

Full Length Research Paper

Effect of *Combretum molle* (Combretaceae) seed extract on hematological and biochemical parameters

Merkin Anato¹, Mesele Bezabih² and Tsige Ketema^{1*}¹Department of Biology, College of Natural Sciences, Jimma University, Jimma, Ethiopia.²Institute of Health Science, Jimma University, Jimma, Ethiopia.

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Combretum molle (Combretaceae) is widely used traditional medicinal plant for treatment of different illnesses in most parts of Africa. However, its *in vivo* toxic effect is not evaluated yet. Thus, the aim of this study was to evaluate cytotoxic effect of *C. molle* seed extract on biochemical and hematological parameters using mice. Accordingly, effect of seed extract of the plant on hematological and biochemical parameters and on tissues of different organs were determined. Blood sample collected on the next day of the last dose administration was used for further analysis. Organs were collected in formalin for histological study. Result of the study showed that seed extract of *C. molle* did not cause significant effects on most red blood cells (RBCs) indices. Also, except eosinophil count, all white blood cells (WBCs) indices showed significant reduction ($P < 0.05$) under use of almost all doses of the seed extract. Level of liver enzymes such as glutamic oxaloacetate transaminase (sGOT) and glutamic pyruvic transaminase (sGPT) showed significant increment ($P < 0.05$) in mice treated with different doses of the plant seed extract, while concentration of albumin was significantly reduced ($P < 0.05$). Similarly, significant increment ($P < 0.05$) in levels of kidney function indicators urea and creatinine was observed. At relatively lower dose (125 mg/kg), the crude extract of *C. molle* seed showed lower effects on haematological, biochemical and immunological parameters. As dose increased, its cytotoxic effect increased.

Key words: *Combretum molle*, hematological, biochemical, histopathology.

INTRODUCTION

Combretum molle is one of the herbal plants belonging to the family Combretaceae which includes 20 genera and about 600 species (Miaffo et al., 2015). This plant is

popularly used in South Africa for the treatment of stomach pains, dysentery, gastric ulcers, abdominal disorders, and other illnesses (Eloff et al., 2008). Also,

*Corresponding author. E-mail: tsigeketema@gmail.com.

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roots of *C. molle* seem to have a variety of uses against hookworm, stomach pains, snake bite, leprosy, fever, dysentery, general body swellings, and abortion as well as for swelling of the abdomen, sterility and constipation (Fyhrquist et al., 2012). This plant bark and root *in vitro* tested in different African countries for treatment of bacteria (Sahlu, 2013; Regassa and Araya, 2012). Also, seed of the plant tested against fungi and bacteria (Masoko et al., 2007; Amare and Tadesse, 2016), and leaf against helminths (Ademola and Eloff, 2010). In addition, *C. molle* bark extract was tested for its *in vitro* antimicrobial activities (Regassa and Araya, 2012; Amare and Tadesse, 2016).

Moreover, different species of *Combretum* have been reported to have *in vitro* anti-plasmodial activity against chloroquine sensitive *Plasmodium falciparum* strains (Benoit et al., 1996; Ancolio et al., 2002; Karou et al., 2003; Sanon et al., 2013). Furthermore, tannin, stem bark, seed and leaf extract of *C. molle* plant have revealed *in vitro* activity against *P. falciparum* (Asres and Balcha, 1998; Asres et al., 2001; Gansane et al., 2010). Almost every part of these plants (roots, leaves, seeds, twigs, and stem bark) has been used in African traditional medicine for the treatment of various ailments and diseases (Miaffo et al., 2015). Similarly, seed of this plant is widely used by traditional medicinal plants practitioners in Ethiopia, mainly among rural residents of Gambella region, for treatment of malaria, HIV and other diseases. While seed of *C. molle* plant widely used by local people for treatment of various illness; its effects on haematological and biochemical parameters were not evaluated yet. Therefore, the objective of the current study was to investigate *in vivo* effect of seed extract of the *C. molle* plant on haematological and biochemical parameters in mice.

MATERIALS AND METHODS

Plant material preparation

The seed of *C. molle* was collected from the vicinities of Gambella region, Agnua zone, Gog woreda (about 886 km south west of Addis Ababa). The voucher specimen [CM7 (*C. molle* No.7)] was identified and deposited at Jimma University Herbarium (JUH), Department of Biology, Jimma University, Ethiopia. Seed of the plant part was collected and dried in the processing room and then powdered, kept at room temperature in a well-closed and amber bottle until extracted. The dried and powdered seed (100 g) was extracted by maceration in 300 mL of 80% methanol for three consecutive days (72 h) at room temperature. The extraction process was facilitated by using frequent shaking.

The mixture was first filtered using cotton wool and then with Whatman filters paper No. 1 (Whatman®, England). The residue was re-macerated for another 72 h twice and filter. The combined filtrate was then dried by rotary evaporator (Buchi Rota Vapor, Switzerland) at a temperature of 40°C and 45 rpm. After dried in water bath, a total dry extract was harvested and the dried extract kept at -20°C in a tightly closed bottle in a refrigerator until used for testing.

Experimental animals

A total of 65 Swiss albino mice were used for acute toxicity and cytotoxic activity testing of *C. molle* seed extract. Accordingly, both sex mice, age 8 to 10 weeks, and weighed of 30 to 40 g were maintained at temperature of 22±3°C, relative humidity of 40 to 50% and 12 h light/12 h dark cycle. The animals were housed in transparent plastic cage with SS sipper 250 mL water bottle. Wood shaving was used as bedding and it was replaced every morning after the cage was cleaned and disinfected with 70% alcohol. The animals were kept under unlimited access to food and water. The mice were allowed to acclimatized to the laboratory environment for at least one week before being subjected to the experiments.

Acute toxicity determination

Mice were randomly arranged into four groups (each group containing 5 non-infected) for acute toxicity testing of the crude extract of *C. molle* seed at different dose levels (500, 1000, and 2000 mg/kg), and the last group, considered as control (Ketema et al., 2015). The acute toxicity was carried out in accordance to OECD guidelines. The mice were acclimatized and then fast for about 3 h. The extract was administered to the animals orally after it dissolved in 20% of Tween 80 in saline. The control group received 0.5 mL of 20% of Tween 80 in saline through the same route. Any acute sign of toxicity and mortality of each group within 24 h after administration of the extract was recorded. Toxicity signs such as death, changes in physical appearance, and behaviour including loss of appetite, hair erection, lacrimation, tremors, convulsions, salivation, and diarrhoea, and other signs of overt toxicity were directly observed for 72 h. The feeding condition (consumption of pellet and water) was recorded for 14 days.

Animal treatment and study protocol

Animals were randomized into four groups (I-IV) each containing four mice grouped for haematological and biochemical study. Doses of the extract used for this assay was 100 mg/kg (group I), 250 mg/kg (group II) and 500 mg/kg (group III) and 0.5 mL 20% of Tween 80 in saline (group IV or negative control). Animals were sacrificed 24 h after the last doses were administered (orally) lasted seven days. The mice at the time of sacrifice were weighed and then terminally anaesthetized. Blood sample was collected through cardiac puncture and collected in EDTA coated tubes. The abdominal cavity was opened through a midline abdominal incision and the liver and kidney were immediately removed and placed in fixative and processed for histological study.

Haematological and biochemical assay

About 0.2 mL blood sample collected in EDTA tube was used for quantification of total WBC, lymphocytes, monocytes, basophils eosinophil, neutrophils, RBCs, Haemoglobin (Hb), Haematocrit (HCT), platelets, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular haemoglobin (MCH) using CBC machine. About 1 mL of the remaining blood sample was centrifuged at 10,000 rpm for 10 min. The supernatant (plasma) was transferred into new Eppendorf tube and followed by measurement of liver enzymes, serum glutamic oxaloacetate transaminase (sGOT), serum glutamic pyruvic transaminase (sGPT), and albumin (Alb); indicators of kidney function such as creatinine (Cr) and urea; inflammation test,

Table 1. Hematological parameters of Swiss albino mice received three different doses of *C. molle* seed extract.

Group	Hematological parameter								
	RBC ($10^3/\mu\text{L}$)	Hb (g/dL)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dL)	RDW-SD (fl)	RDW-CV (%)	Platelet $\times 10^3/\mu\text{l}$
Control	10.44 \pm 1.05	15.36 \pm 1.48	50.9 \pm 0.51	48.7 \pm 0.72	14.7 \pm 1.33	30.26 \pm 0.39	35.4 \pm 1.2	25.06 \pm 2.27	687.33 \pm 57.2
100 mg/kg	9.25 \pm 1.12	14.27 \pm 1.32	45.07 \pm 0.203	48.7 \pm 0.05	15.45 \pm 1.37	31.75 \pm 0.32	36.42 \pm 1.28	24.3 \pm 2.43	734.2 \pm 36.8*
250 mg/kg	9.28 \pm 0.98	14.1 \pm 1.35	45.53 \pm 0.11	48.40 \pm 0.79	15.13 \pm 1.42	31.29 \pm 0.42	35.98 \pm 1.17	24.26 \pm 2.18	746.5 \pm 18.3*
500 mg/kg	8.98 \pm 1.23*	13.54 \pm 1.08*	43.52 \pm 0.22*	48.83 \pm 0.51	15.2 \pm 1.29	31.23 \pm 0.43	38.1 \pm 1.3*	25.13 \pm 2.38	714 \pm 45.7

Values with asterisk are indicating significantly different from values of controls. RBC: Red blood cells, Hb: haemoglobin, HCT: haematocrit platelets; MCV: mean corpuscular volume; MCHC: mean corpuscular haemoglobin concentration; MCH: mean corpuscular haemoglobin.

C-reactive protein (CRP) using LAMBDA 750 UV/Vis/NIR Spectrophotometer.

Histological study

For histological analysis, liver and kidney of the sacrificed mice were collected in 10% buffered neutral formaldehyde; paraffin-embedded liver and kidney tissues were labelled and stained with hematoxylin and eosin. Slides were coded and scored for histological evidence of liver and kidney damages.

Data analysis

All data was analysed using SPSS software (version 20.0) and the results were expressed as mean \pm standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare results among and within groups for differences followed by Turkey's HSD post-hoc test. Sample analysis was repeated at least three times to get solid statistical data. Significance level was considered at $P < 0.05$.

Ethical consideration

The study was ethically approved by the Ethical Review Committee of the College of Natural Sciences of Jimma University, Ethiopia. Mice were handled in humane way

and extreme precautions were taken to avoid stress induced by poor handling techniques. Blood samples were collected from mice under final anaesthesia.

RESULTS

Acute toxicity

Physical characteristics such as body weight change, sign of any toxicity, regular measurement of water and food consumed, and any behavioral changes among the mice were assessed every day. Accordingly, significant body weight differences between treated and control groups over the observed period was not observed. Also, adverse effects that require modification of the procedure were not encountered. Likewise, differences were not seen in amount of pellet and volume of water consumed between treated and control groups.

Haematological parameters test

Outcomes of hematological parameter showed that, use of higher doses of crude extract *C. molle*

seed for seven days cause significant ($P < 0.05$) reduction of count of RBCs, Hb and HCT levels compared to the control group. On the other hand, relatively lower doses of the seed extract (100 and 250 mg/kg) caused significantly ($P < 0.05$) increased count of platelet. However, as the dose increased to 500 mg/kg, platelet count reduced, but not significantly different from the control group. Other hematological parameters such as MCV, MCH, MCHC and RDW-CV were not affected in mice that received the treatment (Table 1).

Biochemical tests

The two kidney function indicators; urea and creatinine were significantly affected under use of higher doses of the seed extract (250 and 500 mg/kg) (Figure 1). Likewise, level of liver enzymes were affected in mice received relatively high dose of the seed extract. At highest dose (500 mg/kg), level of GPT enzyme was significantly ($P < 0.05$) elevated.

While level of the other enzyme, GOT, was significantly increased in mice treated with all

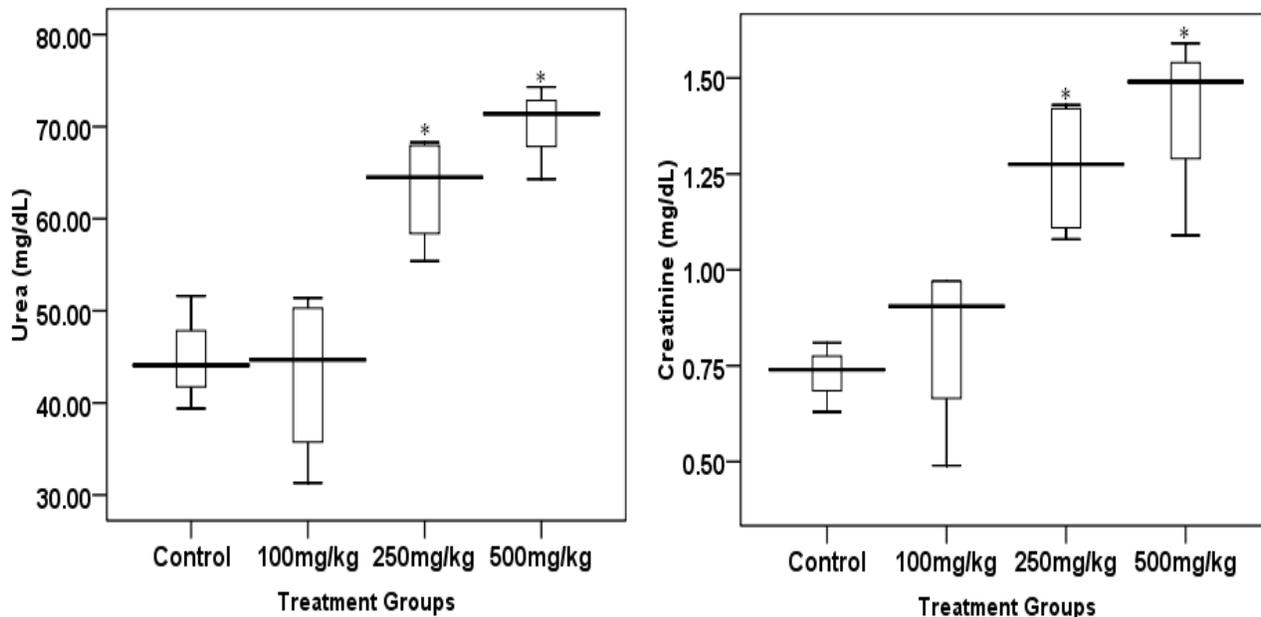


Figure 1. Levels (Mean \pm SEM) of kidney test indicators (urea and creatinine) of Swiss albino mice received *C. molle* seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls.

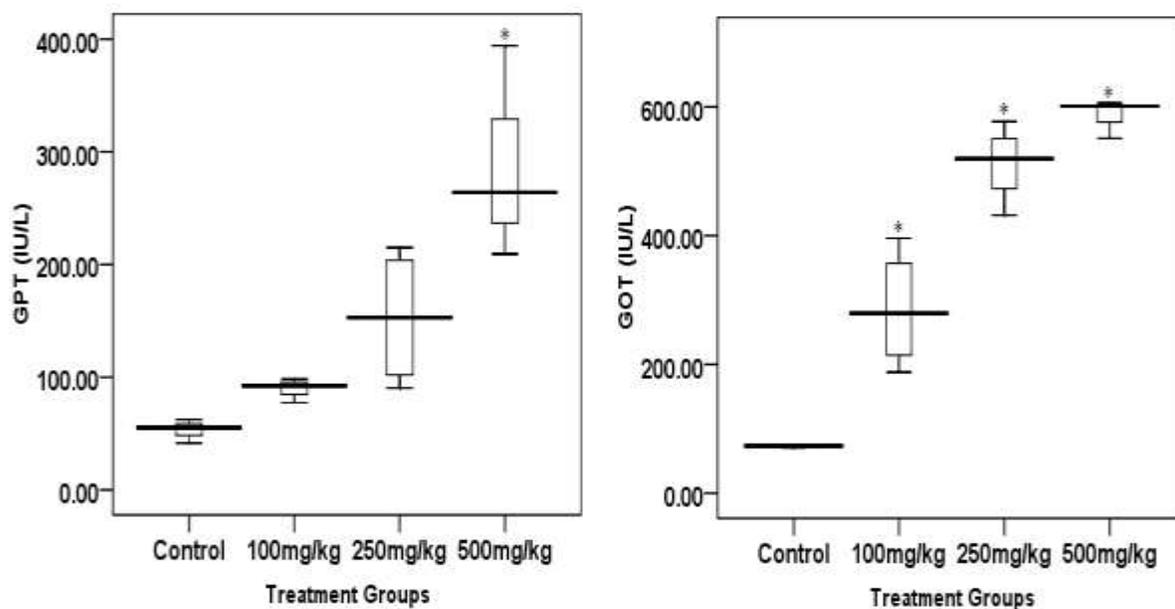


Figure 2. Level of liver enzymes (mean \pm SEM) of Swiss albino mice received *C. molle* seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls.

doses of the extract (100, 250, and 500 mg/kg) (Figure 2). On the other hand, level of albumin showed significant reduction in mice treated with the two higher doses (250 and 500 mg/kg) (Figure 3).

On the other hand, the plant caused substantial production of one of the inflammation reaction indicators. Under all doses (100, 250 and 500 mg/kg), level of C-reactive protein showed significant ($P < 0.05$) increment

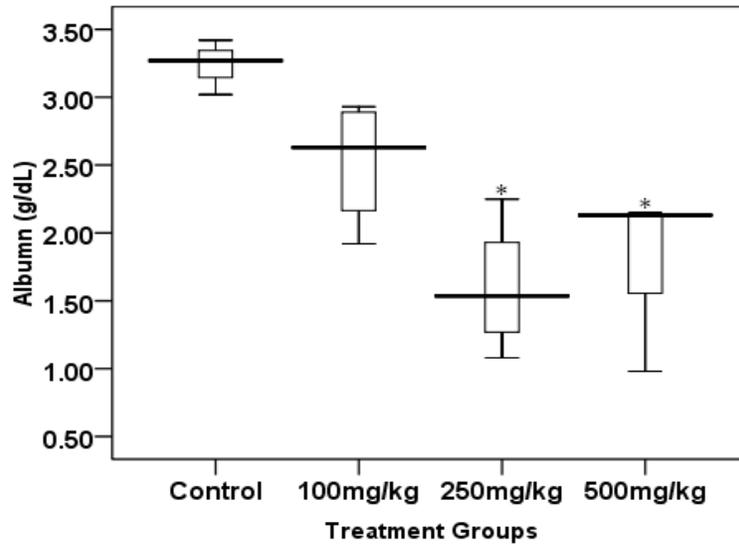


Figure 3. Level of albumin (mean ± SEM) of Swiss albino mice received *C. molle* seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls

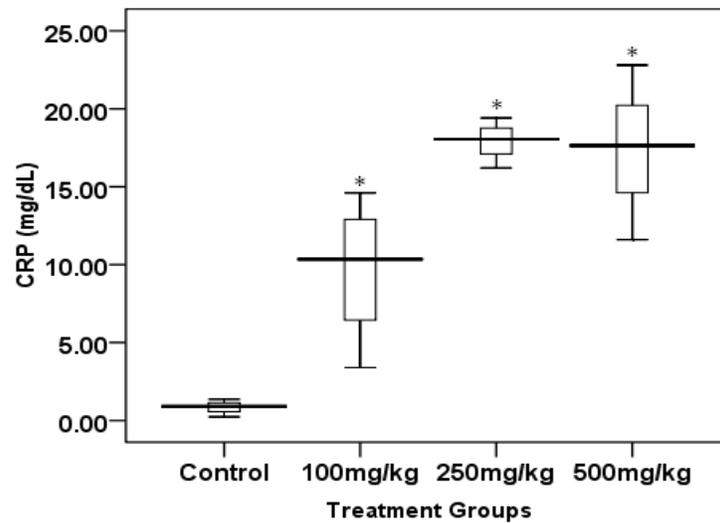


Figure 4. C-reactive protein (CRP) level (mean ± SEM) in Swiss albino mice received *C. molle* seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls.

(Figure 4).

Effect of *C. molle* seed extract on immune cells

Effect of the plant seed extract on innate immune cells

such as basophils showed variability. Under lowest dose (100 mg/kg), percentage of basophils showed significant increment ($P < 0.05$), while as dose increased to 250 and then 500 mg/kg, the level of this immune cell significantly decrease ($P < 0.05$) as compared to the control mice. On the other hand, eosinophils count showed consistent

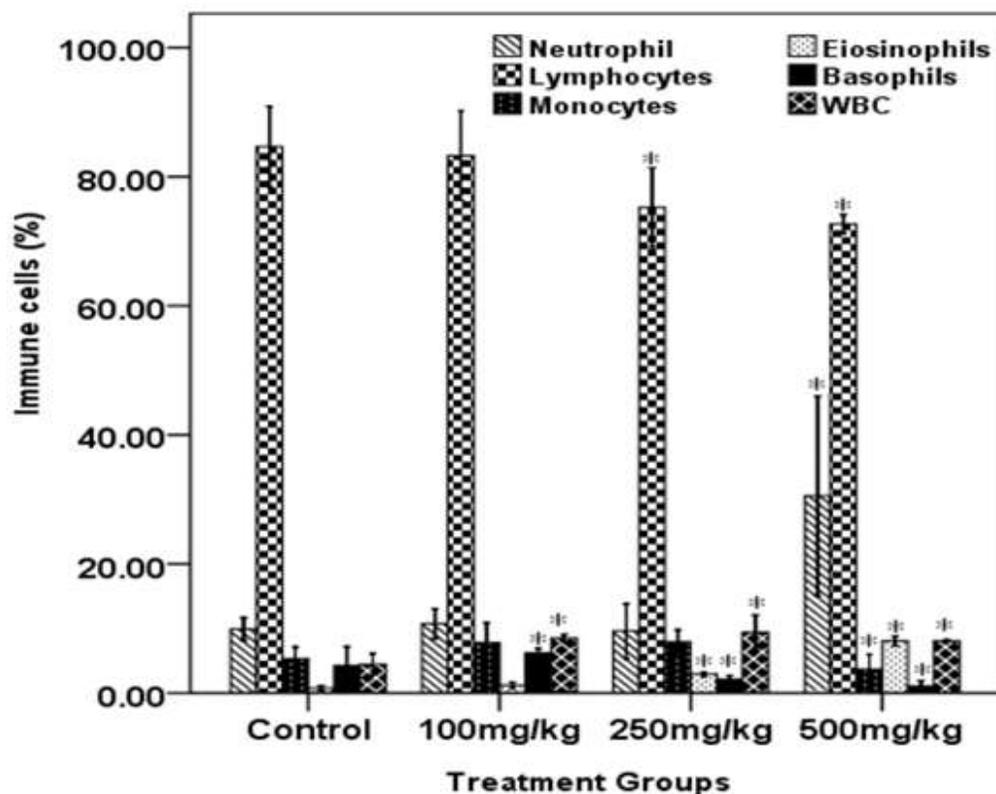


Figure 5. Levels of immune cells (mean \pm SEM) in Swiss albino mice received *C. molle* seed extract for seven days. Values with asterisk are significantly different (ANOVA, Tukey's HSD *post-hoc* test) from values of the controls.

significant increment ($P < 0.05$) at relatively high doses (250 and 500 mg/kg). Except, at the lowest dose (100 mg/kg) for lymphocytes count, under all doses, differences were not observed between the control and treated groups. Moreover, almost all immune cells showed significant ($P < 0.05$) reduction under use of the highest dose (500 mg/kg) of the extract (Figure 5).

Histological analysis

Higher dose of seed extract of *C. molle* (500 mg/kg) caused necrosis of cells of kidney and liver (Figure 6). While, at relatively the lowest doses use (100 and 250 mg/kg), kidney cells were normal, but liver cells showed slight changes such as fatty and water charges.

DISCUSSION

Despite wide use of *C. molle* as medicinal plant for treatment of various ailment including helminthic, protozoal, bacterial and other infectious diseases in different parts of Africa (McGaw et al., 2001; Fyrquist et

al., 2002; Bussmann et al., 2006; Grønhaug et al., 2008), studies conducted on its *in vivo* toxicity test is scanty. According to the traditional practice of the local community, the seed of the plant is used after it is extracted by water as a solvent. Thus, the current study methanol was used as a better solvent for the extraction. Also to mimic human practice in local community, after single oral dose acute toxicity testing, seed crude extract of the plant was administered for seven consecutive days. Accordingly, the crude extract of *C. molle* seed showed less acute toxicity even at highest dose (2000 mg/kg). However, its longer use (seven days) even at low doses (100 mg/kg) had negative impact on proper functioning of kidney and liver. This was observed by excess secretion of liver enzymes (GPT and GOT), and reduction in production of protein (albumin) by liver cells. It is believed that a rise in plasma enzymes (GPT and GOT) levels are the pertinent indicators for liver toxicity (hepatotoxicity) (Singh et al., 2011). The estimation of this enzyme is a more specific test for detecting any liver abnormalities since it is primarily created in the liver (Amacher, 2002). Albumin is the main protein in blood and is made by the liver. Hepatotoxicity leads to decrease in albumin production (Thapa and Walia, 2007). So, that

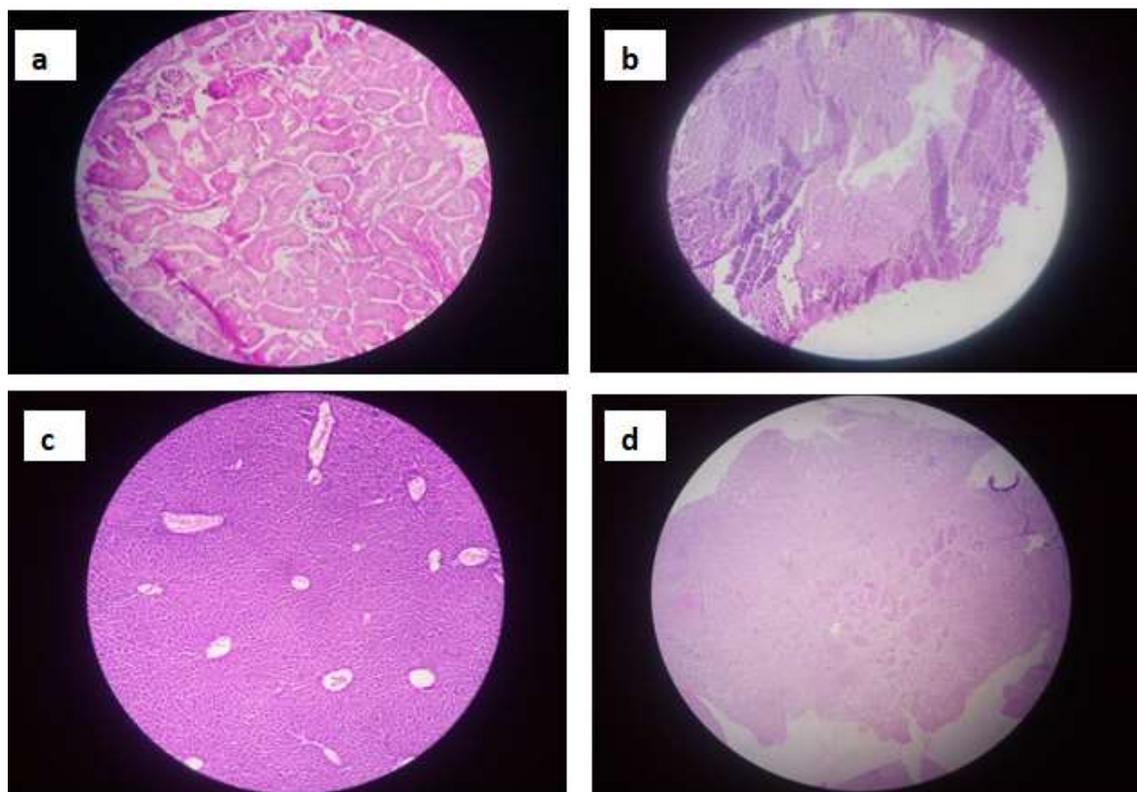


Figure 6. Tissues of kidney (a=control, b= treated with 500 mg/kg seed extract of *C. molle*) and Liver (c=control, d= treated with 500 mg/kg seed extract of *C. molle*) microphotograph of Swiss albino mice received *C. molle* seed extract. Formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and Eosin (HE). The sections were photographed with X40 objective (bars are 100 μ m).

significant reduction in level of albumin is direct indication for failure of liver to produce adequate level of this protein.

Likewise, excess levels of creatinine and urea in plasma of mice treated with the plant extract was detected. Usually, kidneys maintain optimum chemical composition of the body fluids by acidification of urine and removal of metabolite wastes as creatinine, urea, uric acid, and ions (Biyani et al., 2003). Thus, the observed excess secretion of urea and creatinine has implication on the toxic potential of the seed extract on kidney. This revealed that a continuous use of the plant seed extract has toxic effects on vital organs. This was more evidenced by histological study of tissues of different organs, where at the highest dose (500 mg/kg) cause intense necrosis of cells of the organs (liver and kidney). The seed extract of *C. molle* caused excess secretion of CRP, an inflammation indicator in mice treated with even at low doses. This protein is a special type of protein (plasma protein) produced by the liver cells only during episodes of acute inflammation, in response to pro-inflammatory cytokines (Zhang and Jianxiong, 2007). As a result, level of CRP, as indicator of systemic inflammation, in plasma

highly increases during acute phase response to tissue injury, infection, or other inflammatory stimuli (Gruys et al., 2005). Different *in vitro* studies suggested that mollic acid glucoside isolated from *C. molle* leaves has anti-inflammatory and anti-asthmatic activities (Ojewole et al., 2008; Shah et al., 2011; Yeo et al., 2012). However, in the current *in vivo* study, the observed elevated level of CRP, an indicator of systemic inflammation, could be due to the damaged liver, since elevated liver enzymes are associated with higher CRP concentrations and liver is the main produced of CRP (Kerner et al., 2005). Population of Gambella region, Ethiopia (where the plant is collected) has a practice of using this plant for some days (seven days are common) continuously for treatment of different ailments (personal observation). This practice might prone the users for unexpected inflammation reaction even at low doses and increases vulnerability to various diseases, such as rheumatoid arthritis, atherosclerosis and asthma (Mueller et al., 2010).

Some hematological parameters such as RBC count, Hb and HCT levels were significantly reduced in mice treated with the highest dose of the plant seed extract. It shows that at high dose use, seed extract of the *C. molle*

plant could also have toxic effect on RBC indices and increase susceptibility to anemic condition. On the other hand, low doses of the plant seed extract associated with excess production of platelets count. The observed elevated count of platelet in mice treated with 250 mg/kg dose might be related to the organs damage. It appears that platelet homeostasis is controlled by thrombopoietin, a glycoprotein hormone produced by the liver and kidney which regulates the production of platelets. Damaged liver and kidney might increase release of this hormone to the blood and increases production of platelets. This was evidenced by the renal or liver failure conditions associated with the increased platelet production in patients with kidney and liver problems (Linthorst et al., 2002; Makar et al., 2013). This is because of high level of thrombopoietin in blood of patients which causes abnormal production of platelets (Makar et al., 2013).

Conclusion

At relatively lower dose, *C. molle* seed extract did not cause effects on haematological and biochemical parameters. Thus, further isolation and evaluation of active components of the seed extracts for their toxicity and testing against pathogens should be a priority area of investigation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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