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Research

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In vivo antimalarial activity of the crude leaf extract of *Combretum molle* against *Plasmodium berghei* in mice

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Abstract

Background: Malaria is an infectious, hematologic disease causing death and illness in children and adults, especially in tropical countries. The aim of this study was to evaluate the antimalarial activity of *Combretum molle* extract *in vivo* assays against *Plasmodium berghei* in Swiss albino mice.

Methods: *Plasmodium berghei* a rodent malaria parasite was inoculated to healthy Swiss Albino mice age 6–8 weeks either sex, weight 20–33g. 100, 200 and 400mg/kg/day of Crude methanolic extract of Combretum molle were administered. Parameters such as Percent parasitemia, body weight, Body temperature, packed cell volume and survival time were then determined using standard tests. Data were analyzed using one-way ANOVA followed by the Post hoc Tukey HSD test with SPSS software version 24.0 and $P \leq 0.05$ considered as statistically significant.

Results: Chemosuppresive effect exerted by the crude extract ranged between 27-68%. The curative effect of the crude extract was in the range of 25-49% and ptophylactic effect of the crude extract was in the range of 51–76.2%%. The maximum effect in all tests on Chemosuppresive, curative, Prophylactic, prevention of weight loss, body temperature and packed cell volume and an increase in mean survival time was observed at higher doses of the crude extract.

Conclusion: From the present study it can be concluded that the crude extract of *Combretum molle* leaves has been shown promising antimalarial activity. This finding supports the traditional use of the plant for the treatment of malaria in Ethiopia. Thus, it could be considered as a potential source to develop safe, effective and affordable antimalarial agent.

Key Words: Antimalarial, *Combretum molle*, crude extract, *Plasmodium berghei*, sub-acute toxicity

Background

Malaria is an infectious, hematologic disease [1] causing death and illness in children and adults [2]. Malaria is caused by single-cell Plasmodium species that invade, grow and replicate in red blood cells in a cyclic pattern which makes it such a deadly disease[3, 4]. Malaria control requires prevention, primarily vector control, and prompts treatment with effective antimalarial drugs [5]. Malaria is among the most devastating and widespread tropical parasitic diseases in which most prevalent in developing countries [6]. Antimalarial drug resistance results in a global resurgence of malaria making a major threat to malaria control.[7].

From the World Health Organization (WHO) 2018 malaria report 219 million cases of malaria from 90 countries. The WHO African region with 200 million cases (92%) in 2017, especially Sub-Saharan Africa suffers by far the greatest malaria burden worldwide. Almost 93% of all deaths due to malaria in 2017 were from Africa [8].

Seventy percent of malaria caused deaths occurring among the under five-year age group. Nigeria, the Democratic Republic of the Congo, Burkina Faso and India accounted for 47% of all malaria deaths globally [9]. *P. falciparum* species are dominant in Africa and *P.vivax* infection is in Southeast Asia and South America [10]. In pregnant, women *P. falciparum* causes infections because of parasite's ability to massively sequester in the placenta and compromised immune status, mainly in the first and second trimesters of pregnancy [11].

About 60% of the Ethiopian population is at risk of malaria with major transmission seasons from June to December and remain responsible for a massive burden of disease and death [12, 13].

The experimental plant

The Combretaceae is a large family of herbs, shrubs, and trees, comprising about 20 genera and 600 species with tropical distribution around the globe and centers of diversity in Africa and Asia. Simple hairs of indumentum, multicellular or glandular hairs secreting calcium oxalate and forming scales or present beneath cuticle and making leaf blade surface verruculose and sometimes translucent

dotted. Leaves (opposite, sub opposite, whorled, spiraled, or alternate, usually petiolate), flowers are unisexual or bisexual and the seed is usually one-seeded and flattened [14].

Combretum is the largest and most widespread genus of Combretaceae which comprises approximately 200-250 species. *Combretum molle* is a shrub or small, graceful, deciduous tree, 3 - 13 m high, with a crooked or lean trunk, occasionally swollen at the base, and up to 30 cm in diameter. *Combretum molle* (R.Br.ex.G.Don) Engl and Diels are indigenous to Africa and are one of the important medicinal species of the family [15]. Different parts of the *Combretum molle* are widely used as a medicinal plant to treat various diseases such as parasitic, protozoan and other infectious diseases [16].

The Fresh leaf is boiled in water and the decoction is drunk by a cup of tea which was studied from Delanta (Amhara region), and Amaro District (Southern Nations Nationalities People region). Probably leaves are the center of intermediary metabolism leading to biologically active secondary metabolites [17, 18]. The *in vitro* cell culture (K562S human monocyte infected with *P. falciparum* W2) study had shown activity with an IC_{50} value of 5.7 or 7.9µg/mL, respectively from 80% methanol extract of leaves. Ethanol 90% extract of leaves, root bark or stem bark *in vitro c*ell culture with *P. falciparum* K1 showed activity with an IC_{50} value of 4.0 µg/mL[19].

Stem bark *in vitro* cell culture (Trophozoites of *P. falciparum*) of acetone fraction showed activity with IC_{50} value of 8.17µg/mL. MeOH extract of dried stem *in vitro* cell culture with *Plasmodium falciparum* also showed activity with an IC_{50} value of 1.25 µg/Ml [20, 21].

Methods

Plant Material

Fresh leaves of *Combretum molle* were collected from "Tara Gedam", Libo Kemkem District, South Gondar Zone, Amhara region 85 Kilometer away from Gondar city on December 13, 2018. The collected plant material was wrapped and covered with plastic sheets during transportation. The specimen of the plant was authenticated by a botanist, in the biology department, University of Gondar and

was deposited in the herbarium with a voucher specimen (No 001MAS) for future reference.

Preparations of crude methanolic extract

The leaves of *C.molle* were washed with tap water gently to eliminate any dirt and dried at ambient temperature, protected from light, reduced to an appropriate size by hand compression and stored at ambient temperature until extraction. 1500g of leaf powder of *C.molle* was weighed with electric balance and transferred to the Erlenmeyer flasks and 80% methanol (one gram of *C.molle* powdered leaf to ten milliliters of 80% methanol ratio) was added and agitated in between. After 72 hours of maceration extraction was performed using thick layers of 40 mesh gauze and then filtered with Whatman filter paper no1 two times and 2nd extraction was repeated after 72 hours of maceration and the 3rd extraction was repeated another 72 hours later (with a total of nine days). The extracts were combined and concentrated by the dry oven at 40^{0}C and the yield was determined and found to be 287g (19.13%) and stored in a refrigerator at 4^{0}C until use [22].

Phytochemical screening

Secondary metabolites are classes of compounds that are known to show a curative activity against several ailments in humans and then could explain the traditional use of medicinal plants for the treatment of some illnesses. Complex structural molecules such as alkaloids, Phenolic compounds, anthraquinones, resins, saponins, steroids, and terpenoids are found in a more restricted distribution than primary metabolites. They are not indispensable for the plant that contains them; at least their metabolic functions have not been discovered yet[23]. Standard qualitative tests were employed to detect secondary metabolites [24 - 31].

Animals and parasite

Swiss albino mice weighing between 20 - 33g, 6 – 8 weeks of age and either sex were purchased from the Ethiopian public health institute (EPHI) Addis Ababa. The animals were kept in cages and housed in a standard animal house under a natural 12/12h light-dark cycle at room temperature and maintained on a standard pellet diet and water ad libitum. Before the experiment, they were acclimatized to the test environment for one week. The care and handling of mice were according

to international guidelines for the use and maintenance of experimental animals [32, 33].

Chloroquine-sensitive malaria, *P. berghei* was obtained from EPHI in the traditional medicine research department Addis Ababa and transported by infecting the donor mice and maintained the parasite up to the main procedure of the study.

Acute toxicity test

Five mice weighing between 20 - 28 grams, 6-8weeks of age female sex were kept for one week prior to dosing to acclimatize. One mouse was randomly taken for sighting study and food but not water was withheld for 4 hours. Following the period of fasting, the mouse was weighed and the crude methanolic extract of *C. molle* leaf 2000mg/kg body weight was administered by the oral route. Strict observation within 30minutes and periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. Changes in skin and fur, eyes, mucous membranes, respiratory, autonomic and central nervous system changes, somatomotor activity and behavior patterns were observed. Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were evaluated. But the mouse in the sighting test did not show any sign of toxicity for four-days and the main toxicity doses of the remaining 4 mice for methanolic crude extract were given and followed as usual for 14 days [32, 34].

Subacute toxicity test

The subacute toxicity study was conducted on 24 healthy adult female mice divided into four groups of six each. The control group received solvent; the other three groups were given 100, 200 and 400mg/kg/day of *C. molle* crude extract orally for 28 days. The mice were observed every two hours for toxic signs and their weights were measured weekly for five weeks. On the 28^{th} day, mice were anesthetized with ketamine and sacrificed. Their Liver and kidneys were removed and weighed [32, 35].

Tissue processing

Immediately removed liver and kidneys were placed in 10% formalin with phosphate-buffered saline solution overnight at room temperature. After overnight fixation, the tissues were dehydrated with increased concentration of alcohol: 70%, 80%, 90% for five and half hour, then cleared with xylene for seven hours. Finally, the tissues were impregnated for six hours. Then, tissue blocks were prepared by

embedding with paraffin wax in a square metal plate and labeled. Tissue blocks were sectioned with a thickness of 5µm using a rotary microtome. Strips of sections were gently lowered into the surface of a warm water bath at 40^{o}C for sections to be floated, then mounted with coated egg albumin slides and the slides containing paraffin wax within a slide holder were placed in an oven with a temperature of 60^{0}C for 10-15 minutes until they become free of wax.

Sectioned tissues were placed in xylene to deparaffinize and hydrated with decreasing alcohol concentrations, then stained with Hematoxylene for 5 minutes and counterstained by using eosin for one minute. After washing in tap water, the sections were dehydrated with increased alcohol concentration and cleared with xylene.

Finally sections were mounted on microscopic slides by using Canada balsam mount and cover slips and examined the tissue under the compound light microscope with different objectives were taken a photo automatically with magnification of x40 [36-38].

Pharmacologic screening (4-day Suppressive test)

In vivo evaluation of methanolic leaf crude extract of *C. molle* antimalarial activity on rodent malaria parasite *P.berghei*. Donor *P. berghei* infected Swiss albino mice (parasitemia of approximately 30%) were sacrificed by decapitation and, then blood was collected through cardiac puncture with a sterile disposable needle and syringe. The blood was diluted with sterile sodium chloride 0.9% in water in such a way that 0.2ml of blood contained about 10^7 infected RBCs and mice were infected by 0.2ml blood suspension intraperitoneally which was expected to produce steadily rising consistent infection of the required intensity in mice [35]. Then Mice were randomly divided into five groups of six each, weighed, and maintained in a standard diet. The doses of crude extract were adjusted from the safest dose of acute-toxicity study and were 5%, 10%, and 20% (i.e. 100,200 and 400mg/kg) once daily for 4 days. The fourth group received chloroquine base 25mg/kg (positive control) and the fifth group was received solvent which contains 3ml of 96% ethanol + 7ml of Tween 80 + 90ml of distilled water (negative control)[39, 40] and

administered by oral route using oral gavage, starting from 3 hours of parasite inoculation.

Determination of parasitemia

On day 4 (96 hours) post-infection a drop of blood was collected from the mice by vein section of the tail and transferred onto the edge of a microscopic slide and drawn evenly across the second slide to make a thin blood film and allowed to dry at room temperature, fixed with methanol, stained with 10% Giemsa stain for 15 minutes. Slides were viewed using light microscopy with oil immersion (1000 x magnification). Percentage parasitemia was determined by counting the number of PRBCs out of 200 RBCs in random fields of the microscope. Five fields of approximately 200 cells counted and the parasitemia was calculated as the percentage of the total RBCs containing PRBCs [41, 42]. The body weight, body temperature, packed cell volume and mean survival time were determined for each group arithmetically [40].

Percent parasitemia was calculated according to the following formula

$$\%\ \text{paristemia} = \frac{\text{Total number of PRBC}}{\text{Total number of RBC}} \times 100$$

Where:- PRBC: Parasitized red blood cells

RBC: red blood cells

Average percentage of parasitemia suppression was calculated as follows:

$$\text{Average}\,\%\ \text{of paristemia supression} = \frac{\text{Av.}\,\%\ \text{of parasit.in control} - \text{Av.}\,\%\ \text{of parasitemia in test}}{\text{Av.}\,\%\ \text{of parasitemia in control}} \times 100$$

Av = average

Rane's (Curative) test

Evaluation of the curative potential of methanolic leaf crude extract of *C. molle* was carried out according to the method described by curative methods [43]. On Day 0, standard inocula of about 10^7 infected erythrocytes were inoculated in mice intraperitoneally and randomly divided into their respective groups weighed and maintained in a standard diet. Seventy-two hours later they were dosed Crude methanolic extract at 100,200 and 400mg/kg/day, Chloroquine base 25mg/kg/day and solvent which were prepared from 3ml of 96% ethanol + 7ml of Tween 80 + 90ml of distilled water once daily for five days. Giemsa stained thin blood film was prepared from the tail of each mouse daily for 5 days (day 3,4,5,6 & 7) to

monitor the parasitemia level. The body weight, body temperature, packed cell volume and mean survival time were determined for each group arithmetically [40].

The Repository (Prophylactic) Test

In prophylactic test prior to infection, mice in the test groups (three groups with 100, 200 and 400mg/kg of crude extract) were administered once daily orally for three days. The remaining two groups were administered with the standard drug (chloroquine base 25 mg/kg) and vehicle (solvent 10ml/kg/day). On the fourth day, a standard inoculum of *Plasmodium berghei* containing ~$1x10^7$ infected erythrocytes was administered intraperitoneally to each mouse. On the seventh day of infection (seventy-two hours later), thin blood smears (Giemsa stained) were prepared from the tail of each mouse by vein section. Percentage parasitemia as well as the chemo suppression, body weight, body temperature, packed cell volume and mean survival time were determined for each group arithmetically [43].

Measurement of Packed Cell Volume (PCV)

Packed cell volume (PCV) was measured to predict the effectiveness of the test extract and fractions in preventing hemolysis resulting from increasing parasitemia associated with malaria. Heparinized capillary tubes were used for the collection of blood from the tail of each mouse. The capillary tubes were filled with blood up to three-fourth of their volume and sealed at the dry end with sealing clay. The tubes were then placed in a micro-hematocrit centrifuge, with the sealed end outwards and centrifuged for 5 min at 12,000 rpm. The tubes were taken out of the centrifuge and PCV was determined using a standard Micro-Hematocrit Reader [44, 45].

Monitoring of body weight and temperature changes

In Peter's test the bodyweight was measured one hour before parasite infection on D0 and at the end of D3 after treatment completion, while the rectal temperature was measured 1 hour before and 3 hours after parasite infection and then consecutively for 4 days to monitor the consequence of plant extracts on mice's body temperature. For Rane's test, the body weight and temperature were measured three hours before infection on day 0 (D0) and consecutively for five during the treatment period to establish the effect of plant drug on malarial mice. For the prophylactic test, the body weight and temperature were measured three hours

before infection on day 0 (D0) and consecutively from D4–D7 after infection period to establish the effect of plant extract on *P. bergei* infected mice [46].

Data quality control

The quality of data was assured by using randomization during the grouping of experimental animals. Unique codes were used for all frosted microscopic slides during the preparation of blood smears. Parasitized red blood cells were counted blindly by laboratory technologist (Medical laboratory technologist, MSc in medical parasitology).

Data analysis

The results of the study were expressed as the mean ± standard error of the mean (M ± SEM). Comparison of parasitemia and statistical significance was determined by one-way ANOVA (a repeated measure of analysis of variance) descriptive statistics followed by Post Hoc tests for multiple comparisons of Tukey HSD and paired t-test was employed to test significance of the difference between initial and final results within the same and in between groups using SPSS for window (Version 24.0) statistical package. All data were analyzed at a 95% confidence interval ($\alpha = 0.05$) was considered statistically significant [40].

Results

Phytochemical screening

The phytochemical screening of 80% methanol crude extract of *Combretum molle* indicated the presence of alkaloids, cardiac glycosides, flavonoids, phenols, saponins, tannins, and terpenoids.

Acute oral toxicity test

The acute oral toxicity study showed that no death and any sign of toxicity to mice with an oral dose of 2000mg/kg of the crude extract of *C. molle* observed for two weeks. Here it could be deduced that the lethal dose fifty (LD_{50}) was above 2000mg/kg per body weight. The bioactive constituents of the plant are safe for use even though it is difficult to extrapolate the result to humans.

Subacute toxicity study

The crude extract of *C. molle was* administered for consecutive 28 days at doses of 100, 200 and 400mg/kg/day showed a mean loss of 1.01±0.84g ($p = 0.013$),

1.23±0.86g (P = 0.0.012), and 0.45±0.66g (P= 0.065) respectively. Mice treated with solvent gain a mean body weight of 3.05±0.95g.(See table 1).

Table 1: Body weight of mice in subacute toxicity study of *C. molle* crude extract

Treatment group	Body weight (g)					
	Initial weight	**After 1 week**	**After 2 weeks**	**After 3 weeks**	**After 4 weeks**	**Body Wt. dif.**
Control	28.44±0.97	31.52±0.72	31.45±0.68	31.47±0.54	31.48±0.62	3.05±0.95
CM100mg/kg	26.46±1.27	28.29±1.66	28.65±1.27	26.42±1.48	25.45±2.04	-1.01±0.84[a1]
CM200mg/kg	27.31±0.68	27.11±1.69	24.76±1.26	26.68±1.76	25.89±1.52	-1.23±0.86[a1]
CM400 mg/kg	27.47±0.84	27.43±1.04	24.60±0.63	26.70±1.18	26.73±0.90	-0.45±0.66

Results presented as mean ±SEM; n= 6; CM= *Combretum molle*

The mean weight of kidney in subacute toxicity test at 100, 200 and 400mg/kg/day was 0.17±0.02g (P= 0.945), 0.17±0.02g (P=0.945) and 0.13±0.01g (P= 0.180) respectively. There was no significant difference compared to negative control group (0.18±0.01g). (See table 2)

The mean weight of liver in subacute toxicity test at 100, 200 and 400mg/kg/day was 1.80±0.09g (P=0.242), 1.65±0.10g (P=0.028) and 1.55±0.10g (P=0.008) respectively. At doses 200mg/kg and 400mg/kg produced significant differences compared to negative control group ($p<0.05$ and $p<0.01$) respectively compared to the negative control 2.05±0.08g.(See table 2)

Table 2: Absolute organ weight of mice during sub -acute toxicity study of *Combretum molle* crude extract

Treatment group	**Weight (g)**	
	Kidney	**Liver**
Control	0.18±0.01	2.05±0.08
CM100mg/kg	0.17±0.02	1.80±0.09
CM200mg/kg	0.17±0.02	1.65±0.10[a1]
CM400mg/kg	0.13±0.01	1.55±0.10[a2]

Results presented as mean ±SEM; n= 6, [a] compared to control, [1]$p<0.05$, [2]$p<0.01$
CM= *Combretum molle*

Result of histopathology of liver and kidney of mice

Effects of methanolic leaf extracts of *C. Molle* on histology of the liver and kidney tissues.

Microscopic examination of liver sections of mice treated with C. molle leaf crude extract at 100, 200, and 400mg/kg/day showed the normal architecture of structural units of the hepatic lobules, formed by cords of hepatocytes separated by hepatic sinusoids. The central vein and portal area containing branches of hepatic artery, bile duct and portal veins were maintained with their normal appearance similar to the negative control (See figure 1A).

Microscopic examination of kidney sections of mice treated with *C. molle* leaf crude extract at 100, 200, and 400mg/kg/day showed the normal architecture of structural units of the Glomerulus, Urinary space, Proximal convoluted tubule, Distal convoluted tubule, Macula densa, Vascular pole were maintained with their normal appearance similar to the negative control (See figure 1B).

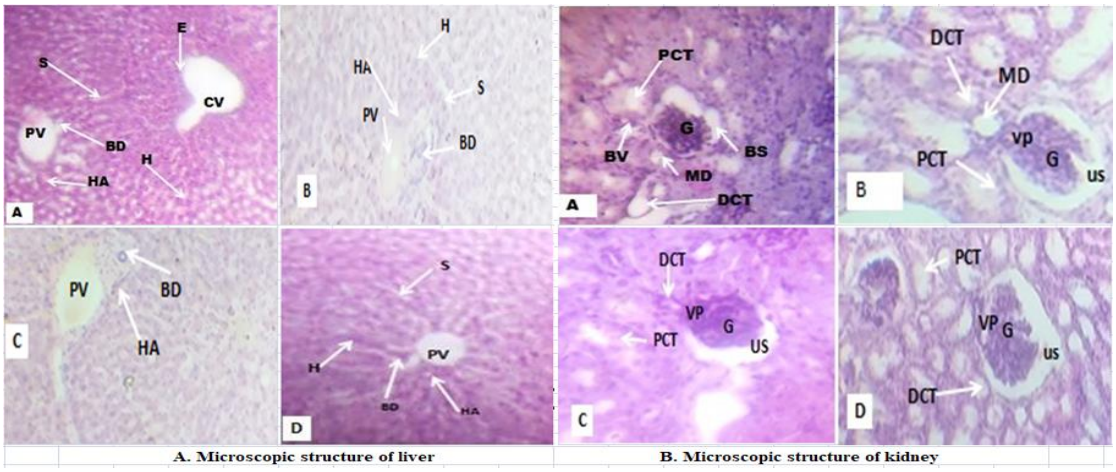

Figure 1: Microscopic architectures of liver and kidney after histopathological procedure (A=Negative control, B=100mg/kg, C=200mg/kg, and D=400mg/kg) of C. molle crude methanolic extract.

Liver: (CV=Central vein, H=Hepatocytes, E=Endothelial cells, S=sinusoids, K=Kupffer cells)

Kidney: (G =Glomerulus, US=Urinary space, PCT=Proximal convoluted tubule, DCT=Distal convoluted tubule, MD=Macula densa, VP=Vascular pole)

Antimalarial suppressive test

Antimalarial suppressive test of methanolic crude extract of *C. molle* leaf and chloroquine base against *P.berghei* infected mice resulted in reduced parasite load as compared to their respective negative control group. Methanolic crude extract at 100, 200 and 400mg/kg/day and Chloroquine bate at 25mg/kg/day showed 33.08, 45.36 and 76.17 95.71 % parasitemia suppression respectively and the results were highly significant($P < 0.001$) compared to the negative control group at day-4.(see table 3)

Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day showed 7.00 ±0.97 (P=0.988), 9.83 ±1.92 (P=0.571), 13.83 ±3.20 (P=0.049) and Chloroquine base at 25mg/kg/day showed 27.00 ±2.03 (P<0.001) days of mean survival time in four day suppressive test respectively. Chloroquine base at 25mg/kg/day showed highly significant (P<0.001) in survival days compared to negative controls (5.67±0.49)(see table 3).

Table 3: Mean Percent Parasitemia and mean percent parasitemia suppression and mean survival time of *P. berghei* infected mice treated with methanolic crude extract of *C. molle* leaf in the 4 day suppressive test.

Extract	% Parasitemia	% Parasitemia Suppression	Mean survival time
CON	33.40±2.43	0.00	5.67±0.49
CM_{100}	22.35 ±1.76	33.08^{a3d3e3}	7.00 ± 0.97^{e3}
CM_{200}	18.25 ± 2.27	45.36^{a3d1e3}	9.83 ± 1.92^{e3}
CM_{400}	7.96 ± 2.26	76.17^{a3b3c1}	13.83 ± 3.20^{a1e2}
CQ25	1.43 ±0.75	95.71^{a3b3c3}	$27.00 \pm 2.03^{a3b3c3d2}$

Data are expressed as mean ± SEM; n = 6, CON = Control (solvent 10ml/kg), CM= crude methanolic extract, CF = Chloroform fraction of *Gardenia ternifolia*, CQ = chloroquine, % par supp= % parasitemia suppression, Numbers refer to dose in mg/kg. a, compared to neg. control; b, to 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to CQ10mg/kg: 1p<0.05, 2p<0.01, 3p<0.001.

Plasmodium berghei infected mice treated in a suppressive test at 100mg/kg/day methanol crude extract loss mean body weight of 0.85 ±0.210g (p = 0.297), 200mg/kg/day loss mean body weight of 1.97± 0.673g (P = 0.991), 400mg/kg/day loss mean body weight of 1.62±0.825g (P= 0.882), and Chloroquine base at 25mg/kg/day loss mean body weight of 0.17 ±0.099g (P= 0.050) and mice treated with solvent loss mean body weight of 2.29 ±0.339g. The extract of *C. molle* leaf prevented a loss of

body weight in infected mice at high doses. Small decrease in body weight was observed in Chloroquine treated group. (See figure 2A)

Plasmodium berghei infected mice treated in suppressive test at 100mg, 200mg, 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day decrease a mean body temperature of 0.82±0.16 ^{o}C (p = 0.106), 0.52±0.13^{o}C (P = 0.003), 0.22±0.17 ^{o}C (P< 0.001) and 0.05±0.03 ^{o}C (P< 0.001) and mice treated with solvent decrease a mean body temperature of 1.35 ±0.18 ^{o}C. The extract of *C.molle* leaf decreases a mean body temperature in infected mice from 200mg/kg/day (P ≤ 0.003). (See figure 2B)

Plasmodium berghei infected mice treated in suppressive test at 100mg, 200mg, 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day loss a mean packed cell volume of 1.75±0.63 % (p = 0.795), 1.25±0.48 % (P = 0.315), 0.80±0.37 % (P=0.60) and 0.83±0.17 % (P= 0.050) and mice treated with solvent loss a mean packed cell volume of 2.40±0.40 %. The extract of *C.molle* leaf did not show a significant difference in a mean packed cell volume compared to the negative control. (See figure 2C)

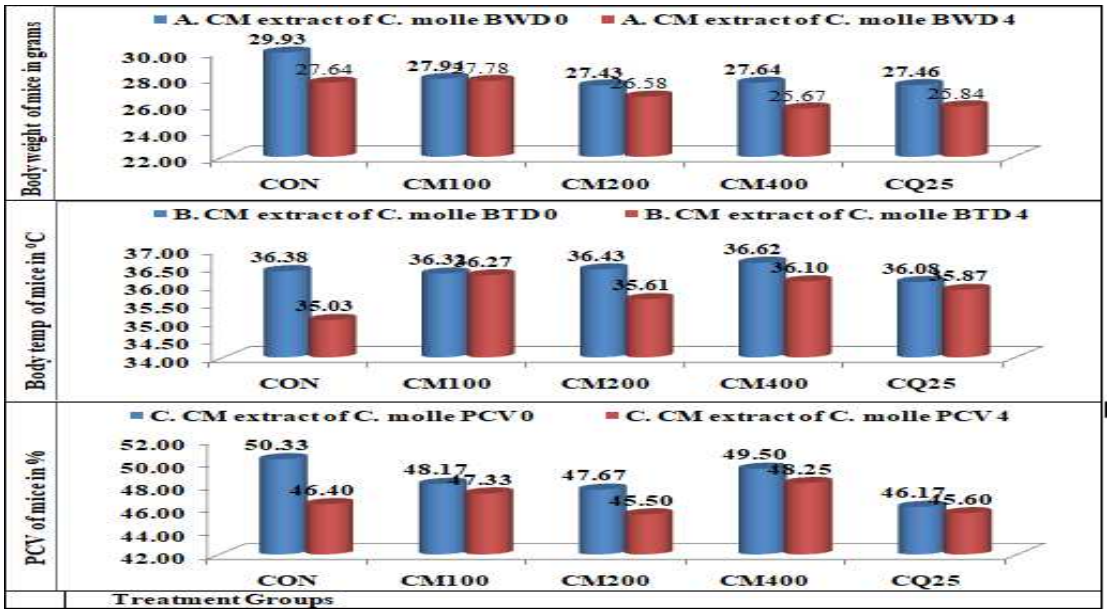

Figure 2: Showed effect of Crude methanolic extract of C. molle leaf *in* P. berghei infected mice in Chemosuppresive test. [CON = negative control (solvent 10ml/kg), CM= chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero,

BWD4= body weight at day 4, PCV= packed cell volume, numbers = dose in mg/kg/day]

Methanolic crude extract in a curative test at 100, 200 and 400mg/kg/day and Chloroquine bate at 25mg/kg/day showed 43.3, 62.98 and 68.48 and 99.05 % parasitemia suppression respectively and the results were highly significant($P < 0.001$) compared to the negative control group at day-7.(see table 4)

Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day showed 7.83±0.40 (P=0.997), 9.50±0.72 (P=0.248), 12.17±1.01 (P<0.001) and Chloroquine base at 25mg/kg/day showed 28.83±0.65 (P<0.001) days of mean survival time in curative test respectively. Crude methanolic extract 400mg/kg and Chloroquine base at 25mg/kg/day showed highly significant (P<0.001) in survival days compared to negative controls (7.50±0.34)(see table 4).

Table 4: Parasitaemia and percent parasitemia inhibition and survival date of *P. berghei* infected mice treated with crude methanolic extract of *C.molle* in the Rane's (curative) test

Extract	%Para Day 3	%Para Day4	%Para Day 5	% Para Day 6	Day 7		Av. Survival date
					%Para	% Parasitemia inhibition	
CON	22.73±0.84	24.95±0.92	27.55±0.73	32.68±0.29	38.76±0.82	0.00	7.50±0.34
CM_{100}	24.77±0.72	24.58±0.73	23.72±0.65	22.77±0.67	21.98±0.76	43.3^{a3c3d3e3}	7.83±0.40^{d3e3}
CM_{200}	22.33±0.60	23.02±0.48	20.48±0.65	17.37±0.79	14.35±1.01	62.98^{a3b3e3}	9.50±0.72^{e3}
CM_{400}	22.57±0.48	23.32±0.50	17.77±0.49	14.03±0.54	12.22±0.49	68.48^{a3b3e3}	12.17±1.01^{a3b3e3}
CQ_{10}	23.17±0.84	17.53±0.97	8.07±1.92	2.37±0.90	0.37±0.27	99.05^{a3b3c3d3}	28.83±0.65^{a3b3c3e3}

Data are expressed as mean ± SEM; n = 6: CQ = chloroquine, CON = Control, CM, crude methanolic extract of *Combretum molle*, % par supp= % parasitemia suppression, Numbers refer to dose in mg/kg. a, compared to neg. control; b, to 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to CQ 25mg/kg: 1p<0.05, 2p<0.01, 3p<0.001.

Plasmodium berghei infected mice treated in a curative test at 100mg/kg/day methanol crude extract increase a mean body weight of 2.41±1.912g (p = 0.011), 200mg/kg/day loss mean body weight of 0.88±2.140g (P = 0.128), 400mg/kg/day loss mean body weight of 4.38±1.014g (P= 0.697), and Chloroquine base at 25mg/kg/day loss mean body weight of 2.42±0.927g (P= 0.315) and mice treated with solvent loss mean body weight of 8.18±3.624g. (See figure 3A)

Plasmodium berghei infected mice treated in a curative test at 100mg, 200mg, 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day decrease a mean

body temperature of 0.95±1.36 ºC (p = 0.673), 0.73±1.05 ºC (P = 0.991), 1.01±1.05 ºC (P= 0.998) and 4.86±1.48 ºC (P< 0.001) and mice treated with solvent decrease a mean body temperature of 1.57±1.63ºC. (See figure 3B)

Plasmodium berghei infected mice treated in a curative test at 100mg, 200mg, 400mg /kg/day methanol crude extract and Chloroquine base at 25mg/kg/day loss a mean packed cell volume of 6.65±1.89% (p = 0.001), 5.41±0.84% (P < 0.001), 0.53±1.24% (P<0.001) and 1.02±0.45% (P< 0.001) and mice treated with solvent loss a mean packed cell volume of 15.02±1.04%. The extract of *C.molle* leaf showed a significant difference in a mean packed cell volume compared to the negative control. (See figure 3C)

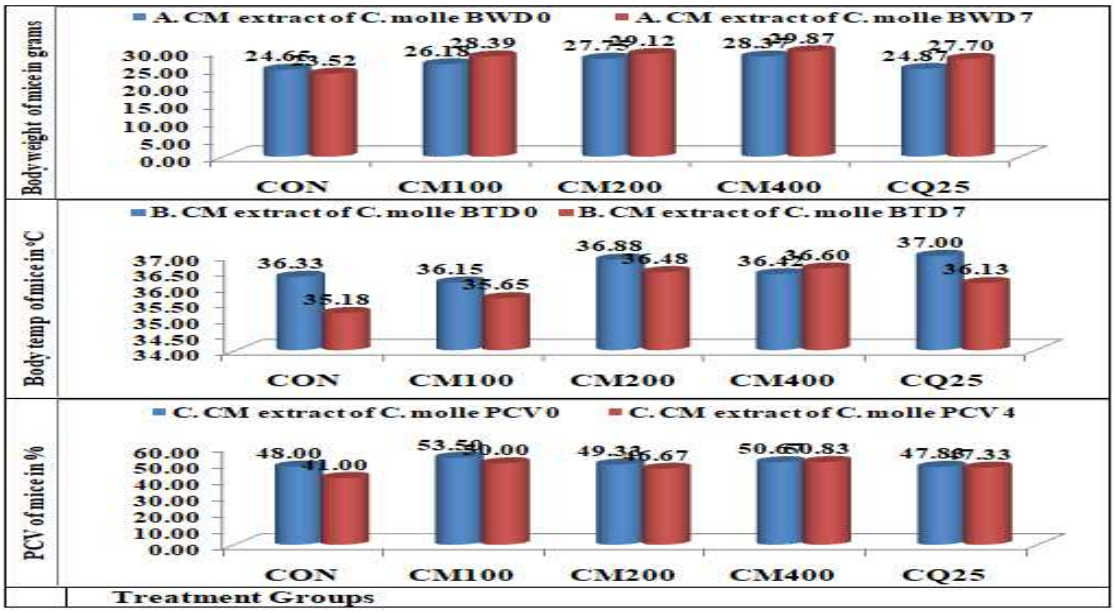

Figure 3: Showed effect of Crude methanolic extract of C. molle leaf *in* P. berghei infected mice in Curative test. [CON = negative control (solvent 10ml/kg), CM= chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero, BWD7= body weight at day 7, PCV= packed cell volume, numbers = dose in mg/kg/day]

Antimalarial repository test of methanolic crude extract of *C. molle* leaf and chloroquine base against *P.berghei* infected mice resulted in reduced parasite load as compared to their respective negative control group. Methanolic crude extract at 100, 200 and 400mg/kg/day and Chloroquine bate at 25mg/kg/day showed 51.00, 62.65, 76.20 and 99.20% parasitemia suppression respectively and the results were highly significant(P < 0.001) compared to the negative control group at day-7.(see table 5)

Methanolic leaf crude extract of *C. molle* leaf at 100, 200, 400mg/kg/day in repository test showed 7.50±0.99 (P=0.977), 10.00±2.31 (P=0.550), 17.67±3.05 (P=0.001) and Chloroquine base at 25mg/kg/day showed 29.00±0.63 (P<0.001) days of mean survival time in seven-day repository test respectively. Chloroquine base at 25mg/kg/day showed highly significant (P<0.001) in survival days compared to negative controls 6.00±0.97 (see table 5).

Table 5: Mean Percent Parasitemia and mean percent parasitemia suppression and mean survival time of *P. berghei* infected mice treated with methanolic crude extract of *C. molle* leaf in repository (prophylactic) test.

Extract	% Parasitemia	% Parasitemia Suppression	Mean survival time
CON	24.90±2.16	0.00	6.00±0.97
CM_{100}	12.20±0.41	51.00^{a3d2e3}	$7.50±0.99^{d2e3}$
CM_{200}	9.30±0.56	62.65^{a3e3}	$10.00±2.31^{d1e3}$
CM_{400}	5.93±0.49	76.20^{a3b2e2}	$17.67±3.05^{a2b2c1e3}$
CQ25	0.20±0.20	$99.20^{a3b3c3d2}$	$29.00±0.63^{a3b3c3d3}$

Data are expressed as mean ± SEM; n = 6: CQ = chloroquine, CON = Control, CM, crude methanolic extract of *Combretum molle*, % par supp= % parasitemia suppression, Numbers refer to dose in mg/kg. a, compared to neg. control; b, to 100mg/kg; c, to 200mg/kg; d, to 400 mg/kg; e, to CQ 25mg/kg: 1p<0.05, 2p<0.01, 3p<0.001.

Plasmodium berghei infected mice treated in a repository test at 100mg/kg/day methanol crude extract of *C. molle* increase a mean body weight of 0.37±1.39g (p = 1.000), 200mg/kg/day increase mean body weight of 1.84±1.08g (P = 0.665), 400mg/kg/day increase mean body weight of 0.56±0.67g (P= 0.988), and Chloroquine base at 25mg/kg/day increase mean body weight of 0.08±0.93g (P = 1.000) and mice treated with solvent loss mean body weight of 0.15±1.01g. (See figure 4A)

Plasmodium berghei infected mice treated in a repository at 100mg, 200mg, 400mg /kg/day methanol crude extract of *C. molle* and Chloroquine base at 25mg/kg/day increase a mean body temperature of 0.75±0.30^{o}C (p = 0.009), 0.27±0.41^{o}C (P = 0.149), 0.62±0.12^{o}C (P< 0.021) and 0.75±0.16^{o}C (P=0.009) and mice treated with solvent decrease a mean body temperature of 0.67±0.27^{o}C. (See figure 4B)

Plasmodium berghei infected mice treated in a repository at 100mg, 200mg, 400mg /kg/day methanol crude extract of *C. molle* decrease a mean packed cell volume of 3.25±0.95% (p = 0.998), 1.25±0.85% (P =0.984) and 9.40±12.23%, (P=0.995)

respectively and Chloroquine base at 25mg/kg/day decrease a mean packed cell volume of 0.50±0.56% (P=0.934) and mice treated with solvent increase a mean packed cell volume of 6.00±0.41%. (See figure 4C)

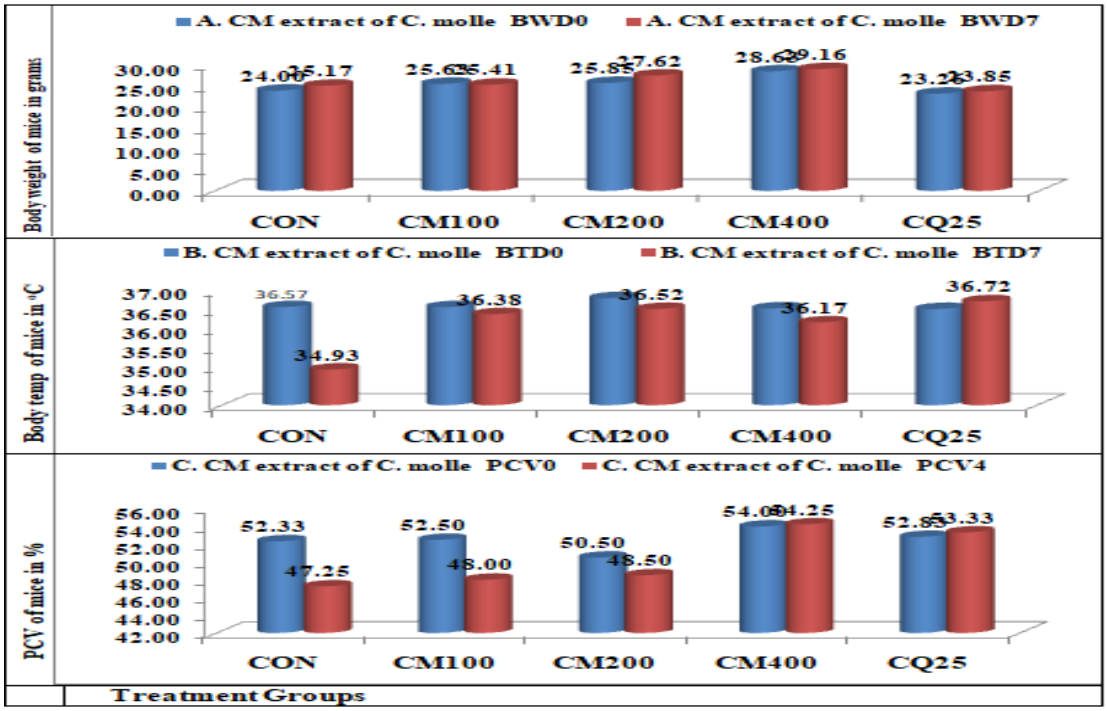

Figure 4: Showed effect of Crude methanolic extract of C. molle leaf *in* P. berghei infected mice in prophylactic test. [CON = negative control (solvent 10ml/kg), CM= chloroform fraction, CQ = chloroquine, BWD0= body weight at day zero, BWD7= body weight at day 7, PCV= packed cell volume, numbers = dose in mg/kg/day]

Discussion

The rodent malaria model in mice is the most extensively used, and ideal for the primary *in vivo* tests of new antimalarials. Mice are easy to raise, maintain, handle, and are small (20-30g), thus requiring a small amount of drugs compared to chickens, rats or monkeys. Mice are also susceptible to several species of malaria parasites isolated in other rodent species. Mice may develop acute fulminating diseases induced by blood-stage parasites, or by sporozoites injected either by mosquito bites or intravenously, being excellent models for testing new drugs.[11].

The leaf of *C. molle* methanolic crude extract phytochemical screening showed the presence of secondary metabolites: alkaloids, Cardiac glycosides, flavonoids, phenols, saponnins, tannins and terpenoids by using specific chemicals and methods [23]. These

compounds are known to show curative activity against several ailments in man and therefore could explain the traditional use of medicinal plants for the treatment of some illnesses [29, 30, 31].

Crude Methanolic extract was non-toxic to test mice, as they did not show signs of acute toxicity for 14 days at the doses of 2000mg/kg body weight, thus the traditional use of the plant material by local people like the decoction of root, leaf, and fruit could be safe provided that the amount used is in the ranges tested in this study. However, species variation would also limit such a straight forward extrapolation of the findings of this study to humans.

The subacute toxicity evaluation of C. molle crude methanolic leaf extract showed a mean body weight decrease from week 0 to week 4 in a decreasing order from 100mg/kg/day to 400mg/kg/day and mice treated with the solvent showed a mean weight gain from week 0 to week 4.

The mean weight of kidneys of mice treated with the three different doses of *C.molle* methanolic crude leaf extract in subacute toxicity evaluation did not show any significant difference compared to the negative control.

The mean weight of liver of mice treat treated with the three different doses of *C.molle* methanolic crude leaf extract in subacute toxicity evaluation showed significant differences at 200mg/kg/day and 400mg/kg/day compared to the negative control.

The histopathological examination of liver and kidney tissues in subacute toxicity of mice treated with 100, 200, and 400mg/kg/day body weight of *C. Molle* crude methanolic leaf extract showed normal histological structures similar to the negative control group.

The crude methanolic extract of dried stem of *C. molle* in *In vitro* cell culture with *plasmodium falciparum* also showed an acivity with IC_{50} value of 1.25 µg/ML [20, 21]. So the methanolic crude extract obtained from *C. molle* showed high *in vitro* activity which helps to proceed *in vivo* study. Therefore, the present study was carried out on *in vivo* antimalarial study of the methanolic crude extract of *C. molle* leaf in *Plasmodium berghei* infected mice. Methanol crude extract showed dose-dependent activity against *plasmodium berghei* in a 4-day suppressive test. In comparing the result obtained from the present study the three doses of crude methanolic extract

showed significant parasitemia suppression compared to the negative control. *In vivo* antiplasmodial activity can be classified as moderate, good, and very good if an extract displayed percent parasite suppression equal to or greater than 50% at a dose of 500, 250 and 100mg/kg body weight per day respectively [12]. Based on this classification, the methanolic crude extract at 200 and 400 mg/kg/day showed 45.36% and 76.17 % suppression against *P. berghei* in mice respectively and exhibited good to moderate antiplasmodial activity. These result indicated that compounds which have antimalarial activity were soluble in the solvent used to extract.

The crude methanolic extract of *C.molle* in a four day suppressive test at all doses did not produce significant difference in body weight and packed cell volume compared to the negative control but they produce significant difference ($P < 0.003$) from 200mg/kg/day in reducing body temperature. The crude methanolic extract at doses of 100, 200 and 400mg/kg/day showed increased mean survival time compared to the negative control but didn't produce a significant difference from the negative control. Mice treated with chloroquine base 25mg/kg/day showed a significant difference compared to the negative control.

In the curative test the average percent parasitemia was increased in the negative control from 3rd day to the 7th day. The *Plasmodium berghei* infected mice treated with crude extract the average percent parasitemia increased from the day -3 to day -4 and showed a decrease in percent parasitemia from the day-5 to day -7 in a dose-dependent manner compared to the negative control. The infected mice treated with Chloroquine base showed a decrease in average percent parasitemia from 3rd day to the 7th day.

In the curative test crude methanolic extract of *C. molle* produced significant difference in percent packed cell volume compared to the negative control. The crude methanolic extract at doses of 100, 200 and 400mg/kg/day showed increased mean survival time compared to the negative control and but only produced significant difference at 400mg/kg/day compared to negative control.

Methanolic crude extract in repository(prophylactic) test at 100, 200 and 400mg/kg/day and Chloroquine bate at 25mg/kg/day showed 51.00, 62.65, 76.20 and

99.20% parasitemia suppression respectively and the results were highly significant(P < 0.001) compared to the negative control group at day-7.

The promising result from the curative and prophylactic tests suggests that the crude extract has therapeutic efficacy against established malarial parasitic infections. The observed significant percent parasitemia inhibition, prevention of body weight reduction, increase in packed cell volume, body temperatue and mean survival time in four-day suppressive test and observed curative potential in established *Plasmodium bergei* infection were expected to be from the effects of the secondary metabolites such as Inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation[25]. Intercalation of DNA and inhibition of DNA synthesis through topoisomerase inhibition and termination of cell division, complexion with extracellular and soluble proteins and disruption of microbial membranes, enzymes, hydrogen bonding and hydrophobic effects, as well as by covalent bond formation, inactivation of microbial adhesins, enzymes, cell envelope transport proteins[27]. Inhibiting growth, and protease activity **[29].** Capable of precipitating gelatinous compounds, phagocytic cells, host-mediated tumor activity [30].

Conclusion

The present study indicates that 80% methanolic crude extract of *Combretum molle* leaf has good antimalarial on the measured parameters. The crude methanolic extract showed significant parasitemia suppression and mean survival time in the 4-day Chemosuppresive, curative and prophylaxis tests but other parameters such as prevention in a reduction of body weight, body temperature, in protecting infected animals from parasite-induced PCV reduction were not significantly different compared to the negative control. The subacute toxicity evaluations showed no toxicity at the three different doses in histopathological examination. Findings suggest that the phytochemicals responsible for antimalarial activity of the plant are soluble in 80% methanol which upholds the earlier in vitro findings as well as the folkloric use in Ethiopian traditional medicine.

Recommendation

We forward the recommendation for investigators to carry out in-depth study to isolate, identify and characterize the bioactive constituents responsible for antimalarial activity of leaf of the *C.molle*. In the present study acute and sub-acute toxicity was done showing that the plant was saf*e.* Therefore, we recommend performing chronic histopathological and biochemical toxicity study of the plant.

Ethical approval:

The animals were handled according to the governance of animal care and use for scientific purposes in Africa and the Middle East [33]. And the ethical clearance was requested and obtained from the Department of Pharmacology, School of Pharmacy, University of Gondar for the use of animals for experiment with Ref. No. 5094/96/2011. And the research was performed as per the agreement.

List of abbreviations: Not applicable

Consent to publish: Not applicable

Availability of data and material: All data generated or analyzed during this study have been attached on the supplementary material.

Competing interests

The authors declare that they have no competing interest to disclose.

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Authors' contributions

MA was involved in developing the proposal and designing of the study. MA, GM, TJ, and EF conducted the actual study and the statistical analysis and in the write up of the manuscript was done by MA and TJ. All authors gave the final approval of the version to be published, and agree to be accountable for all aspects of the work.

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