

Sub-chronic Oral Toxicity Evaluation of Anti-ectoparasitic Formulation Comprising Eucalyptus Globulus Essential Oil and Jatropha Curcas Fixed Oil in Mice

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Abstract

The primary concern of this study was to evaluate sub-chronic toxicity effects of *Eucalyptus globulus* based anti-ectoparasitic formulation after oral administration. Eighteen mice, six animals for each of the two treatment doses and for the control group were used. Two treatment doses of the formulation 1.25% and 3.75% ml/kg body weight were used. Animals were observed for any mortality and signs of toxicity for two hours daily following administration. Weight was measured initially at the beginning of dosing and every seventh day throughout the study period. Blood samples were collected following anesthetization for the hematological and biochemical analyses. Liver and kidney were excised following scarification for gross and histopathological studies at the end of 3 months consecutive administration. No death was recorded and insignificant body weight changes were documented.

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Besides, insignificant biochemical, hematological and pathological changes were observed. However, liver and kidneys of animals treated with dose of 3.75% ml/kg showed minor mononuclear leukocytic infiltrations around portal areas and tubulointerstitial leukocytic infiltrations. sub-chronic toxicity study results revealed that the formulation does not produce apparent toxic effects. However, further toxicological investigation is needed to strengthen this finding.

Keywords: *Eucalyptus globulus*; anti-ectoparasite; livestock; sub-chronic toxicity; mice.

1. Introduction

Livestock farming in Ethiopia contribute significant share to its economy. It is the mainstay for livelihoods and food security, environmental sustainability and public health in the country. Livestock products including live animals, meat, skins and hides are also major source of foreign exchange. However, livestock diseases and ectoparasite infestations are among the principal constraints of its productivity and cause of high economic losses. Parasitic skin diseases caused by external parasites such as mange mites, lice, keds and ticks lead to skin rejection at various tanneries [1,2,3]. ectoparasiticide chemicals such as amidines, macrocyclic lactones, nitroguanidines, carbamates, acaricides, and organophosphates are widely applied to regulate ectoparasites[4] . However, most synthetic antiectoparasites are hazardous and toxic and the relatively safe chemicals are expensive. Therefore searching for more affordable and safer products from natural sources is highly commendable. Essential oils derived from *Eucalyptus globulus* are widely applied as natural pesticides[5] . The Chemical composition of essential oils of *Eucalyptus globulus* is 1,8-cineole, α -pinene, limonene and α -terpinyl acetate. Cineole is the major composition in most *Eucalyptus* species including *Eucalyptus globulus* [6,7]. *In vivo* evaluation for the insecticidal potency of the formulation containing essential oils from *Eucalyptus globulus* showed encouraging results for the control of ectoparasites[8, 9, 10] . These results suggested that essential oils from *Eucalyptus globulus* can substitute the use of chemical insecticides such as diazinon and it is cost effective. However, besides to their wide range of application and cost effectiveness, there is increasing concern about the safety of medicinal plant products. Therefore, the present sub-chronic study was conducted to investigate toxicity of *Eucalyptus globulus* based anti-ectoparasitic formulation prepared using V497 emulsifier after sub-chronic oral treatment in mice.

2. Material and Methods

2.1. Study setup

The study was conducted at Traditional and Modern Medicine Research Directorate [TMMRD] of Ethiopian Public Health Institute [EPHI] and Addis Ababa University, College of Health Sciences, School of Medicine, Department of Anatomy.

2.2. Experimental animal preparation

Healthy adult male and female Swiss albino mice used in this experiment were obtained from the animal house of Ethiopian Public Health Institute [EPHI] . Female mice were nulliparous and non-pregnant. Grouping was done randomly and animals were marked with permanent marker for individual identification.

2.3. Preparation and dilution of the formulation

Essential oil was extracted from *Eucalyptus globulus* leaves using hydro distillation; and fixed oil from *Jatropha Curcas* seed by pressing in Wondogenet Agricultural Research Center [WARC] . The anti-ectoparasitic formulation with active ingredient of 20% *Eucalyptus globulus* essential oil, 3% fixative oil of *Jatropha curcas*, 15% V497 emulsifier and 100 ml distilled water was prepared. Two doses of 1.25% and 3.75% of ml/kg body weight were prepared for administration every day.

2.4. Sub-chronic toxicity study

Animals were fasted from food but not water for 3-4 hours prior to administration of the formulation. Each animal weighed before dosing. A total of eighteen, nine male and nine female Swiss albino mice used for the study. Six [three male and three female] animals were used for each of the two doses and for the control group. Males and females were kept in separate cages. They were grouped into six groups [I, II, III, IV, V and VI] of three mice per group. Groups I, III and V were female mice, while the remaining groups were comprised of male mice. Administrations of the formulation to the treatment groups and the vehicle to the control group were began seven days after acclimatization. At the commencement of dosing, the animals were between 8-12 weeks old. The first two groups, groups I [females] and II [males] were given 1.25% ml/kg of the formulation while group III [females] and IV [males] treated with 3.75% ml/kg body weight doses of the formulation in accordance to their body weight. The 5th [females] and 6th [males] groups were control groups receiving the vehicle, distilled water. Weight was measured initially at the beginning of dosing and every 7th day throughout the study period following 3-4hrs of fasting before administration. Throughout the study period animals were administered every 24hrs with cautious cage side observation for any signs of toxicity.

2.5. Gross pathologic observations

At the end of treatment period, each mouse was anesthetized using diethyl ether, blood samples were collected for hematological and biochemical procedures and humanely sacrificed by cervical dislocation. The whole liver and both right and left kidneys were excised. Detailed gross pathological observation was made for any lesion. The organs were then thoroughly cleaned in distilled water and preserved in a container with 10% neutral buffered formaldehyde solution over 24 hours.

2.6. Histopathological studies

Tissue samples from the liver and both kidneys were immediately fixed in 10% neutral buffered formalin overnight at room temperature. The following morning tissues were washed with tap water, dehydrated in ethanol in step-wise manner passing them through a series of increasing concentrations of 70% and 90% for 120 minutes, followed by absolute alcohol I, absolute alcohol II and absolute alcohol III, each for one and half hours, and absolute alcohol IV overnight. Xylene-I and xylene-II were then applied to clear alcohol for one and half hours and for two and half hours, respectively. The specimens were then infiltrated with three changes of paraffin wax [I, II and III] for one and half hours, two and half hours, and overnight respectively. Finally the tissues were embedded in paraffin wax in square metal plates forming tissue blocks, whereby each tissue block

was labeled and stored at room temperature till sectioned. Tissue blocks were sectioned with a thickness of 5 μ m using Leica rotary microtome [LEICA RM 2125 RT, Germany] . The ribbons of the tissue sections were gently collected and placed onto the surface of a water bath heated at 40 $^{\circ}$ C. After the sections were appropriately spread on the water bath, they were mounted over tissue slides. The slides were arranged in slide racks and were placed in an oven with a temperature of 60 $^{\circ}$ C for 15 minutes to facilitate the adhesion of the specimens onto the glass slides. Specimens were then allowed to cool at room temperature and stained using Hematoxylin and Eosin staining method.

2.7. Hematological and biochemical analyses

Blood samples were withdrawn from the jugular vein at the end of the experiment following anesthetization and prior to scarification of each mouse. Parts of the blood samples obtained from each mouse were collected in separate test tubes with an anti-coagulant substance, EDTA [ethylene diamine tetra-acetic acid] and the remaining parts in plain test tubes with no EDTA. Blood samples from EDTA containing test tubes were immediately processed for hematological parameters using Automated Hematological Analyzer, Sysmex xt-1800i [Sysmex Corporation, Japan] . The hematological parameters White blood cell count [WBC] , Red blood cell count [RBC] , Haemoglobin concentration [HGB] , Hematocrit[HCT] , Mean Corpuscular Volume[MCV] , Mean Corpuscular Haemoglobin [MCH] , Mean Corpuscular Hemoglobin Concentration [MCHC] and Platelet count [PLT] were analyzed. For the biochemical analysis, the blood samples in the plain test tubes were allowed to stand for 3 hours for complete clotting and then centrifuged at 5000 rpm for 15 minutes using a bench top centrifuge [Humax-k, Human-Gmbh, Germany] . The sera were withdrawn and transferred into other clean vials and kept at -20 $^{\circ}$ C until analysis for clinical biochemistry measurements. The concentrations of alanine aminotransferase, aspartate aminotransferase, total bilirubin, urea, uric acid and creatinine were automatically determined using Cobasintegra 400 plus Analyzer [Rochdiagnostics, Japan] .

2.8. Light microscopy and photomicrography

Hematoxylin and Eosin stained tissue sections of the liver and kidney were carefully examined under binocular compound light microscope [Olympus cx41, Japan] . Tissue sections from the treated groups were examined for any evidence of histopathological changes with respect to those of the controls. After examination, photomicrograph of selected slides from both the treated and control groups were taken using [Evos XI, China] automated built-in digital photo camera under a magnification of x20 objective lens.

2.9. Statistical analysis

All data were organized and analyzed using SPSS version 23 statistical software. The values of body weight changes, different hematological and biochemical parameters were analyzed and the results were expressed as $M \pm SE[x]$ [standard error of the mean] . Differences between experimental and control groups were compared using one-way analysis of variance [ANOVA] , followed by Dunnett's T-test to determine their level of significance. Differences at $p < 0.05$ were considered statistically significant.

2.10. Ethical consideration

The study was conducted after approval of the study proposal by the Department of Anatomy, School of Medicine, College of Health Sciences, Addis Ababa University and a recommendation letter was sent to Ethiopian Public Health Institute from where ethical clearance letter was obtained. Animals used in this study were kept from any unnecessary painful and terrifying situations.

3. Results

3.1. Effects of the formulation on physical signs of toxicity, gross pathology and body weight

During the three months of study period no death was observed. Animals which received oral repeated doses of 1.25% ml/kg and 3.75% ml/kg body weight showed no signs and symptoms of toxicity including loss of appetite, diarrhea, dizziness, salivation, restlessness, partial hypo-activity and change in their general behavior in comparison with the control groups. Liver and kidney from all the test animals and control groups evaluated for gross pathological changes suggest no necropsy and lesions. Throughout the study period no significant [$p > 0.05$] body weight changes were observed in all the treatment groups as compared to the control groups. There was, however, body weight gain in both the experimental as well as control groups [figure 1 and 2].

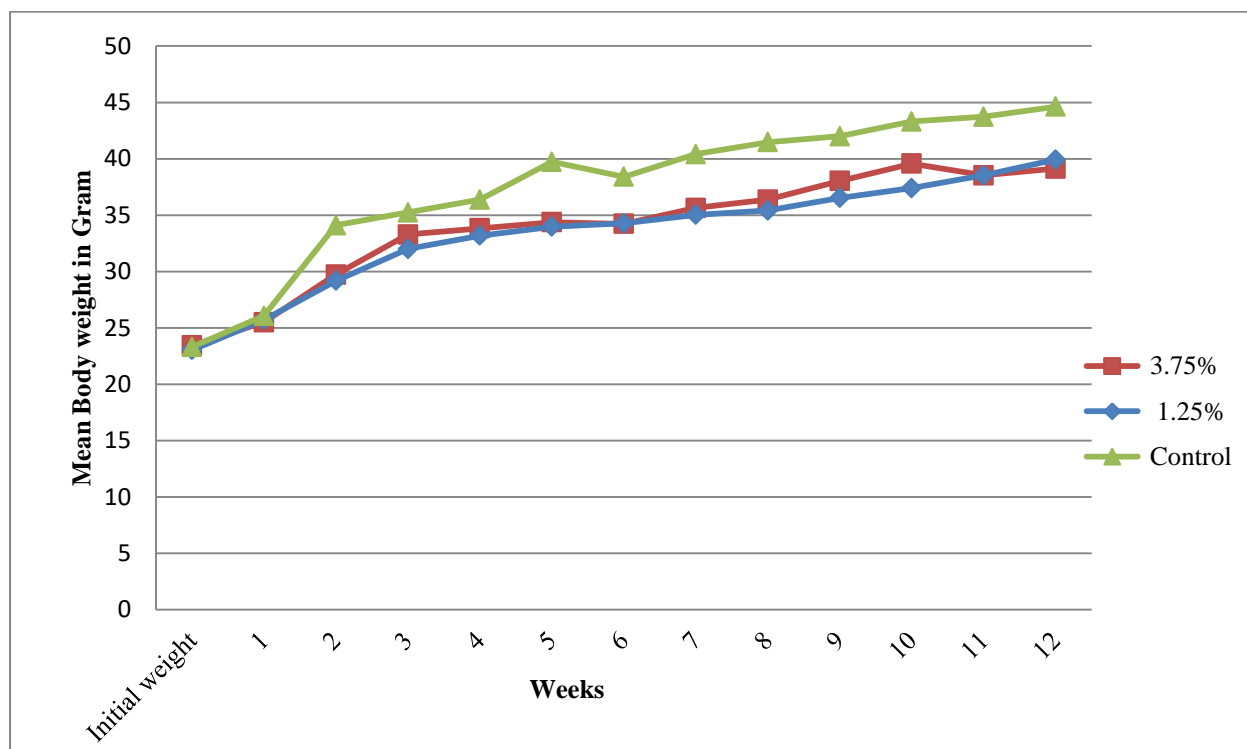


Figure 1: Comparison of mean body weight change between male mice treated with 1.25% and 3.75% 1ml /kg body weight doses of the *Eucalyptus globulus* based anti-ectoparasitic formulation with the control group.

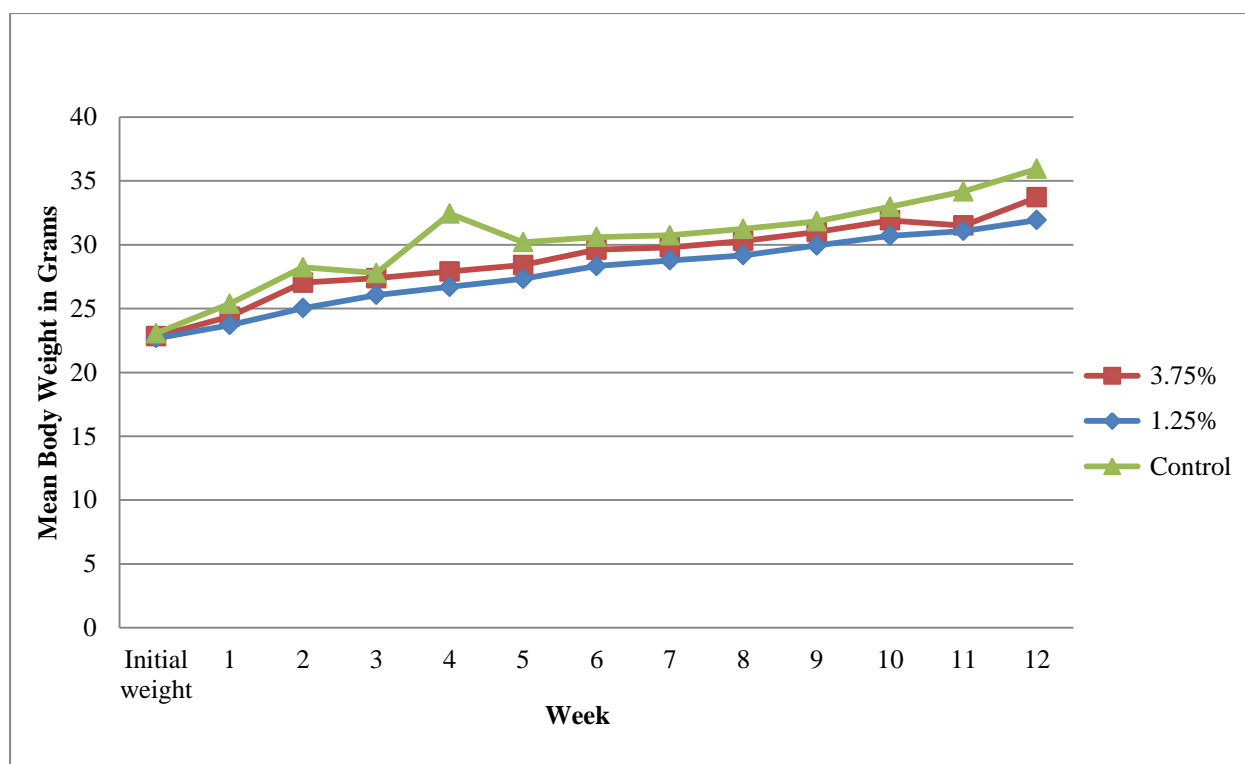


Figure 2: Comparison of mean body weight change between female mice treated with 1.25% and 3.75% 1ml /kg body weight doses of the *Eucalyptus globulus* based anti-ectoparasitic formulation with the control group.

3.2. Effects of the formulation on hematological and biochemical blood parameters

During the three months treatment of the formulation statistically significant [$p > 0.05$] was not observed in any of the hematological parameters in the experimental animals as compared to the control groups [table1] .

Table 1: Effects of the formulation on hematological parameters of treated mice compared to the controls.

Parameters	1.25% [ml/kg]	3.75% [ml/kg]	Control
WBC [$\times 10^3/\mu\text{L}$]	4.61 \pm 0.53[0.87]	4.25 \pm 0.38[0.99]	4.28 \pm 0.64
RBC [$\times 10^6/\mu\text{L}$]	9.00 \pm 0.61[0.27]	9.62 \pm 0.29[0.86]	9.90 \pm 0.32
HGB [g/dL]	16.00 \pm 1.04[0.57]	16.17 \pm 0.31[0.69]	16.83 \pm 0.18
HCT [%]	47.33 \pm 2.01[0.21]	50.03 \pm 1.11[0.96]	50.50 \pm 0.56
MCV [fL]	52.47 \pm 0.39[0.54]	52.57 \pm 0.46[0.64]	53.10 \pm 0.54
MCH [pg]	17.55 \pm 0.19[0.77]	17.35 \pm 0.22[0.94]	17.18 \pm 0.69
MCHC [g/dL]	34.50 \pm 0.38[0.67]	35.13 \pm 0.22[0.11]	34.11 \pm 0.44
PLT [$\times 10^3/\text{iL}$]	743 \pm 139.52[0.56]	734 \pm 122.66[0.53]	919 \pm 137.83

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

3.3. Effects of the formulation on biochemical blood parameters

Three months administration of the formulation showed no statistically significant [$p > 0.05$] change in the biochemical parameters of blood in the treatment groups compared to the controls [table 2] .

Table 2: Effects of the formulation on biochemical parameters of treated mice compared to the controls.

Parameters	1.25% [ml/kg]	3.75% [ml/kg]	Control
ALT [IU/L]	64.43±12.68[0.13]	62.70±10.01[0.11]	98.83±14.95
AST[IU/L]	338.30±43.92[0.68]	370.25±61.64[0.94]	389.13±32.82
ALP[IU/L]	83.85±2.59[0.66]	79.53±15.99[0.78]	66.00±10.48
Urea [mg/dL]	56.52±3.69[0.97]	68.06±12.04[0.35]	54.30±2.97
Creatinine [mg/dL]	0.15±0.004[0.59]	0.13±0.02[0.87]	0.13±0.01
Uric Acid	4.15±0.48[0.41]	4.67±0.99[0.19]	2.97±0.53
Total Bilirubin	0.23±0.1[0.26]	0.10±0.01[0.99]	0.18±0.01

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

3.4. Effects of the formulation on histology of liver

Light microscope examination of liver sections obtained from treated animals showed no significant histopathological architectural differences as compared with the controls [figure 3 and 4] . However, liver of animals treated with 3.75% ml/kg showed small number of portal mononuclear leukocytic infiltration [figure 4]

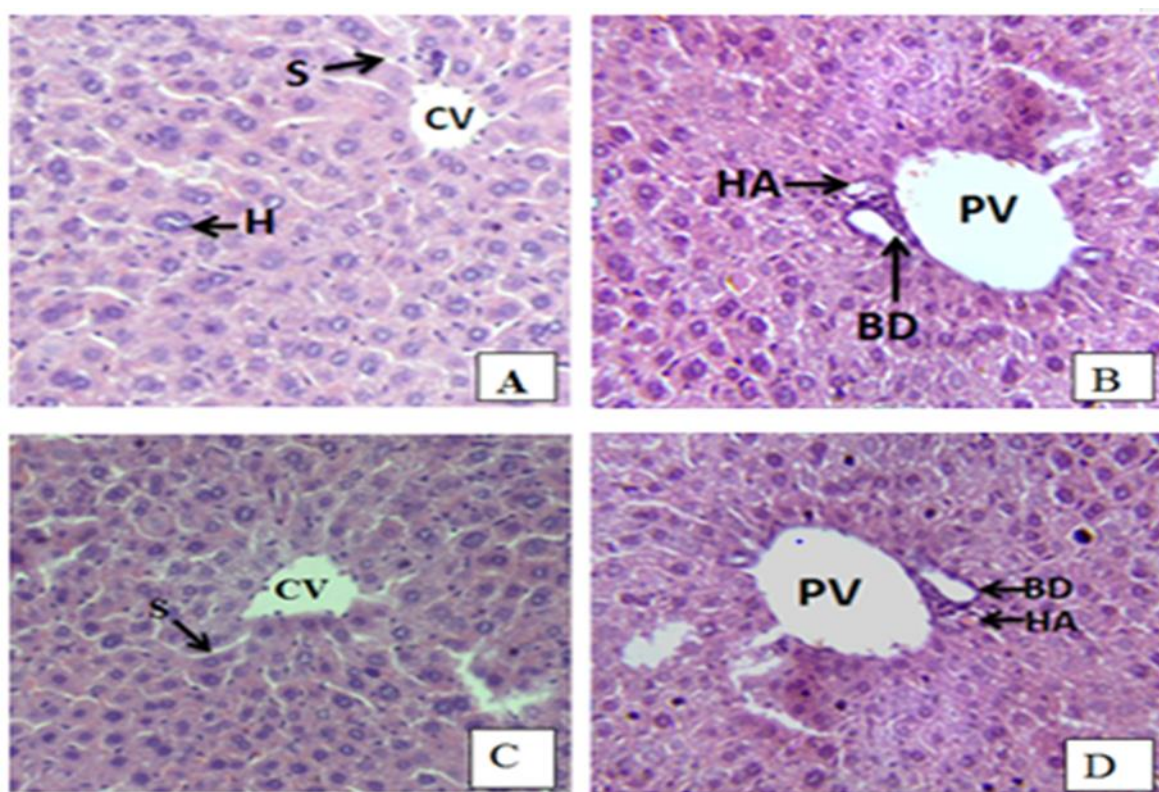


Figure 3: Photomicrographs of liver sections of control mice [A&B] , and mice administered with the formulation at 1.25% ml /kg body weight [C&D] . CV=Central vein, PV = Portal vein, HA = Hepatic artery, H= Hepatocyte, S= sinusoids, BD= Bile duct. [H&E, X200] .

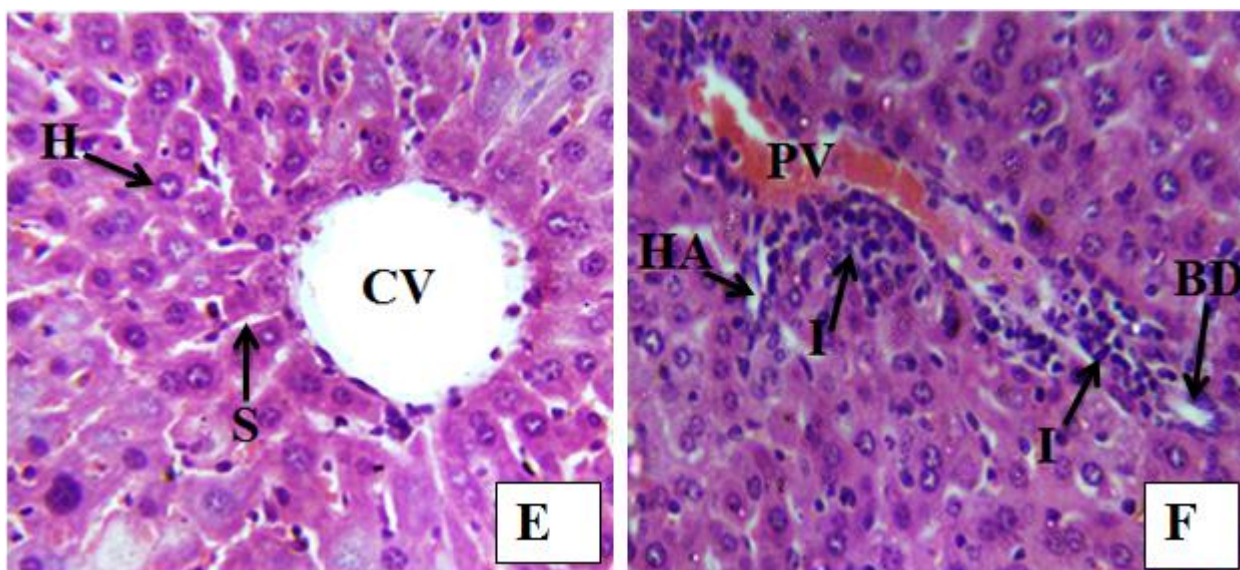


Figure 4: Photomicrographs of liver sections of mice administered with the formulation at 3.75% ml/kg body weight [E&F] . CV=Central vein, H= Hepatocyte, S= sinusoids, PV = Portal vein, BD = Bile duct, HA = Hepatic artery, I= Leukocytic infiltration. [H&E, X200] .

3.5. Effects of the formulation on histology of kidneys

Histological examination of kidney sections of treated animals showed the normal histological structures [figure5] . However, kidneys of animals treated with 3.75% ml /kg body weight showed minor tubulointerstitial leukocytic infiltration [Figure 5C] .

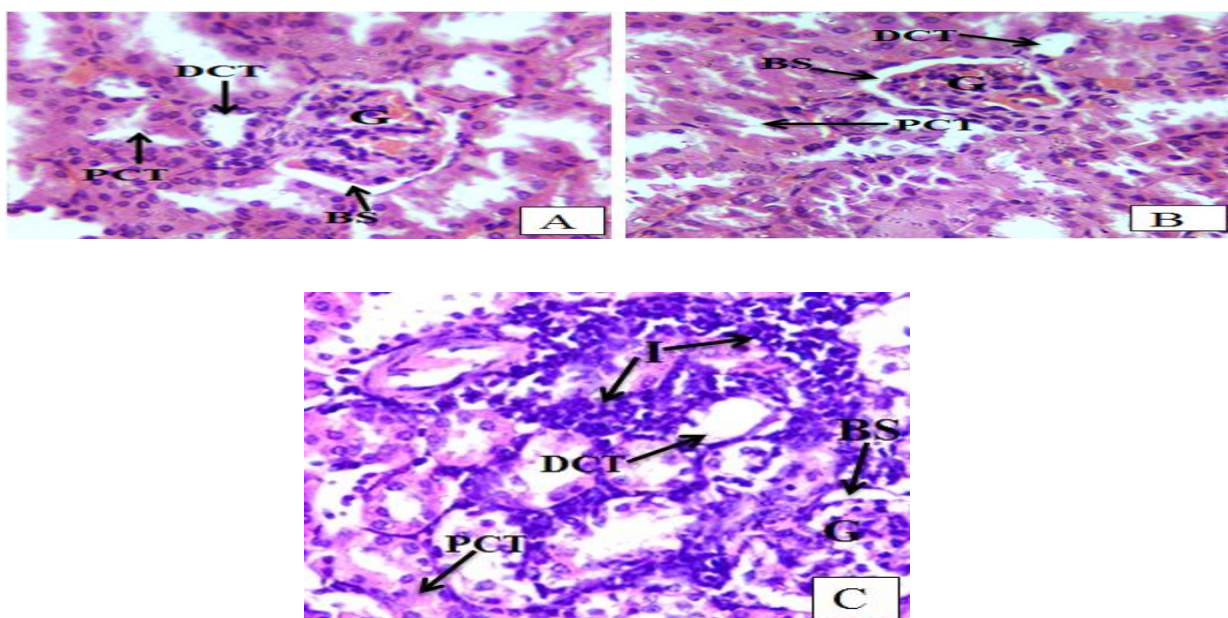


Figure 5: Photomicrographs of kidney sections of control mice [A] and mice administered with 1.25% ml /kg body weight [B] , and 3.75% ml /kg body weight [C] of the formulation. G = Glomerulus, BS= Bowman's space, DCT = Distal convoluted tubule, PCT = proximal convoluted tubule, I = leukocytic Infiltration. [H&E, X200] .

4. Discussion

In the present sub-chronic study, *Eucalyptus globulus* based anti-ectoparasitic formulation was administered daily at 1.25% and 3.75% ml/kg doses to reveal its possible toxicity effect. Toxic medications and pesticides damage body enzymes and affect hormones. Significant body weight deviations and mortality are among the outcomes of their adverse effects[11, 12] . The three months treatment of the formulation did not show any toxicity related gross pathological changes, loss of appetite, diarrhea, restlessness and hypo-activity. No animal was found dead and insignificant body weight changes were documented. In line with this finding Gebremickael and his colleagues[13] reported that acute and sub-chronic oral toxicity evaluation of *Eucalyptus Globulus* essential oil-water emulsion did not result any toxicity related mortalities and body weight change in mice. Hematological analyses provide blood related toxicity of drugs, formulations and plant extracts. Abnormalities in body metabolic processes and hematopoietic disorders can be estimated by assessing hematological parameters[14, 15] . In the present sub-chronic toxicity study the hematological indices WBC, RBC, PLT, HGB, HCT, MCV, MCH, and MCHC of the treated animals showed no significant changes as compared to the control groups. These observations demonstrate that the formulation does not produce toxic effect on hematological parameters at the given doses. In accordance with this finding other studies corroborated, the sub-chronic administration of *Eucalyptus globulus* essential oil- water emulsion does not produce hematotoxicity effect[13, 16] . The current result disagreed with the findings of study done on toxicological affects of essential oils from *Eucalyptus globules* on albino rats at which caused a significant increase in WBC counts and produced a significant decrease in haemoglobin concentration and platelets count[17] . The measurement of biochemical parameters provides information about the wellbeing of major organs such as the liver and kidney. Enzymes are widely used in toxicological studies as markers of detection and evaluation of cell damage. The liver is involved in waste product metabolism and clear potentially toxic substances out of the blood stream. An abnormal serum liver chemistry tests ALT, AST, ALP and total bilirubin levels may signal liver damage or alteration in bile flow. Those enzymes leak into the blood stream higher than normal amounts during hepatocyte injury and liver inflammation[18] . None of those liver chemistry tests in this study was significantly increased suggesting the formulation is not hepato-toxic at the given doses. This result is in agree with findings of related study by Gebremickael and his colleagues[13] . However, this was inconsistent with the findings of study done on the effects of aqueous extract of *eucalyptus glubulus* on lipid peroxidation and selected enzymes of rat liver at which resulted a significant change with the liver enzymes[15] . In synergy with liver the kidneys, filter and excrete toxic substances from the blood. Renal function blood tests aid in evaluating kidney problems and monitoring the response of kidneys to treatments. An abnormal creatinine, urea, and uric acid levels indicate problem with glomerular filtration and renal diseases[19] . Analyses of these parametrs in this study show insignificant variation inferring the formulation does not cause kidney failure. Besides, to its insignificant effect to the kidney function tests in this finding literatures have documented the extract of *Eucalyptus globulus* alleviates kidney damage[20] .

Toxic substances distort not only gross appearance but also cause significant changes in the basic histological architecture of organs and cells. The liver is an organ that performs several important functions in the body, such as degradation of drugs and toxins. In addition to liver function test enzymes liver histopathology examination is used to assess abnormal cells in the liver and tell how well the liver is working[21] . Sub-chronic

administration of the formulation in this study caused no significant histopathological changes in the liver. Although small number of infiltrations were observed around the portal area, hepatocytes were normal with intact cell margins and normal nucleus. The lobular architecture, portal triad, central vein and sinusoids appeared normal and there were no congestion of sinusoids. The observed small number of the mononuclear leukocytic infiltration may be associated with mild inflammation at higher dose of the formulation. In comparable with the liver the kidneys are among the most vital organs in the body that excrete wastes and toxins. Diagnosis and study of their tissues under a microscope provide information on the effects of drugs and formulations and guides treatment. In the present study normal histologic architecture of glomeruli, proximal and distal convoluted tubules were observed in kidneys of treatment and control animals. In line with these findings literatures have documented that *Eucalyptus globulus* leaves methanolic extract does not change the histo-architecture of liver and kidneys in rats and showed hepatorenal and renal toxicity protective properties these animals[22] .

5. Conclusion and Recommendations

During the three months sub-chronic study of the formulation, significant changes in the general behavior and body weight were not observed. Hematological and biochemical parameters as well as the gross and microscopic structures of liver and kidneys were not significantly affected. However, further toxicological investigation of the formulation is recommended on other vital internal organs and other animal models.

6. Limitations of the study

- The histopathological studies were not further investigated by other methods used to study tissue features in different ways such as electron microscopy.
- Reproductive and developmental toxicity investigations which reveal any congenital anomaly associated factor in the use of this formulation during gravidity, particularly at the embryonic/critical period of development were not carried out.

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