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Sub-chronic Toxicity of Anti-ectoparasitic Formulation Comprising Cymbopogon Citrates Essential Oil and Jatropha Curcas Fixed Oil in Swiss Albino Mice

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Abstract

Ectoparasites are found widely distributed in all agro-ecological zones of Ethiopia. The problems they cause can be alleviated through the use of traditional medicinal plants. *Cymbopogon citratus* is one of the traditional herbs commonly used for such purpose. This study was conducted to profile safety of the *C. citratus* based anti ectoparasitic formulation. The toxicity study had shown significant weight change in animal group that received 3.75% of the formulation. There were also significant changes in serum lipid profiles of treated group animals compared with the control. The level of uric acid in animals treated with 3.75% of the formulation showed significant increase compared with the control.

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Liver and kidney histopathology of both treatment group animals revealed few mononuclear leucocytic infiltrations. The blood cell parameters and different biochemical findings had revealed no sign of toxicity. However; other measured parameters like the body weight, histological and some biochemical findings had shown both significant and insignificant sign of toxicities. Therefore; it is important to consider the effect of the formulation in prolonged treatment on body weight, uric acid excretion and lipid metabolism.

Key words: Cymbopogon citratus; anti-ectoparasite; medicinal plants; Sub-chronic toxicity; histopathology.

1. Introduction

The plant based traditional medicines appear most promising; and they are culturally ingrained, accessible, and affordable. About 70% to 95% of population in many developing countries, including Ethiopia use and rely on them as a primary source for both human and veterinary medicine [23]. Ethiopia is considered as having the largest livestock population in Africa and currently considered as the biggest exporter in the continent [14, 22]. Ectoparasites including lice, sheep keds, ticks, fleas and mange mites commonly cause a wide range of health problems on livestock population [11, 24, 21]. Infestations from these ectoparasites induce great economic losses by reducing wool quality, meat and milk yield, and related with cost of treatment [21]. *Cymbopogon citratus* is an economically important plant belonging to Family: *Poaceae*, Genus: *Cymbopogon*, specific epithet: *citratus*. It is commonly called lemon grass in English. Its linear leaves can grow up to 90 cm in height and 5 mm in width. The leaves, stems and roots of *C. citratus* are commonly used in herbal medicine [8, 30]. Fresh *C. citratus* leaves contain about 0.4% essential oil. The essential oil contains citral α (~40%), citral β (~32%), nerol (~4.18%), geranicol (~3.04%), citronellal (~2.10%), terpinolene (~1.23%), geranyl acetate (~0.83%), myrecene (~0.72%), terpinol (~0.45%), methylheptenone (~0.2%), borneol (~0.1-0.4%), lanilylacetate (~0.1%), α-pinene (~0.07%) and β-pinene (~0.04%) [13, 29]. Geographical variations in some chemical constituents of *C. citratus* have been noted. Geraniol is the main substance found in essential oil of Ethiopian *C. citratus;* constituting about 40%, followed by 13% citral and 12% α-oxobisabolene. A variation was also noted in West and East Indian *C. citratus* leaf extract content of myrcene [1, 9]. Ethiopian *C. citratus* based formulation showed a comparable *in-vitro* acaricidal effect on the sheep ked (*Melophagus Ovinus*) as compared with the standard ivermectin at 1.565µl per ml. Superior mange mite mortality was observed at 0.15625% *C. citratus* (*in-vitro*) than diazinon (0.1%) and ivermectine (0.01%). Higher mortality of lice (*Damalinaovis*) was observed at *C. citratus* 0.625% than the standard diazinon 0.1% [2]. A study by Ademuyiwa and his colleagues (2015) concluded as aqueous extract of *C. citratus* exhibits nephroprotective effect in wistar albino rats. A study in Nigeria indicated that aqueous leaves extracts of *C. citratus* has anti hepatotoxic action against cisplatin induced hepatic oxidative damage in rats. This might show free radical scavenging property of the plant leaves extract [10]. The aqueous leaf extract of *C. citratus* at 250 and 500mg/kg resulted in a significant decrease on serum total cholesterol and LDL (Low Density Lipoprotein) in dose related fashion, and also significantly increased HDL compared to the control [7]. Daily treatment for 12 weeks with *C. citratus* based formulation prepared using *tween-80* emulsifier did not show any toxicity related mortalities and changes in general health, behavior, motor activities and growth. The formulation also did not produce toxic effect on hematological and biochemical assays [17]. The formulation was found to have strong acaricidal property against the aforementioned ectoparasites. It is prepared using less costly and easily accessed

excipient in order to minimize the production costs by the manufacturer. This could facilitate affordable quality and safe product for the small scale farmers. Therefore, this study attempts to investigate the safety of the new product on the rational basis to recommend for industrial scale production.

2. Materials and Methods

2.1 Study type: Experimental study

2.2 Study design

Sub-chronic toxicity study for three months was conducted on mice model. The doses of formulation at 1.25% and 3.75% of 1ml/100g were used. All animals were inspected for signs of morbidity and mortality (changes in skin, eyes, occurrence of secretions and excretions, lacrimation, pilorection, pupil size, unusual respiratory pattern and behavioral patterns like observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma). Parameters like body weights, blood cell indices, serum biochemical levels, liver and kidney gross pathology and histopathology were also evaluated.

2.3 Study setting

The study was conducted at Addis Ababa University, College of Health Sciences, School of Medicine, Department of Anatomy (Histology laboratory) and Traditional and Modern Medicine Research Directorate of Ethiopian Public Health Institute (Pharmacology laboratory).

2.4 The formulation: preparation and dilution

Essential oil was extracted from *C. citratus* leaves using hydro distillation; and fixed oil from *J. curcas* seed by pressing in Wondogenet Agricultural Research Center. Anti ectoparasitic formulation with active ingredient of 15% *C. citratus* essential oil, 3% fixative oil of *J. curcas*, 15% *Geronol MS* emulsifier with distilled water was prepared. Doses for administration were prepared every three days using 1.25% and 3.75% concentrations.

2.5 Experimental animal preparation and grouping

Healthy inbreed Swiss albino mice weighing 20 to 25g were obtained from animal breeding facility of Ethiopian Public Health Institute (EPHI). The animals were acclimated to laboratory conditions for seven days before the study started. They were kept at standard EPHI pharmacology laboratory with a recommended temperature and humidity by Organization for Economic Cooperation and Development (OECD). The room light was artificially adjusted at twelve hours light and twelve hours dark. They were fed with conventional laboratory diets with unlimited supply of drinking water [26]. The study was conducted on a total of 18 healthy, non pregnant and nulliparas' female and male adult mice. The animals were assigned randomly in to three groups (GI, GII and GIII) each with six mice (three males and three females). They were kept separately in labeled aluminum cages to facilitate identification of the experimental groups. All mice in the same group and with the same sex were labeled on their tail by permanent marker color for the sake of differentiating one animal from the other.

2.6 Dose selection, dosing and duration of treatment

All animals were restricted from food and water for 3 to 4 hours prior to administration, and their body weight were recorded [26]. The *C. citratus* based formulation (test preparation) and distilled water (control) dose for each animal in both treatment and control group was calculated based on their body weight. The treatment groups: GI and GII were given *C. citratus* based formulation orally by gastric intubation at the dose of 1.25% and 3.75% of 1ml per 100g respectively for 3 months. The control group (GIII) animals were given distilled water 1ml per 100g. Oral toxicity is used by considering incidental ingestions of the formulation by human and other animals.

2.7 Data Collection

After administration, all animals were observed continuously for two hours daily for any sign of toxicity. The body weight of each mouse was recorded on the 1st day (before administration), and then every week throughout the experiment. Blood was collected from jugular vein in to two separate test tubes; with anti coagulant EDTA (Ethylene Diamine Tetra Acetate) and the other without anti coagulant. The blood sample with anti coagulant was immediately mixed with EDTA coated on the wall of the tube. The hematological parameters like: Red Blood Cell (RBC), White blood cell (WBC), Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and platelet count were determined using automated hematological analyzer, Sysmex XT-1800i (Sysmex Corporation, Japan). For biochemical analysis, the blood samples in the test tubes without anti coagulant were centrifuged at 5000 rpm for 15 minutes using a bench top centrifuge Humax-HK (Human-GmbH, Germany). The serum level of Alkaline phosphatase (ALP), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), total bilirubin, totalcholesterol, triglyceride, Low density lipoprotein (LDL), High density lipoprotein (HDL), urea, uric acid and creatinine were determined using automated chemistry machine, Cobas Integra 400 plus Analyzer (ROCH Diagnostics, Japan). The animals were sacrificed by cervical dislocation, and subjected to careful examination of the external surface of the body, abdominal cavities and their contents. The liver and kidneys of the experimental animals were excised and fixed in 10% neutral buffered formaldehyde solution for 24 hours.

2.8 Histological specimen preparation

The liver and kidneys were washed several times with tap water to remove excess fixatives following overnight fixation in 10% formaldehyde solution. Both tissues were dehydrated with increasing concentration of ethanol 70, 80, and 90%, respectively for two hours each, followed by absolute alcohol: group I, II and III, for one and half hour for each, and group IV overnight. The tissue samples were then cleared with xylene-I for two and half hours and xylene-II for one and half hour consecutively. This was followed by impregnation in paraffin wax: wax-I for one and half hours, wax-II for two and half hours, and wax-III overnight. Finally, the tissue samples were embedded into tissue block by putting them in squares of metal plates by carefully pouring molten paraffin over them. The tissue blocks were sectioned at a thickness of 3 to 5μm and stained using routine H&E method.

2.9 Light microscopy and photomicrography

The prepared tissue sections were carefully examined under binocular compound light microscope (Leica DM 750, Germany). Tissue sections from the treated groups were examined for any evidence of changes in their architecture due to toxicities compared to the control. Some of the slides were then selected from both treatment and control groups; and photomicrographs were taken by EVOS microscope with integrated built-in high resolution digital camera (AMG, USA). The images were taken at ×10 and ×20 magnifications for documentation of the findings.

2.10 Statistical analysis

All data were organized and checked for completeness, and analyzed by one way ANOVA, using SPSS version 21 computer software. The possible mean difference in body weight, hematological and biochemical values were identified. Then, it is followed by Dunnett's t-test to compare the difference between control and treated groups, and determine their level of significance. All data are expressed as mean ± SEM (Standard Error of Mean). Differences at $p<0.05$ were considered to be statistically significant.

2.11 Ethical consideration

The study was conducted following the approval by the graduate committee of Department of Anatomy, School of Medicine, College of Health Sciences; Addis Ababa University. Animals used in this study were protected from any unnecessary pain and terrifying situations. They were also protected from any pathogens by keeping in appropriate and safe environment according to OECD guideline.

2.12 Study period

The study were conducted from May, 2016 to July 2017

3. Results

3.1 Effect of the formulation on behavior, gross pathology and body weight

The animals treated with the two doses (1.25% and 3.75%) of *C. citrates* based formulation did not show any toxicity related sign throughout the study period. No change was observed on the general behavior of the treatment groups as compared to the control. No animal death and significant variation in size, texture and color of internal organs (liver and kidney) were noted compared with the control group. Both treatment group animals had shown less mean body weight gain compared with the control. However, mean body weight of animals under the treatment of high dose (3.75%) showed significantly less weight gain ($p< 0.05$) only at week twelve as compared with the control group (Figure 1).

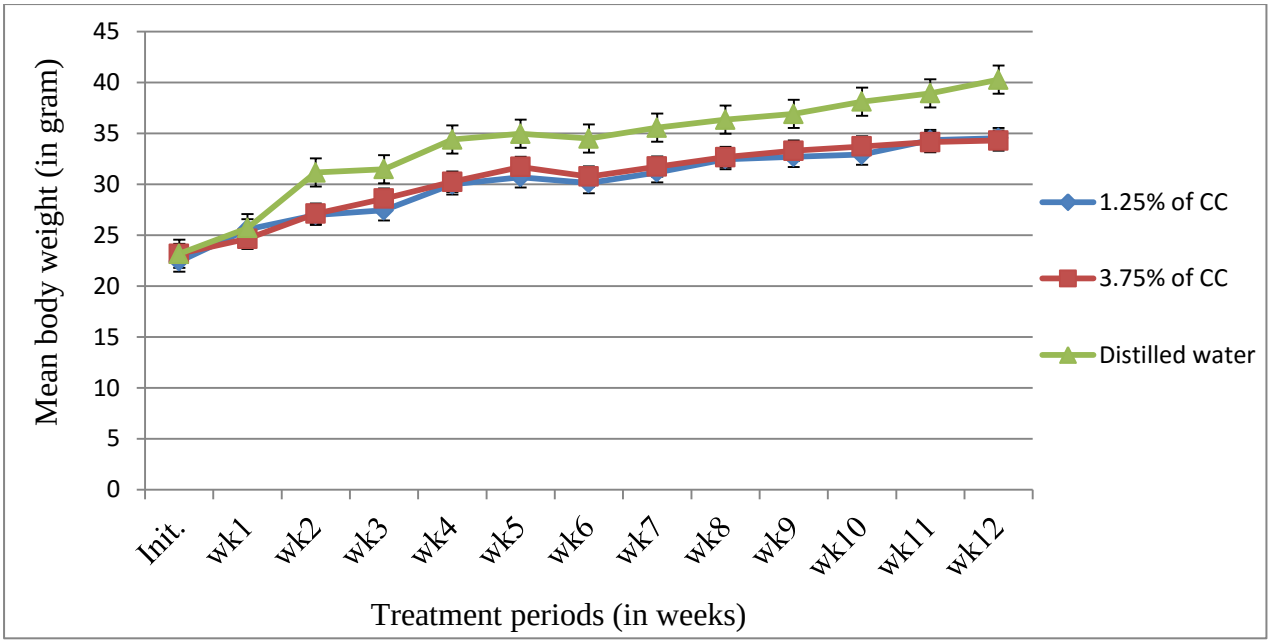

Figure 1: Comparison of effect of *C. citratus* based formulation on body weight of treated and control groups. Note: Significant weight change ($p < 0.05$) in 3.75% of the formulation on week 12 compared to control.

3.2 Effect of the formulation on different blood cell indices

The three months administration of *C. citratus* based formulation at both doses did not produce significant change on any of the blood cell parameters as compared to the control (Table 1).

Table 1: Effect of *C. citratus* based formulation on blood cell parameters of mice in the sub-chronic toxicity study.

	Distilled water	*C. citratus*	
Blood cell parameters	1ml/100g (G-III)	11.25% CC 1ml/100g (G-I)	3.75% CC 1ml/100g (G-II)
WBC ($x10^3$ cells/mm^3)	4.28 ± 0.64	4.45 ± 0.93 (0.97)	4.02± 0.33 (0.94)
RBC ($x10^6$ cells/mm^3)	9.90± 0.31	9.19± 0.55 (0.38)	9.85 ± 0.32 (0.99)
HGB (gm/dl)	16.83± 0.18	16.20± 0.96 (0.68)	17.37± 0.33 (0.76)
HCT (%)	50.50± 0.56	47.10± 2.38 (0.26)	49.72± 1.31 (0.91)
MCV (fL)	53.10 ± 0.53	52.80 ± 0.66 (0.92)	53.03± 0.76 (0.99)
MCH (pg/cell)	17.18± 0.68	17.65± 0.23 (0.68)	17.68± 0.27 (0.65)
MCHC (gm/dl)	34.11± 0.44	34.37± 0.33 (0.83)	35.23± 0.24 (0.06)
Platelet (x103 cells/mm3)	919.00± 137.87	683.00± 203.39 (0.58)	824.00± 210.56 (0.91)

- Values are expressed as Mean ± SEM. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.

3.3 Effect of the formulation on serum biochemical levels

In the serum biochemical levels: total cholesterol level increased respectively by 26.1% and 59.8%, and LDL by 166.8% and 304.1%, at the 1.25% and 3.75% doses of the formulation as compared with the control, which were statistically significant ($p< 0.05$) (Figure 2). On the contrary, levels of creatinine were significantly reduced ($p< 0.05$) by 42.9% and 49.8%, respectively, at both 1.25% and 3.75% doses of the formulation (Table 2). On the other hand, significant reduction ($p< 0.05$) in the serum levels of triglyceride by 24.9% and HDL by 18.89% were observed. Significant increase in uric acid level by 92.93% was recorded at the higher dose (3.75%) of the formulation as compared with the control. In all the remaining biochemical tests investigated, the values were not significantly different from the control group at both low and high doses of the formulation (Table 2 and Figure 2).

Table2: Effect of *C. citratus* based formulation on serum biochemical parameters in the sub-chronic toxicity study.

	Distilled water	*C. citratus*	
Serum biochemical indices	1ml/100g (G-III)	1.25% CC 1ml/100g (G-I)	3.75% CC 1ml/100g (G-II)
ALT (IU/L)	98.83 ± 14.95	101.33± 21.04 (0.99)	50.00 ± 2.81 (0.06)
ALP (IU/L)	66.00± 10.47	53.33± 3.58 (0.30)	55.67± 1.17 (0.43)
Bilirubin (mg/dl)	0.10± 0.01	0.10± 0.009 (0.89)	0.08± 0.002 (0.366)
Creatinine (mg/dl)	0.13 ± 0.01	0.076 ± 0.002 (0)*	0.066± 0.002 (0)*
Urea (mg/dl)	54.30± 2.97	46.80± 2.59 (0.65)	66.03± 10.93 (0.38)
Uric acid (mg/dl)	2.97± 0.52	3.00± .88 (0.99)	5.73± 0.02 (0.009)*

- Values are expressed as Mean ± SEM. The figures in brackets indicate the calculated p values of the treatment groups as compared to the control.
- *: Significantly different ($p < 0.05$) from the control.

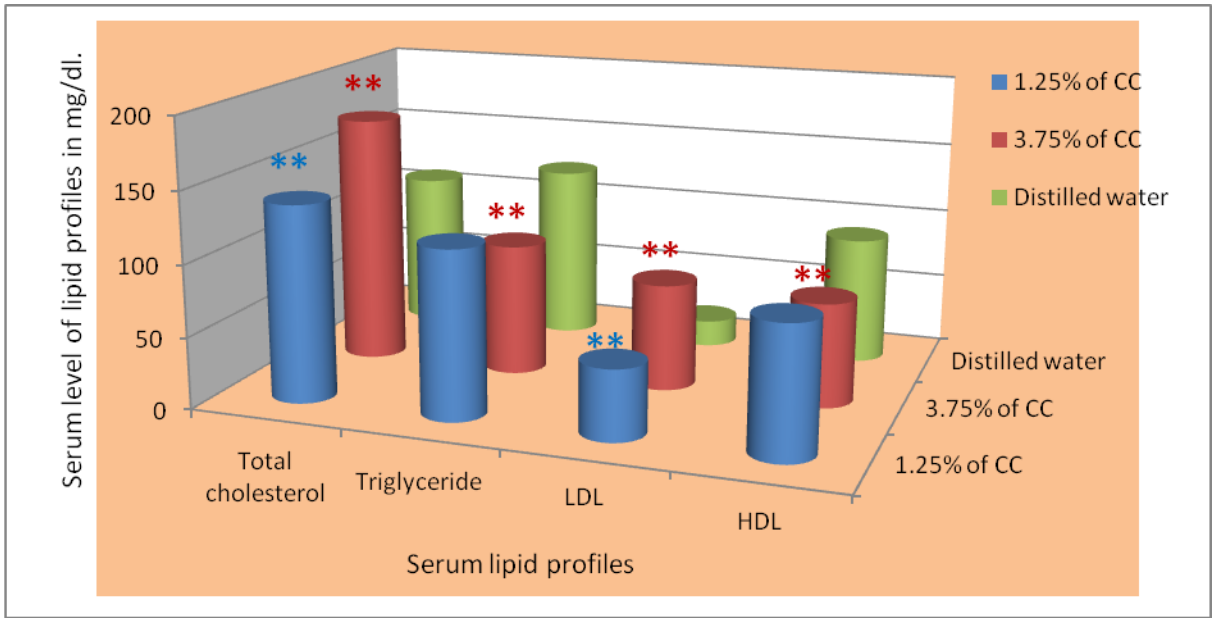

Figure 2: Effect of *C. citratus* based formulation on serum lipid profile level in mice.

**: Significantly different ($p < 0.05$) from the control.

3.4 Effect of the formulation on histology of the liver

Liver section of the control group animals showed normal indistinct lobules with the central vein in the middle. At the corners, there were portal triads, consisting of branches of the hepatic artery, portal vein and bile duct. Hepatocytes with central nuclei were seen radiating away from the central veins to the periphery. There is no blood vessel congestion, necrosis and degenerative conditions of hepatocytes (Figures 3A&3B). In treatment group animals: normal architecture of the central veins, portal triads and hepatocytes were observed in the histopathological examination of the liver. No degenerative conditions and necrotic changes were observed. However, the liver tissue sections of animals in this group showed few pyknotic nuclei; and mononuclear infiltrations around central vein and portal triads (Figures 3 C; 3D; 3 E & 3F).

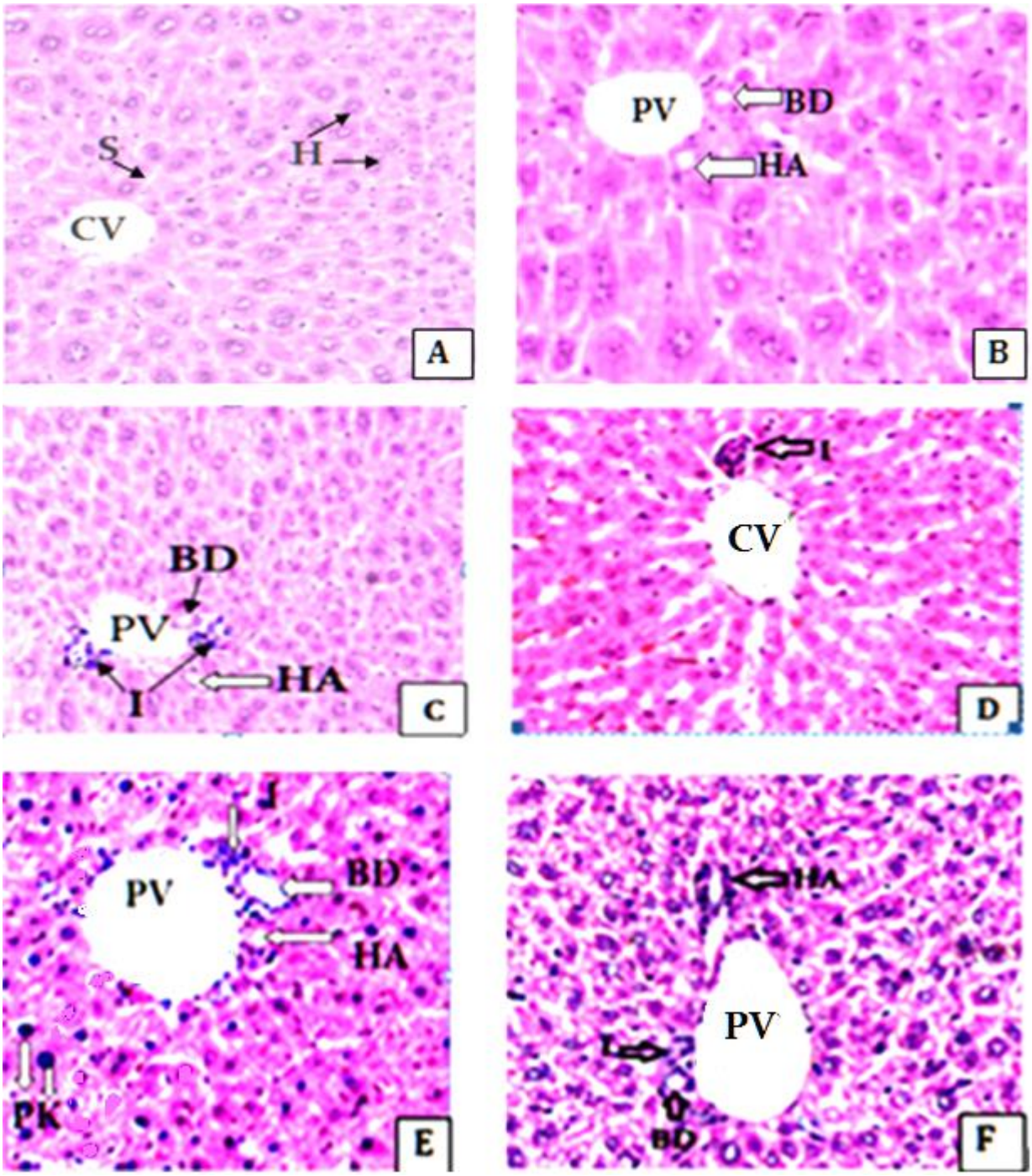

Figure 3: Photomicrographs of liver sections of mice in the control (Figures 3: A & B), mice treated with 1.25% (C & D) and 3.75% (E & F) of the *C. citratus* based formulation.CV = central vein, PV= portal vein, BD= bile duct, HA= hepatic artery, S= sinusoids, I= leucocytic infiltration, PK= pyknotic nucleus. Note: Some mononuclear infiltrations around central vein and portal triads in the mice treated with 1.25% of the formulation (figures 3: C & D). Pyknosis and mononuclear infiltrations adjacent to portal triads in mice treated with 3.75% of the formulation (Figures 3: E & F). (Sections were stained with H&E, x20).

3.5 Effect of the formulation on histology of the kidney

The renal histopathology of the control group animals treated with distilled water showed renal corpuscles with normal size urinary space, normal glomerulus, and tubular structures. There is no congestion and sign of tubular necrosis as observed (Figure 4 A). Normal renal corpuscle with normal glomerulus, urinary space and tubular systems were observed during the histopathological examination of the kidney tissue for animals treated with both doses of the formulation. There was also no tissue necrosis and capillary congestion of renal histology at both doses. However mononuclear leucocytic infiltrations were observed adjacent to tubules and Bowman's capsule in kidney sections of animals with both treatment groups (Figures 4 B and 6 C).

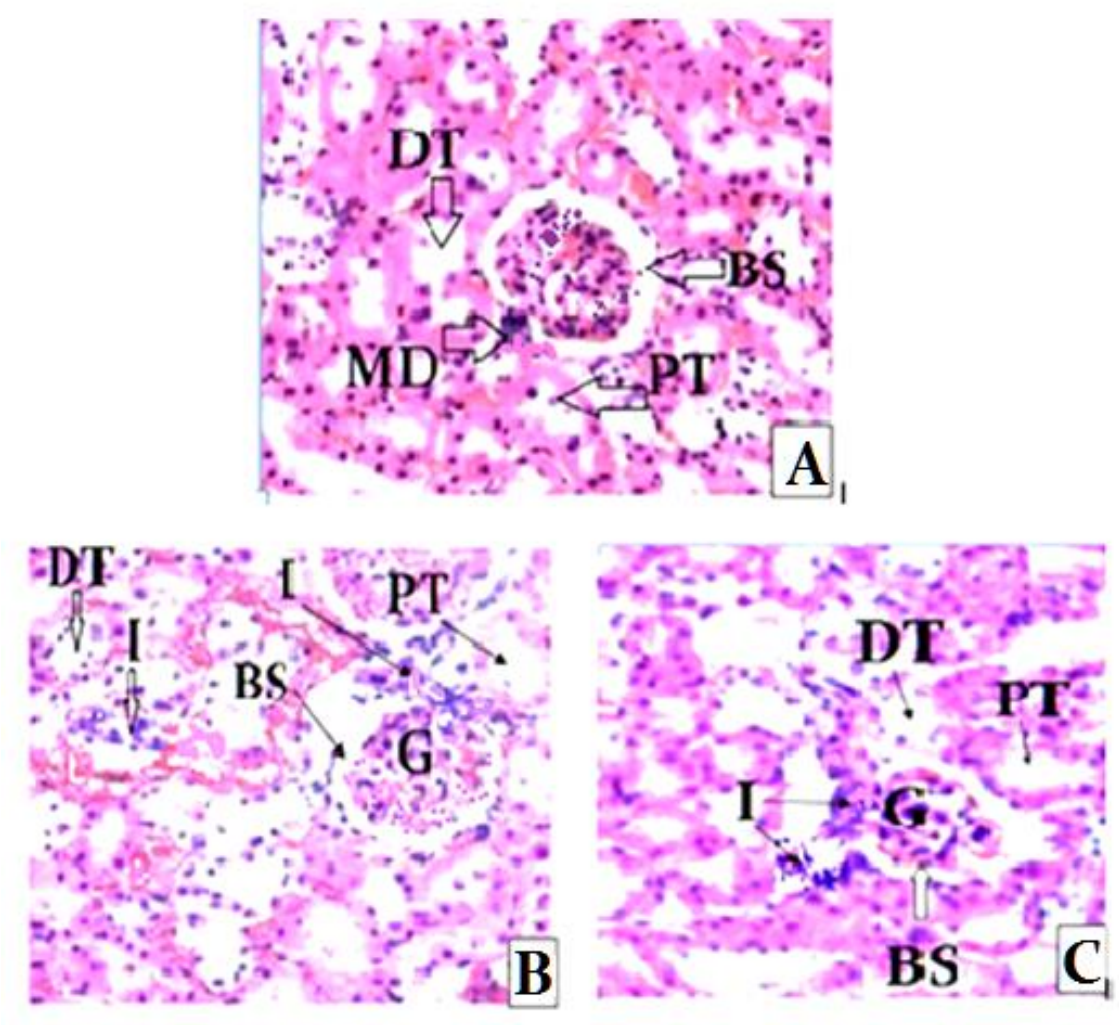

Figure4: Photomicrographs of kidney sections of mice in the control (Fig. 4: A), mice treated with 1.25% (C) and 3.75% (B) of the *C. citratus* based formulation. DT= distal convoluted tubule, PT= proximal convoluted tubule, MD = macula densa, BS = bowman's space, G=glomerulus. Note: Mononuclear infiltration of renal parenchyma adjacent to tubules and Bowman's capsule in the kidney sections of animals with both treatment groups (Figures 4: B & C). (Sections were stained with H&E, x20).

4. Discussion

Different parts of plants, and their products for herbal medicine considered effective are not safe and free of adverse effects [15]. The primary aim of toxicological tests of herbal medicines are identifying adverse effects and determining their safety level [18]. In the current subchronic toxicity study of *C. citratus* based formulation; there was no any toxicity related sign, change on general behavior and animal death observed throughout the study period. No variation of size, texture and color on liver and kidney compared with the control group. This is consistent with G/Mickael and his colleagues (2017) report that employed *C. citratus* formulation with *tween-80* emulsifier. Both treatment group animals showed less weight gain compared with the control. However, only significant weight change ($p< 0.05$) was observed at the end of the study (week twelve) in the group treated by 3.75% of the formulation. The same effect was observed on the study conducted by Adeneye and Agbaje, (2007). This might be due to high content of tannins in the lemon grass which is expected to reduce feed efficiency and weight gain [27], and the effect of *J. curcas* seeds in decreasing total food intake and resulting in lower weight gain [12]. Similar with G/Mickael and his colleagues (2017) the current toxicity study on different blood cell parameters showed no significant difference from the control group animals. These findings may indicate that there is no effect of the formulation on hematopoiesis, destruction and shrinkage of RBCs and immune boosting. In this study serum levels of ALT and bilirubin were slightly increased at 1.25% of the formulation and decreased at 3.75% of the formulation. AST and ALP in both treatment groups showed a decrease from the control. But none of the changes in the above liver function parameters were found significant ($p> 0.05$) compared to the control. A study conducted on rats administered with aqueous extract of *C. citratus* had shown a significant decrease on serum total cholesterol and LDL in dose related fashion, and at the same time significantly increased HDL ($p< 0.05$), compared to the control [7]. Study by Ademuyiwa and his colleagues (2014) on ethanolic extract of the plant resulted in significant decrease ($p< 0.05$) of total cholesterol, triglyceride, HDL and LDL levels compared with the aqueous extract treated and control group rats. However, aqueous extract did show significant decrease in LDL compared with the control. In contrary with all these findings, the current study showed significant increase in total cholesterol and LDL levels at both doses, but the triglyceride and HDL showed a significant decrease only at high dose (3.75% of the formulation) compared to the control. The reason behind all these discordance could be variation in dose, extract type, duration of the treatment, animal type, and geographic variation in chemical constituent of the plant. Nevertheless, the findings from the present study may argue with the recommendations from different researchers that *C. citratus* is important to decrease blood level of bad cholesterols and risk of cardio vascular diseases, and call for further investigation at different conditions. Many results on the effect of different preparations of *C. citratus* on renal system in different type of animals are conflicting [17, 19 25, 28, 30]. In the present study, level of uric acid in animals treated with 3.75% of the formulation showed significant increase ($p< 0.05$) compared with the control. This might show the effect of prolonged treatment with high dose of *C. citratus* based formulation on purine metabolism or renal excretion of uric acid. The difference in clinical parameter indices like creatinine and urea at both doses of the formulation did not show significant increment compared to the control. This is consistent with reports of G/Mickael and his colleagues (2017) and Ademuyiwa and his colleagues (2016). Discrepancies of the findings from different reports may emanate from differences in one or more of: administered doses, duration of treatment, extraction type, age of plant at harvest, and geographical variations in chemical

constituents of the essential oil. The histopathological examination of tissues and organs is best for evaluating treatment related pathological changes which further confirms the alteration in their cellular structure. In the current study, liver histopathologies of both treatment groups and control group animals are almost the same with normal morphology of hepatocytes, central vein and portal triads. There is no sign of necrosis. However, in both treatment group animals' tissue sections, insignificant leucocytic infiltrations were observed adjacent to portal triads and central veins. There were also few hepatocytes with pyknotic nuclei in animals treated with 3.75% of the formulation. The study conducted on rats treated with ethanolic and aqueous extract of *C. citratus*, histopathological results showed no degenerative conditions in the liver [6]. However, essential oil from the plant leaves with doses higher than 1,500 mg/kg showed histological changes in the liver, leading to marked abnormalities in the liver of treated rats [16]. These findings may suggest as *C. citratus* is safe at low doses, but prolonged treatment and high dose could be toxic on this vital organ. According to Ademuyiwa and his colleagues (2016), histological examination of kidney from rats treated with doses of 200, 500, 1000, 2000, 4000 and 5000 mg/kg body weights of ethanolic extract of *C. citratus* showed no adverse effects on the histology of the tissue. Similar to G/Mickael and his colleagues (2017) the current study findings showed no tissue necrosis and capillary congestion, but insignificant peritubular mononuclear cellular infiltrations in both treatment group animals. In agreement with the values of liver and renal function biochemical tests observed; the findings in the histopathological study suggest for no noticeable toxicity of the current formulation on both organs at present settings of study.

5. Limitation of the study

The toxic effect of the formulation on gastro intestinal and other visceral organs, and higher experimental animals is not considered.

6. Conclusion and Recommendations

This sub-chronic toxicity of *C. citratus* based formulation showed no noticeable gross toxicity. There was no significant difference in the blood cell parameters, histopathology and majority of biochemical parameters for liver and kidney functions. However, it is important to consider the effect of the formulation in prolonged treatment on: body weight, uric acid excretion and lipid metabolism. Also it is recommended to consider further toxicological effect of the formulation on different visceral organs, and in higher non rodent species.

7. Conflict of Interest

All authors have reviewed the draft manuscript to be submitted, and declared there is no conflict of interest.

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Appendix

A. Preparation of working solutions

Eq. (A.1). 10% Neutral Buffered Formalin

- 40% formaldehyde---------- 100ml
- Distilled water---------- 900ml
- Sodium di-hydrogen phosphate monohydrate---------- 4gm
- Disodium hydrogen phosphate anhydrous---------- 6.5gm

Eq. (A.2). Harris's Hematoxylin (H)

- Hematoxylin crystals---------- 2.5gm
- Absolute alcohol---------- 25ml
- Potassium alum---------- 50gm
- Distilled water---------- 500ml
- Sodium iodate---------- 0.5gm
- Glacial acetic acid---------- 20ml

Eq. (A.3). 1% Alcoholic Eosin (E)

- Eosin Y, water soluble (CI 45380)---------- 1gm
- 95% Ethanol---------- 100ml
- Glacial acetic acid---------- 0.5ml

Eq. (A.4). 1% Acidic alcohol

- 70% alcohol---------- 500ml
- Hydrochloric acid, concentrated---------- 5ml

Eq. (A.5). Bluing solution

- Sodium bicarbonate---------- 2.5gm
- Distilled water---------- 1000ml

B. Tissue processing procedures

Eq. (B.1). Fixation

- 10% Neutral Buffered Formalin---------- 24 hrs

Eq. (B.2). Washing

- Tap water---------- several changes

Eq. (B.3). Dehydration

- 70% Ethanol---------- 2 hrs
- 80% Ethanol---------- 2 hrs
- 90% Ethanol ---------- 2 hrs
- Absolute alcohol I---------- 1 and 1/2 hrs
- Absolute alcohol II---------- 1 and 1/2 hrs
- Absolute alcohol III---------- 1 and 1/2 hrs
- Absolute alcohol IV---------- overnight

Eq. (B.4). Clearing

- Xylene- I ----------1 and 1/2 hrs
- Xylene- II---------- 2 and 1/2 hrs

Eq. (B.5). Infiltration

- Paraffin wax- I…….. 1 and 1/2 hrs
- Paraffin wax- II---------- 2 and 1/2 hrs
- Paraffin wax- III---------- overnight

C. Hematoxylin and Eosin, Tissue Staining Protocol

Eq. (C.1). De-paraffinization

- Xylene- I---------- 4 min
- Xylene- II---------- 4 min

Eq. (C.2). Rehydration

- Absolute alcohol I---------- 4 min
- Absolute alcohol II---------- 4 min

- 95% Ethanol---------- 3min
- 80% Ethanol---------- 3 min
- Rinse in distilled water---------- 5 min
- Stain in Hematoxylin---------- 10 min
- Rinse in running tap water---------- 5 min
- Decolorize in acid alcohol---------- 1-3 sec
- Rinse in running tap water---------- 5 min
- Immerse in Sodium bicarbonate solution---------- 1 min
- Rinse in running tap water---------- 5 min
- Counter stain in Eosin---------- 1 min

Eq. (C.3). Dehydration

- 80% Ethanol---------- 3 min
- 95% Ethanol---------- 3 min
- Absolute alcohol II---------- 3 min
- Absolute alcohol I---------- 3 min

Eq. (C.4). Clearing

- Xylene II---------- 3 min
- Xylene I---------- 3 min

D. Dilution of stock solution and Preparation of volume for administration

Eq. (D.1). Dilution of stock solution

➢ C1V1=C2V2

1. C1……Concentration of stock solution

2. V1……Volume required

3. C2……Concentration to be prepared

4. V2……Volume of solution to be prepared

- C1= 15%
- V1=?
- C2= 1.25%
- V2= 10ml

- 15% × *V1 = 1.25 × 10ml*
- 0.8ml of stock solution + 9.2ml of distilled water.
- C1V1=C2V2
  - C1= 15%
  - V1=?
  - C2= 3.75%
  - V2= 10ml
- 15% × *V1 = 3.75% × 10ml*
- 2.5ml of stock solution + 7.5ml of distilled water.

Eq. (D.2). Preparation of volume for administration

- The formulation for administration is 1ml/100g body weight of mouse.