a 45 ˚C water bath to allow the crinkled part to spread before being floated on a glass microscope slide for proper adherence. The slides were stained with hematoxylin and counterstained with eosin using a stan-

dard protocol (Kadir, 2014). Slides were examined using a light microscope (CX21FS1, Philippines).

Data analysis

Data were recorded in Microsoft Excel and subjected to statistical analyses using SPSS Software, version 23.0 (IBM Corp, 2015). Results were presented as Mean±SEM, and values were compared using one-way analysis of variance (ANOVA). A post hoc test was performed using the Tukey HSD test, and a $P \leq 0.05$ was considered statistically significant.

Results

Effects of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination in Wistar rats (toxicity studies)

Single oral treatment of female Wistar rats with 2000 mg/kg body weight of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination did not cause any mortality in the female rats throughout the 14-day duration of the acute toxicity study. In addition, no visible signs of toxicity or behavioral changes were observed in the animals.

The administration of 1000 and 2000 mg/kg of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination also produced no mortality in Wistar rats of both sexes dosed orally daily during the 28 days.

No toxic effect was observed as a result of a single dermal treatment of female Wistar rats with 1000 mg/kg body weight of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination from day 1 to 14 PT. There were no abnormal changes observed on the shaved areas of the dorsum as well. Additionally, no histopathological changes were observed in the skin sections of either the test or control groups (Figure 1).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on hematological parameters of Wistar rats

There was a statistically significant (P=0.00) increase in PCV in rats given 1000 and 2000 mg/kg oral doses of *C. citratus* (55±1.05%, 55.2±1.66%) and *C. citratus* (54.8±1.02%, 55.6±0.93%), compared to the control group (47.2±2.5%). There was no statistically significant difference (P=0.65) in PCV between the groups that received *C. citratus* EO and *E. globulus* EO. Still, there was a statistically significant (P=0.04) increase in PCV between the groups that received *E. globulus* and *C. citratus* EOs alone and the groups that received the EO combination at 1000 and 2000 mg/kg (50.8±1.39% and 46.8±0.73%, respectively) (Table 1).

There was a statistically significant (P=0.03) increase in Hb concentrations in the experimental groups compared to the control, except for the *C. citratus* + *E. globulus* EO combination at 2000 mg/kg (15.64±0.22 g/dL) that was not statistically different from the control (15.78±0.8 g/dL) (P=0.24). There was a statistically significant (P=0.05) increase in RBC count in rats given 1000 and 2000 mg/kg oral doses of *C. citratus* and *E. globulus* compared to the control group. There was also a statistically significant (P=0.01) increase in RBC count at 1000 and 2000 mg/kg oral doses of *C. citratus* ($9.36\pm0.25\times10^{12}$/L) and *E. globulus* ($9.26\pm0.16\times10^{12}$/L) compared with the groups that received the EO combination ($7.9\pm0.16\times10^{12}$/L) (Table 1).

There was no statistically significant difference (P=0.78) in total WBC count among all treated groups and the control. There was a significant (P=0.04) increase in eosinophils in the animals given 1000 mg/kg *C. citratus*, 1000 and 2000 mg/kg *E. globulus*, and the group given *C. citratus* + *E. globulus* EO combination at 1000 mg/kg compared to the control (Table 1).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on serum lipid profile of Wistar rats

The changes in the serum lipid profile are indicated in Table 2. There was a significant increase in TC observed in test groups that were administered oral doses of *E. globulus* EO at 1000 and 2000 mg/kg (79.76±4.98 mg/dL) and 2000 mg/kg of *C. citratus* + *E. globulus* EO combination (69±2.26 mg/dL) compared to the control (59.4±7.01 mg/dL). There was a significant (P=0.00) decrease in HDL in the groups that were administered with 1000 mg/kg *C. citratus* (24.9±4.28 mg/dL) and 1000 mg/kg *C. citratus* + *E. globulus* EO combination (29±2.08 mg/dL) compared to the control (31.48±5.49 mg/dL) and the other test groups. There was, however, no statistically significant difference (P=0.45) in LDL, very low-density lipoprotein (VLDL), and TAG between the test groups and the control (Table 2).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on serum protein profile and liver function of Wistar rats

There were no significant differences (P=0.06) in total serum protein, albumin, and globulin values across the test and control groups. There was a significant (P=0.01) decrease in serum AST value in the test groups that received 2000 mg/kg *C. citratus* EO (59±4 µ/L),

Table 1. Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on hematological parameters of Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
PCV (%)	47.2±2.5^{b}	55±1.05^{a}	55.2±1.66^{a}	54.8±1.02^{a}	55.6±0.93^{a}	50.8±1.39^{b}	46.8±0.73^{b}
Hemoglobin (g/dL)	15.78±0.8^{b}	18.46±0.36^{a}	18.9±0.4^{a}	18.3±0.3^{a}	18.68±0.34^{a}	17.02±0.5^{a}	15.64±0.22^{b}
RBC count (×10^{12}/L)	7.88±0.42^{b}	9.2±0.19^{a}	9.36±0.25^{a}	9.22±0.2^{a}	9.26±0.16^{a}	8.38±0.28^{b}	7.9±0.16^{b}
MCV (FL)	59.9±0.15^{a}	59.8±0.14^{a}	59.98±0.08^{a}	59.76±0.2^{a}	60.08±0.05^{a}	59.68±0.48^{a}	59.38±0.39^{a}
MCH (pg)	200.38±0.7^{a}	200.68±1.18^{a}	200.84±0.96^{a}	200.24±0.65^{a}	202.44±0.62^{a}	199.88±1.26^{a}	198.76±1.87^{a}
MCHC (g/dL)	3.24±0.8^{a}	33.56±0.17^{a}	33.4±0.14^{a}	33.26±0.1^{a}	33.56±0.09^{a}	33.5±0.08^{a}	33.16±0.22^{a}
WBC count (×10^{10}/L)	11.28±1.39^{a}	10.96±1.31^{a}	10.72±0.81^{a}	9.52±0.65^{a}	8.72±0.82^{a}	8.66±1.02^{a}	9.28±0.35^{a}
Neutrophils (×10^{10}/L)	3.18±1.88^{a}	3.05±0.66^{a}	2.92±0.37a	2.74±1.18^{a}	2.48±0.75^{a}	2.6±0.87^{a}	2.77±1.21^{a}
Lymphocytes (×10^{10}/L)	7.81±0.97^{a}	7.61±0.75^{a}	7.63±0.37^{a}	6.51±0.4^{a}	6±0.97^{a}	5.82±1.24^{a}	6.35±0.98^{a}
Eosinophils (×10^{10}/L)	0.02±0.2^{b}	0.11±0.32^{a}	0±0^{b}	0.11±0.37^{a}	0.09±0.32^{a}	0.09±0^{a}	0±0^{b}
Basophils (×10^{10}/L)	0.11±0.32^{a}	0.02±0.2^{a}	0.04±0^{a}	0.04±0.2^{a}	0.03±0.24^{a}	0.02±0.2^{a}	0.09±0.32^{a}
Monocytes (×10^{10}/L)	0.16±0.24^{a}	0.18±0.4^{a}	0.13±0.37^{a}	0.13±0.24^{a}	0.12±0.24^{a}	0.14±0.24^{a}	0.07±0.2^{a}

Abbreviations: SEM: Standard error of mean; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.

$^{a, b}$Significant difference along the row (P<0.05).

and 1000 and 2000 mg/kg of *C. citratus* + *E. globulus* EO combination (57±1.30; 55.6±1.57 µ/L), compared to the test group that received 1000 mg/kg *C. citratus* EO (71.6±6.83 µ/L), and 1000 mg/kg (72±2.68 µ/L) and 2000 mg/kg *E. globulus* EO (75.8±5.22 µ/L). No significant difference was observed for serum ALT, ALP, TBL, and DBL values across groups (P=0.15) (Table 3).

Table 2. Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on serum lipid profile of Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
T. Cholesterol (mg/dL)	59.4±7.01^{c}	50.38±6.05^{c}	56.44±4.66^{c}	75.96±4.94^{a}	79.76±4.98a	59.46±6.68^{c}	69±2.26^{b}
Triglycerides (mg/dL)	55.12±4.58^{a}	54.1±2.86^{a}	45.98±1.6^{a}	50.36±1.29^{a}	55.12±3.03^{a}	52.12±2.48^{a}	49.32±0.46^{a}
HDL (mg/dL)	31.48±5.49^{a}	24.9±4.28^{b}	39.1±6.9^{a}	42.76±1.61^{a}	44.82±1.17^{a}	29±2.08^{b}	40.04±2.71^{a}
LDL (mg/dL)	16.94±1.09^{a}	16.32±2^{a}	24.62±3.71^{a}	21.94±4.05^{a}	27.46±1.81^{a}	16.66±2.93^{a}	20.72±2.62^{a}
VLDL (mg/dL)	11.62±0.92^{a}	10.7±0.5^{a}	10.04±0.22^{a}	10.66±0.59^{a}	11.04±0.69^{a}	10.48±0.5^{a}	10.38±0.49^{a}

Abbreviations: SEM: Standard error of mean; T: Total; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein.

$^{a, b, c}$Significant difference along the row (P<0.05).

Table 3. Effects of *C. citratus*, *E. globulus,* and *C. citratus* + *E. globulus* EO combination on serum protein profile and liver function of Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
Total protein (g/dL)	6.04±0.61[a]	6.6±0.45[a]	6.79±0.45[a]	6.64±0.35[a]	6.48±0.38[a]	6.56±0.67[a]	6.36±0.15[a]
Albumin (g/dL)	3.82±0.47[a]	4.1±0.45[a]	4.5±0.42[a]	4.18±0.26[a]	4.08±0.26[a]	3.92±0.31[a]	3.82±0.04[a]
Globulin (g/dL)	2.22±0.3[a]	2.5±0.21[a]	2.29±0.36[a]	2.46±0.4[a]	2.4±0.41[a]	2.64±0.37[a]	2.54±0.16[a]
AST (µ/L)	85±3.33[a]	71.6±6.83[b]	59±4[c]	72±2.68[b]	75.8±5.22[b]	57±1.3[c]	55.6±1.57[c]
ALT (µ/L)	48±2.97[a]	45.6±3.82[a]	43.4±3.25[a]	50.2±3.65[a]	49±7.73	37±3.44[a]	36.4±0.68[a]
ALP (µ/L)	49.12±4.64[a]	50.8±2.83[a]	54.1±3.59[a]	52.36±3.94[a]	55.04±5.02[a]	61.46±4.42[a]	57.04±5.55[a]
TBL (mg/dL)	1.27±0.48[a]	0.79±0.29[a]	1.66±0.06[a]	1.1±0.41[a]	1.26±0.36[a]	1.07±0.11[a]	1.08±0.08[a]
DBL (mg/dL)	0.11±0.03[a]	0.15±0.07[a]	0.12±0.05[a]	0.12±0.03[a]	0.11±0.02[a]	0.15±0.04[a]	0.18±0.04[a]

Abbreviations: SEM: Standard error of Mean; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TBL: Total bilirubin; DBL: Direct bilirubin.

[a, b, c]Significant difference along the row (P<0.05).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on kidney function of Wistar rats

There was no statistically significant difference (P=0.09) in serum creatinine and BUN levels between the test groups and the control group (Table 4).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on antioxidant enzymes in Wistar rats

A significant (P=0.01) increase in CAT enzymes was seen in the groups that received 2000 mg/kg *C. citratus* EO (1.77±0.17 µ/L), compared to the control (1.33±0.06 µ/L) (Table 5). Likewise, a significant increase (P<0.001) in serum MDA was observed in animals that received a 1000 mg/kg oral dose of *C. citratus* compared to the control group. There was no significant (P=0.52) difference in SOD and GPx between the test groups and the control (Table 5).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on hormonal profile in Wistar rats

The results are presented in Table 6. There was a statistically significant (P=0.05) decrease in serum progesterone across treatments, except for those treated with 1000

Table 4. Effects of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination on kidney function of Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
Creatinine (mg/dL)	0.46±0.05[a]	0.78±0.2[a]	0.54±0.04[a]	0.66±0.12[a]	0.86±0.05[a]	0.72±0.14[a]	0.66±0.12[a]
BUN (mg/L)	16.52±0.89[a]	16.54±1[a]	19.2±0.62[a]	15.6±0.89[a]	16.68±1.7[a]	17.08±1.62[a]	16.7±0.59[a]

Abbreviations: SEM: Standard error of mean; BUN: Blood urea nitrogen.

[a, b]Significant difference along the row (P<0.05).

Table 5. Effects of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination on antioxidant enzymes in Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
CAT (µ/L)	1.33±0.06^{b}	1.41±0.08^{b}	1.77±0.17^{a}	1.43±0.13^{b}	1.34±0.04^{b}	1.57±0.17^{a}	1.95±0.06^{a}
SOD (µ/L)	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}	0.01±0^{a}
GPx (µ/L)	10.68±2.48^{a}	11.14±0.56^{a}	7.88±1.14^{a}	9.66±0.75^{a}	10.32±1.07^{a}	8.96±1.31^{a}	5.94±1.25^{a}
MDA (µ/L)	3.31±0.8^{b}	6.1±1^{a}	4.43±0.83^{a}	2.52±0.91^{b}	1.83±0.48^{c}	6.59±0.94^{a}	5.78±0.22^{a}

Abbreviations: SEM: Standard error of mean; CAT: Catalase; SOD: Superoxide dismutase; GPx: Glutathione peroxidase; MDA: Malondialdehyde.

$^{a, b, c}$Significant difference along the row (P<0.05).

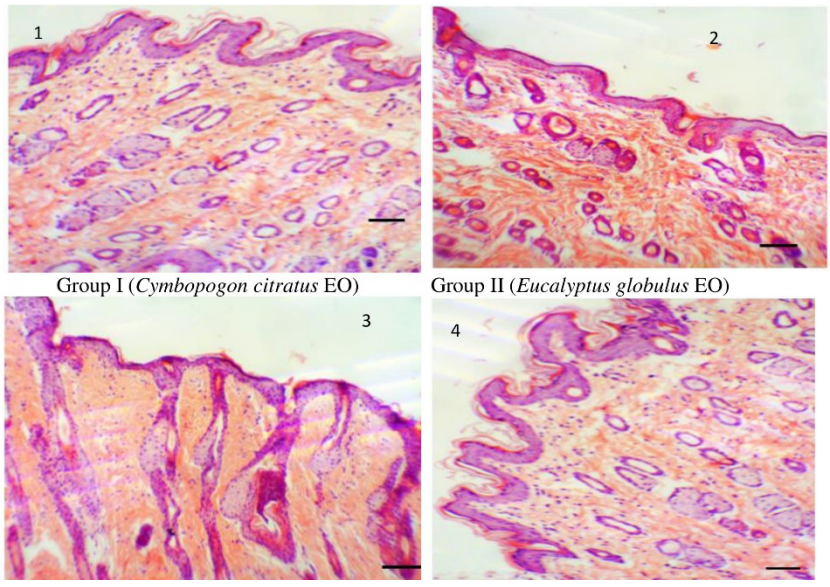
mg/kg *C. citratus* and *E. globulus* EO alone. A significant increase in serum estrogen (P=0.05) was observed in both males and females across the treatment groups. Also, the serum testosterone values were significantly higher (P=0.02) in males who received 1000 and 2000 mg/kg oral doses of *C. citratus* + *E. globulus* EO combination. Serum LH was significantly decreased (P=0.05) in all the test groups that were treated with 1000 mg/kg *E. globulus*, and 1000 and 2000 mg/kg *C. citratus*. There was a decrease in serum FSH values across all test groups (P=0.01).

Table 6. Effects of *C. citratus*, *E. globulus*, and *C. citratus* + *E. globulus* EO combination on reproductive hormones in Wistar rats

Parameter	Control	Mean±SEM					
		C. citratus		*E. globulus*		*C. citratus + E. globulus*	
		1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg	1000 mg/kg	2000 mg/kg
LHF (IU/mL)	4.13±0.09^{a}	0.11±0.02^{b}	0.12±0.02^{b}	0.12±0.02^{b}	0.11±0.05^{b}	0.12±0.05^{b}	0.12±0.03^{b}
LHM (IU/mL)	4.13±0.09^{a}	3.13±0.16^{b}	3.33±0.08^{b}	1.07±0.1^{b}	4.13±0.26^{a}	3.86±0.18^{a}	3.56±0.09^{a}
FSHF (IU/mL)	3±0.07^{a}	2.9±0.02^{a}	0.5±0.01^{b}	0.8±0.02^{b}	2±0.05^{b}	0.5±0.05^{b}	0.5±0.03^{b}
FSHM (IU/mL)	3±0.07^{a}	0.5±0.17^{b}	0.8±0.062^{b}	0.3±0.09^{b}	0.5±0.02^{b}	0.3±0.11^{b}	0.3±0.07^{b}
PF (ng/mL)	43.66±3.09^{a}	28.99±3.18^{b}	17.9±0.49^{b}	36.67±0.8^{b}	27.7±0.71^{b}	25.08±0.62^{b}	14.67±0.7^{b}
PM (ng/mL)	43.66±3.09^{a}	16.59±0.67^{b}	18.06±0.09^{b}	13.35±0.39^{b}	11.62±0.26^{b}	16.24±0.68^{b}	18.69±0.09^{b}
EF (ng/mL)	17±0.38^{a}	24±0.87^{b}	23±0.26^{b}	20±0.24^{b}	22±0.7^{b}	23±0.06^{b}	23±0.01^{b}
EM (ng/mL)	17±0.38^{a}	25±1.63^{b}	24.58±1.03^{b}	19.09±1.11^{a}	21.35±0.65^{b}	24.21±0.07^{b}	24.18±1.3^{b}
TF (ng/mL)	0.36±0.07^{a}	0.35±0.02^{a}	0.33±0.02^{a}	0.5±0.06^{a}	0.35±0.03^{a}	0.43±0.11^{a}	0.34±0.03^{a}
TM (ng/mL)	0.36±0.07^{a}	0.61±0^{a}	0.81±0.01^{a}	0.35±0^{a}	0.68±0^{a}	1.05±0.06^{b}	1.31±0.01^{b}

Abbreviations: SEM: Standard error of mean; LHF: Luteinizing hormone female; LHM: Luteinizing hormone male; FSHF: Follicle-stimulating hormone female; FSHM: Follicle-stimulating hormone male; PF: Progesterone female; PM: Progesterone male; EF: Estrogen female; EM: Estrogen male; TF: Testosterone female; TM: Testosterone male.

$^{a, b}$Significant difference along the row (P<0.05).



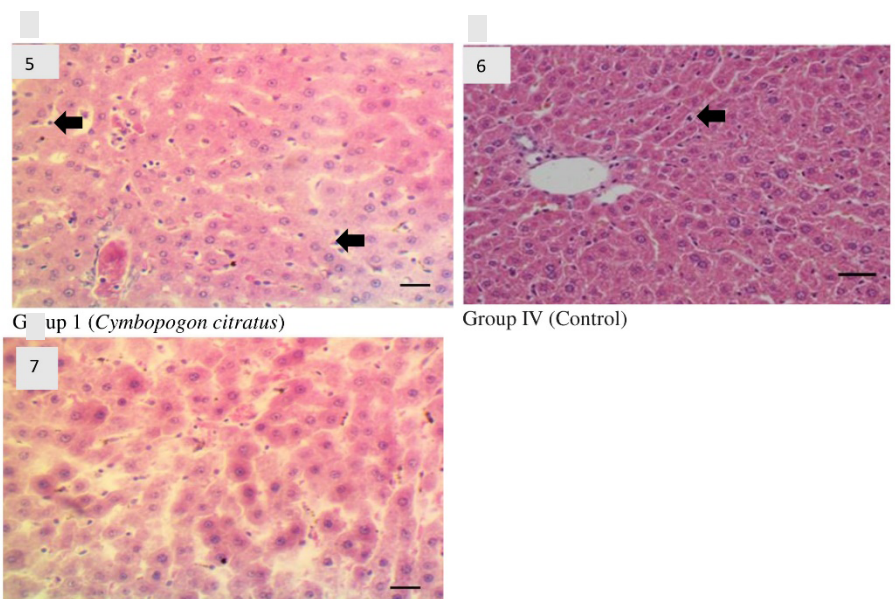
Figures 1-4. Sections of the skin appearing apparently normal (arrow) (H & E; scale bar: 20 µm).

Effects of *C. citratus*, *E. globulus*, and *C. citratus*+ *E. globulus* EO combination on organ histopathology in Wistar rats

Based on the OECD guidelines, high doses (1000 and 2000 mg/kg) were administered for the toxicity studies. In all test groups, mild proliferation of Kupffer cells was observed in the liver (Figure 2). There was a mild presence of proteinaceous materials in the tubular lumen with a mild distention of the Bowman›s capsule, causing the glomerulus to appear atrophied (Figure 3). The cardiac myocytes appear normal, with no visible pathology observed in any of the test groups.

Discussion

Synthetic acaricides have a low margin of safety, which can cause several toxic episodes from accidental ingestion or percutaneous absorption in livestock after topical application. Studies have reported the toxicity potentials of synthetic acaricides (Agwunobi et al., 2021); however, studies on the toxicity of pesticidal plants are evolving. The toxicity of plant EOs has been reported,



Figures 2. Sections of liver showing with mild proliferation of Kupffer cells present across the test groups (H&E stain; scale bar: 20 µm).

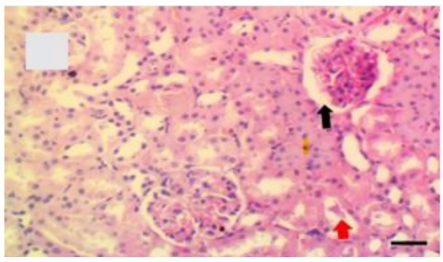


Figure 3. Section of the kidney showing mild atrophy of the Bowman's capsule (black arrow) with proteinaceous materials in the tubular lumen (red arrow) (H&E stain; scale bar: 20 µm)

but occurs only at high concentrations (Zárybnický et al., 2017; Tabarraei et al., 2019).

In this study, no mortality or signs of toxicity were recorded among animals that received the highest dose of the *C. citratus*/ *E. globulus* EO combination. Thus agreeing with findings from previous studies on *C. citratus* in Mice and *E. globulus* EO (Shalaby et al., 2011; Lima et al., 2017; Lulekal et al., 2019). The LD_{50} for *E. globulus* ranged between 1750 mg/kg and 3811.5 mg/kg, indicating the plant's safety (Hu et al., 2014; Mengiste et al., 2020).

Incidents of toxicity due to the topical application of plant EOs have been reported (Mathew et al., 2021). However, in this study, no observable signs of toxicity, mortality, or dermal irritation were recorded after a single dermal application of the EOs on the shaved portion of the skin in Wistar rats. This observation aligns with reports from the literature, which explain the safety of plant EO for topical application, even at doses as high as 2000 mg/kg (Mekonnen et al., 2019).

In this study, there was an increase in the PCV values in the EO treatments. This increase suggests possible hematopoietic stimulation of the bone marrow. These findings, however, are not in agreement with those reported by Hu et al. (2014). Their study on eucalyptus oil-water emulsions in rats caused no significant change in PCV, and Shalaby et al. (2011) reported a decrease in PCV values in their study on *E. globulus* and *Eugenia caryophyllus* EOs.

Drug biotransformation is one of the primary functions of the liver; therefore, hepatic cells are the primary targets of toxicity from all classes of xenobiotics (Zárybnický et al., 2017). Certain biochemical markers, including bilirubin, AST, ALT, and ALP, have been used as indicators of liver injury. Elevations in serum enzyme levels are considered relevant indicators of liver damage, while a rise in both total and conjugated bilirubin levels is a measure of overall liver function (Singh & Jialal, 2020). Decreases in serum AST observed in the test groups indicate that the EOs at the test concentrations used in this study may possess hepatoprotective properties.

Contrary to existing literature, BUN and serum creatinine levels did not significantly change across the treatments (Shalaby et al., 2011; Hu et al., 2014). However, two studies reported no significant elevation in these parameters at the same doses tested (Gebremickael, 2017; Lulekal et al., 2019). Elevated TC and a decrease in HDL

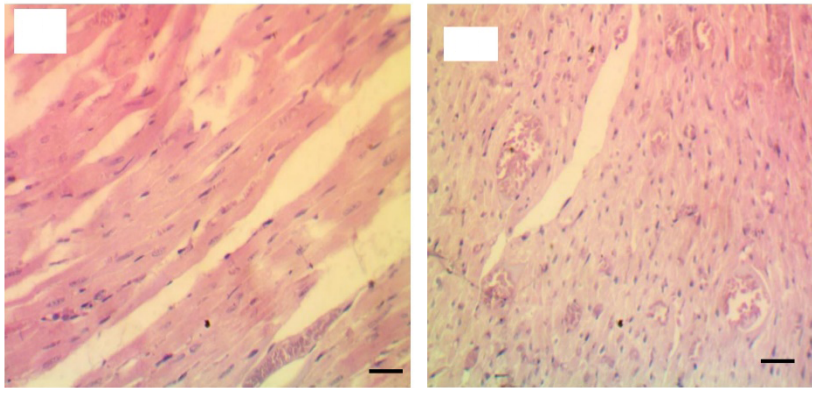


Figure 4. Sections of the heart showing normal histology (H&E stain; scale bar: 20 µm)

values were observed in the animals that received a 2000 mg/kg oral dose of *C. citratus* and *E. globulus* in this study, which could indicate that these EOs may cause an increase in serum cholesterol levels (Rossouw, 2015).

Biomarkers of oxidative stress are relevant in evaluating disease status and the health-enhancing effects of antioxidants. The metabolism of xenobiotics increases the production of reactive oxygen species (ROS) and their intermediates, resulting in an accumulation of ROS that exceeds the capacity of protective antioxidant systems (Marrocco et al., 2017). Some reports document findings on the antioxidant properties of *E. globulus* and *C. citratus* EO (Salem et al., 2018). Elevated catalase and reduced MDA levels were observed in this study. Both responses could be attributed to the protective effect of the cells against oxidative stress, induced or activated by the EO, through the induction of antioxidant enzymes (Garcia et al., 2020).

Few reports have associated the use of synthetic acaricides with alterations in reproductive hormones, infertility, and abortion in animals (Panuwet et al., 2018; Pandey et al., 2020). This study demonstrated that the EOs resulted in a decrease in serum levels of progesterone, FSH, and LH. This FSH/LH response could be attributed to the possible presence of phytoestrogenic compounds in the EO. There was an increase in serum testosterone values in male Wistar rats that received oral doses of 1000 and 2000 mg/kg of the *C. citratus* + *E. globulus* EO combination. This finding shows that a combination of both EOs could synergistically stimulate the production of testosterone. Thus, agreeing with the findings of Saleh et al. (2013), who reported an increase in serum testosterone levels following daily oral administration of the aqueous leaf extract of *C. citratus*.

Conclusion

EOs from *C. citratus* and *E. globulus* were found to be safe at a very high concentration of 2000 mg/kg. Thus, establishing the safety of these EOs as topical acaricidal agents on animals and the environment.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Research Ethics Committee of the College of Veterinary Medicine at the Federal University of Agriculture, Abeokut, Nigeria (Code: FUNAAB/COLVET/CREC/2020/05/01). Additionally, it was ensured that this study adhered to the ARRIVE guidelines for in vitro animal research.

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Authors' contributions

Conceptualization: Olubukola T. Adenubi; Methodology: Timothy Salihu, Olubukola T. Adenubi, and Olushola E. Adeleye; Investigation: Timothy Salihu and Oluwatodimu A. Adekoya; Funding acquisition and writing the original draft: Timothy Salihu; Review, and editing: Tahjudeen A. Afolabi, Dominic O. Odulate, and Johnny O. Olukunle; Resources: Fakilahyel M. Mshelbwala; Supervision: Olubukola T. Adenubi, Foluke A. Akande. and Johnny O. Olukunle.

Conflict of interest

All authors declared no conflict of interest.

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