The Effect of Crude Leaf Extracts of *Osyris quadripartita* Salzm. ex Decne against *Plasmodium berghei* inSwiss Albino Mice

By

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Abstract

Continuous emergence of multi-drug-resistant malaria parasites and their rapid spread across the globe warrant urgent search for new anti-malaria chemotherapeutics. Traditional medicinal plants have been the main sources for screening active phytochemicals against malaria. Accordingly, this study was aimed at evaluating the anti-malarial activity of *Osyris quadripartita* Decn.which is used for malaria treatment by local people around Fiche, north-central Ethiopia. Using aqueous, chloroform and methanol crude leaf extracts of the plant have been prepared and tested for toxicity and anti-malarial efficacy in *Plasmodium berghei*-infectedSwiss albino mice. Methanol solvent gave the highest yield of the plant extract. Acute toxicity study results indicated that the plant extract did not show any sign of toxicity up to 2000mg/kg. To assess the effect of the plants on the parasite, a 4-day suppressive standard test was performed. Data were analyzed using paired t-test and ANOVA. Both aqueous and methanol extract of *O.quadripartita* significantly ($P<0.05$) suppressed parasitemia and prevented packed cell volume (PCV) reduction and body weight gain in dose dependent manner.In addition, they prolonged the mean survival time. On the contrary, chloroform extract of *O. quadripartita* significantly ($P<0.05$) inhibited parasitemia, prevented body weight loss, prevented PCV reduction and prolonged survival time in all doses. The most efficacious extract was that of chloroform, which showed the highest parasitaemia suppression (41.26%) at 600mg/kg whereas its methanol extract caused 24.4% suppression at 200 mg/kg dose tested. Furthermore, aqueous extract of the plant showed 21.67% suppression at the same dose tested. The finding supports the traditional use of the plants for the treatment of malariathough parasite clearance was not achieved.Further evaluation of this plant is, however, needed before it is recommended for the control of malaria.

Key words: Antimalarial activity, *Osyris quadripartita,invivo, Plasmodium berghei,* Swiss albino mice, Ethiopia

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List of abbreviations and acronyms

% Para	Percentage parasitaemia
% Supp	Percentage Suppression
AAU	Addis Ababa University
ACTs	Artemisinin-based Combination Therapies
AMRF	African Medical and Research Foundation
ANOVA	Analysis of variance
BWT	Body weight
CDER	Center for Drug Evaluation and Research
CQ	Chloroquine
dH_2O	Distilled water
EAAM	European Alliance against Malaria
FMOH	Federal Ministry of health
Hb	Hemoglobin
IC_{50}	Inhibitory concentration 50
IP	Intraperitonial
IRS	Indoor residual spraying
ITNs	Insecticide-treated mosquito nets
LD	Lethal Death
MST	Mean Survival Time
NIAID	National institute of Allergy and Infectious Diseases
NIH	National Institute of Health
OCED	Organisation for Economic Cooperation and Development
PCV	Packed Cell Volume
PMI	President's Malaria Initiatives
RBC	Red blood cells
RITM	Research Initiative on Traditional antimalarial Method
Rpm	Revolution per minute
SEM	Standard Error of Mean
SNNP	Southern Nations, Nationalities, and People's

SPSS	Statistical Package for the Social Sciences
TM	Traditional Medicine
UNICEF	United Nations Children's Fund
WBC	White Blood Cell
WHO	World Health Organization

1. INTRODUCTION

Malaria is a disease caused by protozoan parasites belonging to the genus *Plasmodium*of phylum Apicomplexa.Our understanding of the malaria parasite begins in 1880with the discovery of the parasite in the blood of malaria patients byAlphonse Laveran and its transmission by mosquitoes was revealed by Ronald Ross in 1898 (Bruce-Chwatt, 1980).The most common*Plasmodium* species that cause malaria in humans are *Plasmodium falciparum* and *P.vivax* although two other distinct species, *P. malariae*and *P. ovale,* are known to cause human malaria.In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi* – a species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia (Tang *et al*., 2010).

According to the latest estimates, released in December 2014, there were about 198 million cases of malaria in 2013 (with an uncertainty range of 124 million to 283 million) and an estimated 584 000 deaths (with an uncertainty range of 367 000 to 755 000). Malaria mortality rates have fallen by 47% globally since 2000, and by 54% in the WHO African Region. Malaria has been and still is among the leading causes of human morbidity and mortality with about 90% of the cases and deaths occurring in Africa (WHO, 2014).

From economic point of view,there are direct and indirect costs incurred due to malaria. Since about 40% of the global population lives inmalariaendemic areas malaria-related effects on levels of productivity, national growth and development are very high. Currently the disease is endemic in over 100 countries in different regions of the world, including India, Southeast Asia, and Central and South America, although sub-Saharan Africa bears the biggest burden. Ethiopia suffers from unstable and cyclically epidemic malaria(FMoH, 2012).Although countrywide surveys suggest malaria epidemics in Ethiopia have substantially decreased in 2012 compared to

the baseline year of 2004, several focal outbreaks were reported nationwide in the last five years (PMI,2013).

2. MALARIA CONTROL

On the one side malaria appears to shrink as a result of scale-up of integrated control interventions and several endemic countries embarked on malaria elimination programs (WHO 2014). On the other hand, due to climatic changes and human-related factors (coupled human and natural factors) malaria epidemiology is progressively changing putting more people at-risk. Moreover,there is rapid emergence of drug-resistant plasmodium strains. For instance, resistance has already been developed against the latest first-line drug of *P. falciparum* (arteminisinin-based combination therapy ACT) in Asia (O'Brien *et al*., 2011). Malaria control efforts are further complicated by the increased resistance of mosquito vectors to insecticides (Oduola *et al*., 2010) together with challenges of having effective anti-malaria vaccines. Thus, there is an urgent need to search for effective, available, affordable and safe alternative ant-malaria drugs that can be integrated into existing malaria control intervention to successfully control the disease.

2.1. Traditional medicinal plants as anti-malarials

It is well-known that plants have been and are the mainstay of traditional medicine against malaria and other diseases in resource-limited settings, as over one-third of the population in low-incomecountries lack access to essential medicines (WHO 2007). However, the claimed potency of medicinal plants has to be scientifically evaluated and safety and toxicological studies should be done. Rigorous *in vitro* and *in vivo* toxicological investigationsare required to determine the type and degree of toxicity, safety and efficacy of plant products in malaria drug research and ultimate discovery as well as to recommend or discourage the plants' traditional use.

To this end, various studies have been conducted to evaluate the antimalarial effects of traditionally used plants in Asia and Africa.For example, Ramazani et al.,(2010) worked on ten plant species but only - *Berhavia elegans*,*Salanum surattense* and *Prosopis juliflora* –showeda promising anti-plasmodial activity *in vitro*and *in vivo* with no toxicity. Verma*et al.*(2011) reported that *Holarrhena antidysenterica (HA-2)and Viola canescens*(VC-1*)) in vitro* showed anti-plasmodial activity.From sub-Saharan Africa in Burkina Faso (**Jansen et al. 2012)**, the best *in vitro* anti-plasmodial results were obtained for the plant *Dicoma tomentosa*with the dichloromethane, diethylether, ethylacetate and methanol extracts, which exhibited a high activity (IC_{50} [less than or equal to] 5 ug/ml). Hot water and hydroethanolic extracts also showed a good activity (IC_{50} [less than or equal to] 15 ug/ml). The activity was also confirmed *in vivo* for all tested extracts**.** Likewise, ethanol extract of leaves of *Helianthus annus* in Swiss albino mice, observed to have the mean percentage chemo suppression as high as 98.1% and 98.3% in mice which received 2g/kg and 4g/kg day, respectively (Ejebe *et al*. 2011).Aqueous and methanol extracts of 15 plants traditionally used for treatment of malaria in Meru District, were tested (Gathirwa *et al*., 2007).Of the plants tested *in vitro,* 25.0% were highly active (IC_{5o}<10g/ml), 45.59% moderately active (IC_{5o}10-50 g/ml), 16.18% had weak activity of 50-100g/ml while 13.24% were not active (IC_{5o}>100 g/ml).

Despite their wide use in the traditional health care, the work that has been done to evaluate the safety and efficacy of Ethiopian traditional medicinal plants is relatively not extensive. Nonetheless, there are some reports ontheanti-malarial properties of selected Ethiopian traditional medicinal plants by and large using *P. berghei* in mice model as *in vivo* data from animal studies are more indicative of toxicity and may be considered to be better safety markers (WHO 2000). For example, it was shown that in Swiss albino mice crude aqueous and ethanol

extracts of *Aloe* sp., *Azadaichata indica* and *Tamarindusindica* had high anti-malarial activity at 650mg/kg (Mesfin et al. 2012). Similarly, Deressa *et al.* (2010) found that the crudemethanol extract of *Clerodendrum myricoides*, *Dodonea angustifolia* and *Aloe debrana* exerted 82.5, 84.52 and 73.95 percent suppression, respectively in an *in vivo* system.The authorsalso observedthat water extract of *A. debrana* induced 54.36%suppression of parasitaemia. In addition, treating Swiss albino mice with crude extracts from the roots and aerial parts of the traditional medicinal plant *Asparagus africanus* inhibited *P. berghei* parasitaemia by 46.1% and 40.7%, respectively (Dikasso *et al.* 2006a). Likewise, Getie (2010) evaluated the antimalarial activity of methanol and aqueous extract of seeds of *Dodonaea angustifolia* and leaves of *Entada abyssinica* against *P. berghei* in Swiss albino mice and found highest parasite suppressions (86.21%) at 600mg/kg. Mengistie *et al*. (2012) also described that the extracts of *Dodonaea angustifolia* and *Bersama abyssinica* significantly inhibited parasitaemia and increased the survival time of the infected mice. More recently, the anti-malarial activity of hydromethanolic leaf extract of *Calpurnia auriea* wasevaluated and found the highest parasite suppression (51.15%) at 60mg/kg (Eyasu *et al*., 2013).

Concerningthe safety and antiplasmodial activity of *Osyris quadripartita,* a plant whichislocally called '*keret*' in Amharic and is commonly used in Ethiopian traditional medicinal practices, little data is available. For instance, the leaf of this plant is used to treat malaria (Belayneh and Bussa, 2014).Similarly, the leaf of the plant is used to treat cancer (Enyew *et al*., 2014) (Wahiba and Malika, 2014). The aim of this study was, therefore, to assess the anti-plasmodial activity and safety of this plant in an attempt to contribute towards screening traditional medicinal plants for malaria control.

3. HYPOTHESIS

The hypothesis of this study was stated as 'The leaf extracts of *O.quadripartita*have anti-malarial effect and are safe.'

4.OBJECTIVES

4.1. General objective

The general objective of the study was to evaluate the anti-malarial effect and safety of the crude extracts of the leaves of *O.quadripartita*against *P.berghei* in mice.

4.2. Specific objective

The following were the specific objectives of the study.

1. To investigate the anti-malarial activity of aqueous, methanol and chloroform crude leaf extracts of *O. quadripartita* against *P. berghei*in Swiss albino mice.

2. To assess the toxicity of *O. quadripartita*crude extracts on female Swiss albino mice.

3. To determine the level of effective *P. berghei*suppression dose of *O. quadripartita*leaf extracts in mice.

5. MATERIALS AND METHODS

5.1. Plant Description and Sample collection

The genus *Osyris* whichincludes more than 34 species (Ref) belongs to the family Santalacea. *O. quadripartita*(Salzm. ex Decne) is an evergreen shrub or a tree reaching a height of 1-7m with many branches and the branches sometimes pendant (Ref). The plant is hemiparasitic ever green

shrub,meaning although they can survive and grow by themselves, they also opportunistically tap into the root systems of nearby plants and parasitize them.Detailed morphological description of the plant is available elsewhere (Hedberg and Edwards 1989). The picture of the plant which is commonly known as wild tea plant is indicated in figure 1.

Figure 1.Osyris quadripartita by Krish Dulal(From Wikimedia Commons, the free media repository [http://commons.wikimedia.org/wiki/File:Osyris_quadripartita.jpg]

Leaf samples of the experimental plant were collected in December 2013 from *Wertu kebele* around Fiche District, North Showa Zone of Oromial Regional State , 110 km North of Addis Ababa. The plant was identified and authenticated by Dr Mirutse Giday who is an ethnobotanist at Aklilu Lemma Institute of Pathobiology, Addis Ababa University and the voucher specimen was deposited in the National Herbarium of the Addis Ababa University with voucher number?.

5.2. Preparation of crude plant extracts

The leaves of *O. quadripartita* were washed thoroughly with running tap water and each plant material was reduced to small fragments. The washed leaves were dried at room temperature under shade in the Biomedical Sciences Laboratory, Department of Microbial, Cellular and Molecular Biology, College of Natural Sciences, Addis Ababa University and were ground into fine powder using blander and kept in tightly closed brown bottle until used for extraction. The leaf crude extracts of aqueous, methanol (98%) and chloroform were prepared by cold maceration technique (O'Neill, 1985). The 100 g plant extractmaterial was refluxed in 1000 ml of each anaqueous, methanol and chloroform solvent and the respective mixture was placed on orbital shaker, (GFL, model 3020 Germany) at 160 rpmfor 72 hours. The mixture was first filtered using cotton and then the filtrate was passed through Whatman filter paper (No.3, 15cm size with retention down to 0.1µm in liquids) (Whatman LTD, England). The methanol (98%) and chloroform extracts were concentrated in a rotary evaporator (Buchi type TRE121, company, Switzerland) at a temperature of 45^0C; whereas the water extract was freeze-dried using centrifugal freeze drier (model 5 PS, Company, England). All the extracts were stored in screw cap vials at -20^{o}C until they were used for the experiment. The water and methanol (98%) extracts were dissolved in 10ml of distilled water (dH_2O) and the chloroform extract was dissolved in 3% Tween-80 for the experiment.

5.3. Experimental animals and parasite inoculation

Mice are considered to have a comparable genetic model to human, up to 99% degree of genomic conservative (Pennacchio 2003). *In vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites from which *P. berghei* is the most widely used in

the prediction of treatment outcomes. Hence it was an appropriate parasite for this study. As this parasite is sensitive to chloroquine (CQ), this drug was employed as a standard drug in this study (David et al. 2004). Swiss albino mice, 5-7 weeks of age from Addis Ababa University, College of Natural Sciences, Department of Microbial, Cellular and Molecular Biology in Biomedical Sciences Laboratory were used for the test. For the *in vivo* antimalarial assays of plant extracts, CQ sensitive strain of *P. berghei* was maintained at the animal house.

Female albino mice previously infected with *P. berghei* having variable parasitaemia were used as donor animals. The parasitaemia of the donor mice was first determined and parasitized erythrocytes were obtained by cardiac puncture using ethyl ether as anesthesia and sodium citrate (0.5%) diluted in physiological saline (0.9%) as an anticoagulant. The dilution was made based on the parasitaemia of the donor mice and the RBC count of normal mice in such a way that 1ml blood contains $5x10^7$ infected erythrocytes (Moll *et al*. 2008). Each mouse was inoculated by intraperitoneal injection with a blood suspension (0.2ml) containing $1x10^7$ parasitized erythrocytes. The parasite was maintained by serial passage of blood from infected mice to non-infected ones on a weekly basis.

5.3.1. Acute toxicity test for the plant materials

The crude methanol extract of *O. quadripartita*intended for the anti-malarial test against *P. berghei* was evaluated for its toxicity in non-infected Swiss albino mice aged 6-8 weeks (mean age is 7.4) and weighing 23-38g (mean weight is 28.35) according to OECD guide line No 425(OECD 2008). Twenty mice were used by randomly dividing them into four groups of 5 mice per cage. The mice were starved 3-4hrs before the experiment begun with only water

allowed and 1-2hrs after the extract were given (OECD 2008). Then, the mice in groups 1, 2 and 3 were given orally 1000, 1500 and 2000mg/kg respectively, body weight in single dose volume of 0.2 ml of the extract. The mice in the control group received 0.2 ml of distilled water and Tween-80. Then, the mice were observed continuously for 1 hour, intermittently for 4 hours and for a period of 24 hours for gross behavioral changes such as rigidity, sleep, mortality and other signs of acute toxicity manifestations and were followed for 14 days (OECD 2008).

5.4.*In vivo* anti-malarial assays of plant extract

5.4.1. Anti-malarial activity of the extracts

In this study a total of 60 mice were randomly assigned into nine treatment groups and three control groups (two negative and one positive control) with five mice per group. In screening of the plant extracts, the standard four-day suppressive method was used (David et al. 2004). *P. berghei* was obtained from Drug Research Department of Ethiopian Public Health Institute (EPHI). Albino mice previously infected with *P. berghi* having variable parasitaemia were used as donors (redundant!!). The parasitaemia of the donors was first determined. These mice were then sacrificed by head blow and blood was collected in a Petri dish with anticoagulant (0.5%trisodium citrate) by severing jugular vein. The blood was then diluted with physiological saline (0.9%) based on the parasitaemia of the donor mice and the RBC count of normal mice in such a way that 1ml blood contains $5x10^7$ infected erythrocytes (Ryler and Peter,1970).Each mouse received 0.2ml of diluted blood containing $1x10^7$*P. berghei* infected erythrocytes by intraperitoneal route. To avoid variability in parasitaemia, the blood collected from all donor mice was pooled together, and the parasite maintained by weekly passage to other mice.

The experimental mice were infected with 10^6*P. berghei* and randomly divided into nine test groups and three control groups (each for CQ as the standard antimalarial drug and DH_2O and Tween 80(3%) as a negative control). The test extracts were prepared in three different doses (200mg/kg, 400mg/kg, and 600mg/kg of body weight) and CQ at 25mg/kg in a volume of 0.2ml and vehicles at 0.5ml/mouse. The extracts were administered as a single dose per day. Both the extract and the drug were given through intra-gastric route by using standard intra-gastric tube to ensure safe ingestion.

Treatment was started after 3 hrs of infection with *P. berghei* on D0 and was continued daily for four days (i.e. from D0 to D3). On the fifth day (D4) blood sample was collected from the tail snip of each mouse. Thin smears were prepared and stained with a 10% Giemsa solution. Then, each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups and the parasitaemia was determined by counting a minimum of five fields per slide(Ref). Percentage of suppression (% suppression) and percent parasitaemia (% parasitaemia) were calculated using the formulas below as indicated in Innocent et al. (2009) and Assefa et al.(2007).

% Suppression and

% Parasitaemia

5.4.2. Determination of body weight change

For toxicity test, individual weights of mice were determined and recorded shortly before and after the plant is administered (OECD 2008). The body weight of each mouse in all the groups was measured before infection (D0) and on D4 in case of treatment, by using a sensitive digital weighing balance (A and D Electronic Balance, A and D company Limited, Japan) and mean body weight per group was calculated using the formula;

Mean body weight.

5.4.3. Determination of packed cell volume

Packed cell volume (PCV) was determined using blood collected from tail of each mouse in heparinized microhaematocrit capillary tubes and filled up to 3/4thof the tube with blood, sealed one end with crystal seal and place the open end of the tube to the center and the sealed end out wards of the microheamtocrit centrifuge. The blood was centrifuged at 12,000 rpm for 5 minutes using a microhematocrit centrifuge (Hawsksley Microhaematocrit Reader, Hawsksley & sons LTD, England) and then volume of the total blood and the volume of erythrocytes were measured using a ruler. Measurement was done before infection and on D4 after infection.PCV was calculated as:-

$$\textbf{PCV} = \frac{\text{Volume of total erythrocytes in a given volume of blood}}{\text{Total blood volume}} \times 100$$

5.4.4.Determination of mean survival time

Mortality was monitored daily and the number of days from parasite inoculation up to death was recorded for each mouse in the treatment and control groups throughout the follow-up period. The mean survival time (MST) for each group was calculated as follows using formula

MST (Mengistie et al. (2012).

6. Ethical considerations

This study was conducted after the necessary ethical clearance was obtained from Addis Ababa University College of Natural Sciences Ethical Clearance Committee.The living area for mice allowed them to satisfy their basic needs including the ability to eat, drink, urinate, defecate and also regulate their body temperature.

7. Data Analysis

The PCV and BW of *P. berghei*-infected mice in each group who were treated with the same extracts and dose were compared between D0 and D4, the results were expressed as mean plus or minus the standard error of the mean by one way analysis of (ANOVA) and 2- tailed Student's t-test using SPSS Version 15 software. The suppressive test were analyzed by (ANOVA) followed by Tukey-multiple comparison test to compare the level of parasitamia, and survival times of the infected mice between the control group and the extract treated groups at a fixed time. All the data were analyzed at a 95% confidence interval ($P < 0.05$ was considered as statistically significant and $p < 0.01$ as highly significant).

8. RESULTS

8.1. Extraction Yield of Plant Materials

The methanol extract of *O. quadripartita* gave the highest yield as compared to aquoues and chloroform extract, which is followed by aqueous and chloroform extracts respectively(Table 1).

Table 1 Yield of aqueous, methanol and chloroform leaf extracts of *O. quadripartita*

Solvent	Plant powder (g)	Extraction solvent (ml)	Yield (g)	Yield (%)
Aqueous	100	1000	6.60	6.60*
Methanol	100	1000	32.75	32.75*
Chloroform	100	1000	1.447	1.447*

Key=Values are presented asml=milliliter; g=gram; %=percentage; * = there was a significant difference between them(P<0.05).

8.2. Acute Toxicity Test

The experimental mice did not show any gross physical and behavioral changes including hair erection, reduction in feeding and motor activity, weight loss, lacrimation, diarrhoea, depression and abnormal secretion for when continuously monitored for 24 hours. No mortalities occurred within the observation period of two weeks.

8.3. Anti-malarial Activities of the Extracts

8.3.1. **Effect of Crude Extract on BW and PCV**

Aqueous leaf extract of *O. quadripartita* demonstrated a moderate dose-dependent effect on PCV. As the dose increased PCV reduction also increased. At 600mg dose, there was a significant PCV reduction b/n D0 and D4 ($p<0.05$), however there is a reduction of PCV in 200 and 400mg but it is not significant. Similarly, when we compare the result with negative control, only 600mg of the aqueous extract showed a significant reduction. Administration of the three doses of the aqueous extract (200, 400 and 600mg) brought a significant BW change b/n D0 and D4 in all dose but as compared to the negative control,only in 200 and 400mg doses of miceshowed a significant change (Table 2).

Similarly, the evaluation of PCV using the leaf methanol extract of *O. quadripartita* demonstrateda moderate dose-dependent effect. But in methanol, as the dose increases PCV reduction were decreased, only 200mg dose showed a significant change as compared to the control group. When we compare b/n D0 and D4, the 400 and 600mg doses showed a significant reduction in PCV. Administration of the three doses of the methanol extract (200, 400 and 600mg) brought a significant BW change b/n D0 and D4 in all dose but as compared to the negative control,only400mg dose showed a significant change (Table 2).

On the other hand, the chloroform extract of *O. quadripartita* showed PCV reduction in 200, 400 and 600mg due to parasitaemia, but it was not significant as compared to the control group. And also, there were no significant reduction in PCV b/n D0 and D4 in three doses. Administration of the three doses of the chloroform extract (200, 400 and 600mg) brought a significant BW change in all doses of mice and also b/n D0 and D4. (Table 2).

Table 2 Effect of crude aqueous, methanol and chloroform leaf extracts of *O. quadripartita* on PCV and BW of *P. berghei* infected mice

Description	Dose(mg/kg /day)	PCV		BW	
		D0	D4	D0	D4
Aqueous extract	200	58.6±2.02	56.8±1.98*	31.2±0.37	23.6±0.50* [a]
	400	56.6±1.28	54±1.14	25.2±0.37	21.8±0.58* [a]
	600	60.4±0.6	56±0.89* [a]	28.6±0.4	22.6±0.50 [a]
Methanol extract	200	59.4±0.4	55.8±0.73*	28.2±0.37	23.6±0.50 [a]
	400	57.0±1.6	52.2±1.2 [a]	25.6±0.4	23.2±0.37* [a]
	600	58.4±1.77	52.8±1.46 [a]	30.6±0.4	22.6±0.60 [a]
Chloroform extract	200	57.2±1.59	54.2±1.31	27.6±0.24	26.2±0.37* [a]
	400	56.4±0.75	55±0.54	27±0.31	29.6±0.40* [a]
	600	54.8±1.24	53.80±1.52	28.6±0.5	30.8±0.58* [a]
DH_2O (NC)	0.2ml	56.8±1.93	52.2±1.71 [a]	29±0.31	23.6±0.24 [a]
T-80 (NC)	0.2ml	58±1.51	52.4±0.92 [a]	28.8±0.48	23±0.44 [a]
CQ	25	56.8±0.96	56.8±0.89*	29.8±0.48	33.2±0.37* [a]

Key=Values are presented as M±SEM; n=5 (Number of mice/ group in a single experiment); D0=Day 0; D4=Day 4; PCV=Packed Cell Volume; BW=Body Weight; mg/kg/day= One mill gram of the extract per one kilogram of mice for one day; NC = negative control; T-80= Tween 80; DH_2O=Distilled water; CQ= Chloroquine Phosphate;*=Significant comparedwith that of negative control.

8.3.2. Effect of Crude Extract on Parasitaemia, Suppression and Mean Survival Time

The results of the 4 day suppressive study of the aqueous and methanol extracts of *O.quadripartita* showed a moderate dose dependent chemosuppressive effect at the three dose levels in mice infected with Plasmodium berghei malaria parasite. However, only methanol at 200mg showed a significant difference as compared to the negative control.The MST of the aqueous and methanol extracts of all (except aqueous at 600mg) doses showed a significant differenceas compared to the negative control (DH_2O).

On the contrary, the results of the 4 day suppressive study of the chloroform extract of *O. quadripartita* showed the highest result as compared to aqueous and methanol extract but it was also a dose dependent chemosuppressive effect at various doses in mice infected with Plasmodium berghei malaria parasite. The highest extract suppression of parasitaemia was observed at the dose of 600mg/kg body weight of mice with a mild suppression of parasite in 400mg/kg and 200mg/kg body weight of mice when compared to the negative control group (3%T-80) with the highest % para sitaemia (Table 3). Percentage suppression was observed to increase as extract concentration increased. From Table 3, the extract caused a statistically significant ($P<0.05$) chemosuppression of 31.7%, 37.5% and 41.26% for the 200, 400 and 600 mg/kg/day respectively when compared to the control, but it was not significant b/n the dosesThe

MST of the chloroform extracts of all doses showed a significant reduction as compared to the negative control (3% T-80).

Table 2. Suppressive Effect and MST of crude aqueous, chloroform and methanol leaf extracts of *P. berghei* infected mice

Description	**Dose(mg/kg/day)**	%Parasitaemia+ SEM	**%Suppresion**	***MST***
Aqueous extract	200	51.26±2.05	21.67	8.2±0.37*
	400	54.62±2.20	16.53	7.4±0.51*
	600	63.02±3.91	0.37	6.2±0.37
Methanol extract	200	49.48±2.06*	24.4	8.4±0.24*
	400	50.94±1.24	22.16	8.2±0.37*
	600	55.92±0.84	14.5	7.4±0.51*
Chloroform extract	200	44.48±10.18*	31.7	8.0±1.14*
	400	40.7±9.66*	37.5	9.8±0.58*
	600	38.26±8.78*	41.26	11±0.63*
DH_2O(NC)	0.2ml	65.44±1.7	0.00	5.6±0.24
T-80 (NC)	0.2ml	65.14±1.7	0.00	5.8±0.37
CQ	25	0.00	100	ND

Key=Values are presented as M±SEM; n=5 (Number of mice/ group in a single experiment); D0=Day 0; D4=Day 4; PCV=Packed Cell Volume; BW=Body Weight;mg/kg/day= One mill gram of the extract per one kilogram of mice for one day; NC = negative control; T-80=

Tween 80; DH_2O=Distilled water; CQ= Chloroquine Phosphate; ND=No Deathwithin the follow-up interval (28-days)

9. Discussion

In the extraction of the crude plant leaf extract, the highest extraction efficiency was that of the methanol extract of the plant while chloroform extract product was the lowest. A high yield of methanol is an indication of the extracting ability of the solvent used with respect to both polar and nonpolar components (Tadesse, *et al*., 2003).Another probable reason for this variation could be due to high concentration of polar compounds like phenolics and flavonoids in the leaf of the plant species that betterdissolvedin methanol which is a polar solvent (Rached, *et al.*, 2010). The quality and quantity of phytochemicals extracted from plant materials differ depending on, among other factors, the solvent type used. The comparative ability of extraction solvents of penetrating the cellular membrane to extract the intracellular ingredients from the plant material may impact an extract yield. Some reports show that methanol extracts more number and types of compounds in plant materials than other extraction solvents such as aceton, chloroform, ether,water and even ethanol (Tiwari et al. 2011).For example, According to Taddese *et al*. (2003)they use five different species and extract them using methanol. From the five plant extracts only methanol showed the highest yield.Methanol releases more diverse phytochemicals such as anthocyanins, tannins, saponins, terpenoids, xanthoxylines, totarol, quassinoids, lectones, flavones, phenols and polyphenols (Cown 1999). Form this study, the difference between the yields are significantly different($P<0.05$).

Oral administration of aqueous, methanol and chloroform extracts of *O. quadripartita* did not show changes in the general appearance or behavioral pattern of the experimental mice till the end of 28 days.Further, no death was observed in the animals receiving the extract up to a dose of 2000 mg/kg body weight, which is about 10 times the minimum effective dose tested (200 mg/kg). If a test substance has a lethal dose (LD_{50}) higher than 3 times the minimum effective dose, it can be a good candidate for further studies (Krettli et al. 2009). Therefore, absence of mortality up to an oral dose of 2000mg/kg could indicate that the test extract is safe and this could explain the safe use of the plant by the local people who have been using it in traditional management of malaria.

Absence of BW loss among *P. berghei*-infected mice that ingested crude leaf extract of *O. quadripartita*,in all the three solvents even in the lowest dose administered (200 mg/kg), compared to the untreated controls suggests the effect of the extract in preventing malaria-related weight loss. It is well-established that BW loss is one feature of rodent malaria (Perlmann and Troye-Blomberg, 2007 cited in Okokon et al. 2005) (Please review the literature pertaining to the impact of malaria on host BW in rodent, non-human primate and human malaria.). Other similar studies that reported mice BW loss or contrasting findings using different plant products are….

The influence of malaria on hematological parameters isextensively investigated and PCV reduction is considered a hallmark of both human and rodent malaria (reviewed in Lamikanraet al. 2013). *P. berghei*-infected mice suffer from severe malarial anemia because of rapid RBC destruction, either by parasitaemia and/or spleen reticulo-endothelial cells(Chinchilla et al. 1998). For instance, in one study it was noted that within an estimated 48 hours of post-infection rodent

PCV was depleted to 43-44%(Taylor and Hurd 2001). Further, *P.berghei* increasedRBC fragility and led to subsequent reductionof PCV in infected-mice (Iyawe and Onigbinde 2009). Multiple other *in vivo*studies on rodentmalariausing diverse plant speciesfrom Ethiopia as well as abroad reported similar results (Amelo et al. 2014, Ejeb et al. 2011, Eyasu et al. 2013, Mohammed et al. 2014, Ramazani et al. 2010, Tesfaye and Alamneh 2014).Scarcity of previous reports pertaining to anti-malarial activity of *O. quadripartita*including the relative composition and predominance of its leaf chemicals made it difficult to present the current result from comparative perspective.

Another parameter used to evaluate the efficacy of antimalarial plant extracts in this study is mean survival time.A prolong MST and a significant difference were recorded for mice treated with the extract of *O. quadripartita* at 200 and 400 mg/kg of aqueous and 200, 400 ,600mg/kg of methanol but notat600 mg/kg dose of aqueous extract. On the contrary, the chloroform extractsprolonged MST at all doses administered and also the three doses(200,400 and 600mg) have a significant differencewith control group.

The chemosuppressiveactivity of the plant against *P. berghei* and its overall pathologic effect though the extracts were short of clearing the infection.The 4-day suppressive test is a standard test commonly used for *in vivo*anti-malarial phytochemical screening in which ≥30% reduction in parasitaemia following treatment makes a product to be considered active (Fidock et al. 2004, Krettli et al. 2009). Accordingly, both the aqueous and methanol extract of all doses, (except at 200mg) of *O. quadripartita*didn'texhibit a significantchemosuppressive activity on *P. bergheiev*enat the highest dose delivered (600 mg/kg),Thisat least undetectable level of antimalarial activity of aqueous and methanol crude leaf extractof *O.quadripartita*may be an indication that the active ingredients extracted by these solvents might have less potent anti-

malarialproperty. This can be explained by the fact that some plants may contain chemicals that are more soluble in polar solvents such as water, ethanol and methanol while others contain chemicals that are more soluble in non-polar solvents such as chloroform (Paiva et al.2010). Thus the crude aqueous ormethanol extract in the current study were higher in terms of yield they contained little compound effective at least against *P. berghei.*

On the contrary, the chloroform extractof *O. quadripartita*which showed31.7% suppression at the lowest, 37.5% at the medium and 41.26% at the highest doses can be classified as active. However, the difference within a group is not significant. The dose-dependent variation in chemosuppression could be attributed to the low concentration of schizocidal compounds in natural products(Krettli et al. 2009) and as such their activity may be undetectable in lower doses. This increased percentage suppression of parasitaemia with increased dose was observed by several other studies for different plant species (Muzemil 2008,…).

Nevertheless, the plant extract was less effective when compared to that of CQ, the standard drug against *P. berghei*. CQ treatment (10mg/kg) during the infection radically cleared parasitaemia. Rodent malaria clinical manifestation like diarrhea, lethargy, piloerection, reduced locomotor activity, etc were non-existent among CQ-treated showing that the parasitological cure was clinical as well. The 100% clearance of parasitaemia following the CQ chemotherapy indicates that the *P. berghei* strain used in the study was highly sensitive to the drug and lends support that this rodent malaria model system remains effective for in vivo anti-malarial testing.

10. Conclusions

The methanolic crude extract yield of the dried leaves of *O. quadripartita* was highest (32.75%) followed by DH2O (6.65%) and Chloroform extracts (1.447%) implying that most of the compounds in the leaf tissue of the plant were having similar chemical property with methanol. When orally administered no adverse effects were noted for all extractsranging from 1000-2000 mg/kg doses signifyingthe safetyof the extracted phytochemicals in mice by the oral route. The extracts of methanol and aqueous showed parasite suppressive effects on *P. berghei*-infected mice in a dose-related fashion and the chloroform extract was observed to have the stronger activity though its effect was not comparable to that of the standard drug which achieved 100% parasite clearance. Antiplasmodial activities as well as the lack of toxicity of the extract found in the present study which, is the first of its kind to report experimental findings for the plant, may partly confirm the claim by traditional practitioners about the use of the plant against malaria and may serve as a potential source for further investigation.

11. Recommendations

In thisstudy the *in vivo* anti-plasmodial activity of crude aqueous, methanol and chloroform extracts of the leaves of a local medicinal plant *O. quadripartita*was demonstrated for the first time. However, the finding is onlypreliminary; confirmatory studies followed byisolation and identification of the active anti-malarial compound(s) of the plant that are responsible for the

observed parasite suppression thereby resulting in increased MST, BW loss prevention and PCV reduction in the *P. berghei*-infected mice is recommended.Further,the anti-malarial activities of the plant need to be confirmed in CQ-resistant strains of model malaria parasites and afterward evaluation against human malaria parasite.

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17.

DECLARATION

I, the undersigned, declare that this Thesisis my original work and has not been presented for a degree in any other university. All sources of materials used for the Thesis are justly acknowledged.

Name: Senait Girma

Signature:

Submission date:

SUPERVISORS' STATEMENT

We, the undersigned, confirm that this Thesis is approved for submission.

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