Effect of the Crude Extract of the Leaves of *Osyris quadripartita* on *Plasmodium berghei* in Swiss 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 *in vivo* antimalarial activity against chloroquine-sensitive *Plasmodium berghei* of *Osyris quadripartita* Salzm. Ex Decne, locally called 'keret' in Amharic, which is used for traditional malaria treatment by local people in different parts of Ethiopia. The plant part was collected and identified by a botanist. Crude leaves were extracted using aqueous, methanol and chloroform solvent extracts. Methanol 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 test parasite, a 4-day suppressive standard test was performed. Data were analyzed using paired t-test and analysis of variance (ANOVA). Both aqueous and methanol extract of *O. quadripartita* significantly ($P<0.05$) suppressed parasitemia and prevented packed cell volume (PCV) reduction in dose dependent manner and body weight gain in all dose levels. In addition, they prolonged survival time in all doses. 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. Chloroform extract of the plant 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 malaria. Further evaluation of this plant is, however, needed before it is recommended for the control of malaria.

Keywords Antimalarial activity, *Osyris quadripartita, in vivo, Plasmodium berghei,* Swiss albino mice, Ethiopia

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List of abbreviations/acronyms

AAU	Addis Ababa University
ANOVA	Analysis of variance
BW	Body weight
CQ	Chloroquine
D0	Day zero
D4	Day four
dH_2O	Distilled water
DMCMB	Department of Microbial, Cellular and Molecular Biology
IC	Inhibitory concentration
LD	Lethal dose
MST	Mean survival time
NC	Negative control
OCED	Organization for Economic Cooperation and Development
PCV	Packed cell volume
SEM	Standard error of the mean
SPSS	Statistical package for the social sciences
SSA	Sub-Saharan Africa
T-80	Tween-80
WHO	World Health Organization

1. Introduction

1.1 Malaria parasites

Malaria is a protozoan disease caused by species belonging to the genus *Plasmodium* of phylum Apicomplexa. *Plasmodium* was discovered by Charles Louis Alphonse Laveran in a human blood in 1880, and its transmission by mosquitoes was revealed by Ronald Ross in 1898 (Lonc and Płonka-Syroka 2007). The most common *Plasmodium* species that cause human malaria are *Plasmodium falciparum* and *P. vivax* although two other distinct species, *P. malariae* and *P. ovale,* are known to cause human malaria in different parts of the world (http://www.cdc.gov/malaria/about/history/). According to this same *Center for Disease Control* information, *P. knowlesi* was first described by Robert Knowles and Biraj Mohan Das Gupta in 1931 in a long-tailed macaque and the first documented human infection with this species was in 1965. In recent years, *P. knowlesi* was reported to be a wide spread and lethal human infection in certain forested areas of South-East Asia (Singh et al. 2004).

1.2 Global malaria burden

Though malaria mortality rates have fallen by 47% globally and by 54% in the World Health Organization (WHO) African Region since 2000; about 198 million cases and 584 000 deaths occurred in 2013 showing that malaria is still among the leading human infectious diseases with about 90% of the cases and deaths occurring in Africa (WHO 2014a). This same WHO document ascertains that from economic point of view malaria incurs huge direct and indirect costs. Currently about 40% of the global population is at-risk of malaria - in over 100 countries in different regions of the world with sub-Saharan Africa (SSA) bearing the biggest burden. Thus malaria-related effects on levels of productivity, national growth and economic development are estimated to be very high. Although evidences suggest a substantial decline in malaria cases and deaths in recent years compared to the baseline year of 2004, the occurrence of several isolated outbreaks were reported nationwide and the disease remains a major cause of outpatient consultation in Ethiopia (FMoH 2014).

1.3 Malaria control

On the one side malaria appears to shrink as a result of scale-up of integrated control interventions and several endemic countries are embarked on malaria elimination or pre elimination programs (WHO 2014a). On the other hand, due to climatic changes and human-related factors (coupled-human-natural factors) malaria epidemiology is progressively changing putting more people at-risk. Moreover, there is a rapid emergence of drug-resistant plasmodium strains. For instance, resistance has already been developed against the latest first-line anti-malaria drug, artemisinin, in Asia (O'Brien et al. 2011, WHO 2014b, Ashley et al. 2014). Malaria control efforts are further complicated by the increased resistance of mosquito vectors to insecticides (Oduola et al. 2010, WHO 2012) together with challenges of having effective anti-malaria vaccines. Thus, there is urgent need to search for effective, easily available, affordable and safe alternative anti-malaria drugs that can be integrated into the existing malaria control interventions to successfully curtail the disease and for its eventual elimination or eradication.

1.3.1 Traditional medicinal plants as anti-malarials

It is well-known that plants have been and are still the mainstay of traditional medicine against malaria and other diseases in resource-limited settings as over one-third of the population in such countries lack access to essential medicines (WHO 2007). However, the claimed potency of medicinal plants has to be scientifically verified and their toxicity tested. Rigorous *in vitro* and *in vivo* toxicological investigations are 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 a plants' traditional medicinal use.

To this end, various studies on safety and anti-malarial effects of traditionally used plants in Asia and Africa have been conducted. For example, Ramazani et al. (2010) identified three of the ten Iranian plant species, but only - *Berhavia elegans*, *Salanum surattense* and *Prosopis juliflora* – will a promising anti-plasmodial activity *in vitro* and *in vivo*, andwith no toxicity. Like wise Verma et al. (2011) reported that *Holarrhena antidysenterica* and *Viola canescens* exhibit *in vitro* anti-plasmodial activity in Himalaya; Jansen et al. 2012, From SSA in Burkina Faso obtained the best *in vitro* anti-plasmodial results for the plant *Dicoma tomentosa* with the dichloromethane,

diethylether, ethylacetate and methanol extracts, which demonstrated a high activity. In the same study hot water and hydroethanolic extracts also showed a good activity and the activity was also confirmed *in vivo* for all tested extracts**.**

Likewise, ethanol extract of leaves of *Helianthus annus* in Swiss albino mice was observed to have mean percent chemosuppression ofas high as 98.1% and 98.3% in mice which received 2 and 4g/kg/day, respectively in Nigeria (Ejebe et al. 2011). Aqueous and methanol extracts of 15 plants traditionally used for treatment of malaria in Kenya were screened (Gathirwa et al. 2008) and of the plants tested *in vitro,* 25.0% were highly active (having a half maximal inhibitory concentration (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).

In Ethiopia despite the wide use in the traditional healthcare, the work that has been done on evaluation of the safety and efficacy of Ethiopian traditional medicinal plants is relatively less extensive. Nonetheless, there are some reports on the anti-malarial properties of selected Ethiopian traditional medicinal plants largely using *P. berghei*-infected Swiss albino 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).

It was shown that crude aqueous and ethanol extracts of *Aloe* sp., *Azadaichata indica* and *Tamarindus indica* had high anti-malarial activity at 650mg/kg (Mesfin et al. 2012). Similarly, Deressa et al. (2010) found that the crude methanol extract of *Clerodendrum myricoides*, *Dodonea angustifolia* and *Aloe debrana* exerted 82.5, 84.52 and 73.95 percent suppression, respectively in the mice-*P.berghei* system. The authors also observed that aqueous 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. 2006).

Likewise, Getie (2010) evaluated the anti-malarial activity of methanol and aqueous seed extracts of *Dodonaea angustifolia* and leaves of *Entada abyssinica* against *P. berghei* and found the highest parasite suppressions (86.21%) at 600mg/kg. Mengistie et al. (2012) also described that the

extracts of *D. 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* was evaluated and found the highest parasite suppression (51.15%) at 60mg/kg (Eyasu et al. 2013).

1.4. Rationale of the study

O. quadripartita Salzm. ex Decne. (locally called *keret* in Amharic and *watto* in Afaan Oromoo) is traditionally used in Ethiopia treat malaria (Belayneh and Bussa 2014). The plant is also commonly used in Ethiopian traditional medicinal practices (Mirutse et al. 2007, Mesfin et al. 2009, Lulekal et al. 2008, 2013) and elsewhere in Africa (Rimbach 1977, Watt and Breyer-Brandwijk 1962) in treating different ailments. For example, in Ethiopia, the plant is used to treat evil eye, epilepsy and as anaphylactic (Agize et al., 2013), skin injury (Getaneh, 2011), eczema (Kefyalew and Kelbessa, 2015) and cancer (Enyew et al., 2014). *O. quadripartita* is also reported to be used against cancer in Algeria (Wahiba and Malika 2014). According to study by Tadesse et al. (2011), the plant has demonstrated some *in vitro* antibacterial activity. Despite the report of the traditional use of *O. quadripartita* against malaria in Ethiopia, it has so far not been evaluated for its antiplasmodial property. The aim of this study was, therefore, to assess the anti-plasmodial activity and safety threshold of this plant in an attempt to contribute towards screening traditional medicinal plants for malaria control.

1.5 Hypothesis

The hypothesis of this study is that **'**The crude leaf extracts of *O. quadripartita* have antiplasmodial effect and are safe in animal model.'

2. Objectives

2.1 General objective

The general objective of the study was to evaluate the antiplasmodial effect and safety margins of crude leaf extracts of *O. quadripartita* against *P. berghei* in Swiss albino mice.

2.2 Specific objectives

The following were the specific objectives of the study.

1. To investigate the anti-plasmodial 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 leaves extracts on female Swiss albino mice.
3. To determine the level of effective *P. berghei* suppression dose of crude leaf extracts of *O. quadripartita*.

3. Materials and methods

3.1 Plant description and sample collection

The genus *Osyris* which includes more than 34 species belongs to the family Santalaceae. The species *O. quadripartita* Salzm. ex Decne. is an evergreen, dioecious tree or shrub reaching a height of 1-7m with many branches and the branches sometimes pendant (Hedberg and Edwards 1989). The plant is native to Africa (e.g. Algeria, Morocco, Ethiopia, Somalia, Kenya, Tanzania, Rwanda, Malawi, Botswana, Lesotho, Namibia, South Africa), southwestern Europe (Portugal, Spain) and Asia (Saudi Arabia, Yemen, Bhutan, India, Nepal, Sri Lanka, Laos, Myanmar, Thailand, China) (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?423626). In Ethiopia the plant is widely distributed (Hedberg and Edwards 1989). It is hemiparasitic and sometimes opportunistically taps into the root systems of nearby plants and parasitizes them although it can freely grow and survive (Herrera 1984, 1988). The picture of the plant which is commonly known as wild tea plant is indicated in figure 1 below.

Figure 1 *O. quadripartita* around Fiche town, the photo by author, December 2014

Leaves samples of the experimental plant were collected from its natural habitat in *Wertu kebele*, N $09^0$43.780' and E $038^0$48.370' and an altitude of 8415 feet, around Fiche town, 110 km North of Addis Ababa in December 2014. Previous ethno botanical studies characterized Fiche, its floristic composition in relation to traditional medicinal values (Enyew et al. 2013, 2014; d'Avigdor et al. 2014). The collected plant sample for this study was identified and authenticated by Dr Mirutse Giday who is an ethnobotanist at Aklilu Lemma Institute of Pathobiology, Addis Ababa University (AAU) and the voucher specimen was deposited in the National Herbarium with voucher number designated as Senait 001/2014.

3.2 Preparation of crude plant extracts

The leaf tissues were washed thoroughly with running tap water and each leaves of the *o.quadripartita* was reduced to smaller fragments. The washed leaves were air dried at a room temperature under shade in the Biomedical Sciences Laboratory, Department of Microbial, Cellular and Molecular Biology (DMCMB); College of Natural Sciences (CNS), AAU and were ground into fine powder using a blander and kept in a tightly closed brown bottle until used for extraction.

The leaves crude extracts of aqueous, 98% methanol and chloroform were prepared by the established cold maceration technique (O'Neill 1985). Briefly, a 100g plant extract material was refluxed in 1000ml of each of aqueous, methanol or chloroform in separate flasks and the respective mixture was placed on an orbital shaker (GFL, model 3020, Germany) at 160 revolutions per minute for 72 hours. The mixture was first filtered using cotton and then the filtrate was passed through Whatman filter paper №3, (15cm pore size with retention down to 0.1µm in liquids) (Whatman LTD, England). The methanol and chloroform extracts each separately were concentrated in a rotary evaporator (Buchi type TRE121, Switzerland) at a temperature of 45^0C; whereas the aqueous extract was freeze-dried using centrifugal freeze dryer (model 5 PS, Christ, England). All the extracts were stored in screw cap vials at -20^oC until used for the *in vivo* experiment.

3.3 Acute toxicity test

The aqueous and methanol extracts of the plant material were dissolved in 10ml of distilled water (dH_2O) and the chloroform was dissolved in 3% Tween(T)-80 prior to the actual experiment, the chloroform extract failed to dissolve in dH_2O. Individual body weights (BW) of mice were determined and recorded shortly before and after the plant was administered to evaluate its toxicity as per the Organization for Economic Co-operation and Development (*OECD)* Guidelines for the Testing of Chemicals *(OECD 2008). For this purpose non-infected female mice* aged 6-8 weeks (mean age 7.4 weeks) and weighing 23-38g (mean weight 28.35g) were used.

Twenty mice were used by randomly dividing them into four groups of 5 mice per cage. The mice were starved for 3-4 hours before the experiment begun with only water allowed and 1-2 hours after the extracts were given. Then, the mice in groups 1, 2 and 3 were orally given 1000, 1500 and 2000mg/kg BW in single dose volume of 0.2ml of the extract, respectively. The mice in the negative control group received 0.2ml of dH_2O or T-80. Then, the mice were monitored continuously for 1 hour, intermittently for 4 hours and for a period of 24 hours for any gross behavioral changes such as rigidity, sleep, mortality and other signs of acute toxicity manifestations, and the follow-up continued for 14 days (OECD 2001).

3.4 Parasite inoculation and anti-malarial assays

3.4.1 Parasite inoculation and maintenance

Mice are considered to have a comparable genetic model to human, up to 99% degree of genomic conservative (Pennacchio 2003). *In vivo* evaluation of anti-malarial 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. When working on chloroquine (CQ)-sensitive *P. berghei* CQ is employed as a standard positive control (Fidrock et al. 2004) and in this study a similar study design was followed. Previously maintained Swiss albino mice 6-8 weeks of age were used for the test. CQ-sensitive strain of *P. berghei* previously maintained in the DMCMB animal house were used for the experiment.

Female mice having variable parasitaemia level were used as donor animals. The parasitaemia of the donor mice was first determined and parasitized erythrocytes were obtained by cardiac puncture using ethylether 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 erythrocyte 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 intra-peritoneal 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.

3.4.2 Anti-malarial activity

In screening of the plant extracts the standard four-day suppressive method was used (Fidrock et al. 2004). The experimental mice infected with $1x10^7$ *P. berghei* each were randomly divided into nine test groups and three control groups (each for CQ as the standard anti-malarial drug and dH_2O and 3% T-80 as negative controls (NC). The test extracts were prepared in three different doses (200, 400 and 600mg/kg BW and CQ at 25mg/kg, and all including the vehicles were given in a volume of 0.2ml per 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 a standard intra-gastric tube to ensure safe ingestion.

3.4.2.1 Suppression test of the extract

Treatment was started after 3 hours of infection with *P. berghei* on day 0 (D0, inoculation day) and was continued daily for four days (i.e. from D0 to D3). On the fifth day (D4) a blood sample was collected from the tail snip of each mouse. Thin smears were prepared, stained with 10% Giemsa solution and scanned under the light microscope with an oil immersion objective of 100x magnification power following established procedure (WHO 2010). Then, 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. Percent of suppression (% suppression) and percent parasitaemia (% parasitaemia) were calculated in comparison to control using the method described by the modified Peters and Robinson formula (1992):

$$\% \text{ Suppression} = \frac{\text{Parasitemia in negative control} - \text{parasitemia in treated group}}{\text{Parasitemia in negative control}} \times 100\ \%$$

% Parasitaemia = $\frac{\text{Number of Parasitized RBC}}{\text{Total RBC counted}}$ X 100 %

3.4.2.2 Determination of BW change

The BW 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&D Company LTD, Japan) and mean BW per group was calculated using the formula:

Mean BW=$\frac{\text{Mean body weight of mice in a group}}{\text{Total no of mice in that group}}$

3.4.2.3 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 $3/4^{th}$ of the tube with blood, sealed one end with crystal seal and placed with the open end of the tube to the center and the sealed end outwards of a microhaematocrit reader (Hawsksley & sons LTD, England). The blood was centrifuged at 12 000 revolutions per minute for 5 minutes and then the 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 using the formula described by Gilmour and Sykes (1951):

PCV = $\frac{\text{Volume of total erythrocytes in a given volume of blood}}{\text{Total blood volume}}$ x100

3.4.2.4 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 the formula:

MST= $\frac{\text{Sum of survival time (days) of all mice in a group}}{\text{Total number of mice in that group}}$

3.5. Ethical considerations

This study was conducted after the necessary ethical clearance was obtained from CNS Institutional Ethics Review Board, AAU. 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. Overall there was a humane handling and sacrificing of the experimental animals.

3.6 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 (Mean $\pm$SEM) of the mean by one way analysis of (ANOVA) and 2-tailed Student's t-test using statistical package for the social sciences (SPSS) version 15 software (SPSS IBM, Chicago, IL). The suppressive test results were analyzed by ANOVA followed by Tukey-multiple comparison test to compare the level of parasitaemia, 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.

4. Results

4.1 Extraction yield

The methanol extract of the plant material was the highest yield followed by the aqueous and chloroform extracts (Table 1). The differences between the yields were significant.

Table 1 Yield of aqueous, chloroform and methanol crude 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: ml=milliliter; g=gram; %=percentage; * = there was significant difference between these values ($P<0.05$).

4.2 Acute toxicity test

The experimental mice who ingested crude leaf extracts of the three solvents did not show any indications of gross physical or behavioral changes such as hair erection, reduction in feeding and motor activity, weight loss, lacrimation, diarrhea, depression or abnormal secretions within the 24 hours monitoring period. No fatalities occurred within the observation period of 14 days.

4.3 Anti-malarial activity

4.3.1 Effect of crude leaf extracts on PCV and BW

The aqueous, chloroform and methanol leaf extracts of *O. quadripartita* demonstrated a dose-dependent effect on the mean PCV value of *P. berghei*-infected mice (Table 2). With increasing dose percentage change in PCV reduction was lowered for all the three extracts. Also, irrespective of extract type dose there was reduction on mean PCV on D4 though the difference was significant only for the 200mg/kg the aqueous, and 200 and 400mg/kg methanol extracts. But, when the results were compared with the NC only 600mg of the aqueous extract showed a significant mean PCV reduction ($p = 0.01$).

A loss in BW was noticed for all groups of *P. berghei*-infected mice four days post-extract-inoculation except those that ingested 400 and 600mg/kg chloroform extract. However, the percentage change in BW loss decreased in a dose-dependent manner for all the three extracts at the three doses. In the three groups of infected mice that ingested the three different doses of the aqueous and methanol extracts a significant BW loss was recorded on D4 compared D0, but for the chloroform the difference was not significant. On the other hand, while compared to the NC the change in BW, for all the three doses, was significant for chloroform extract it was not so for the other two. The infected mice that treated with 400 and 600mg/kg chloroform significantly gained BW.

Table 2. Effect of crude aqueous, methanol and chloroform leaf extracts of *O. quadripartita* on PCV

Description	**Dose (mg/kg/day)**	**PCV**			
		D0	**D4**	**% change**	***p*-value****
Aqueous	200	60.4±0.60	56.0±0.89[a]	-7.28	0.02, 0.05
	400	56.6±1.28	54.0±1.14	-4.59	0.17, 0.34
	600	58.6±2.02	56.8±1.98*	-3.07	0.34, 0.01
Methanol	200	58.4±1.77	54.2±1.46 [a]	-7.19	0.02, 0.29
	400	57.0±1.60	53.2±1.20 [a]	-6.67	0.04, 0.59
	600	59.4±0.40	55.8±0.73	-6.06	0.06, 0.06
Chloroform	200	57.2±1.59	54.2±1.31	-5.24	0.11, 0.29
	400	56.4±0.75	55.0±0.54	-2.48	0.46, 0.34
	600	54.8±1.24	53.8±1.52	-1.82	0.59, 0.17
dH_2O (NC)	0.2ml	56.8±1.93	52.2±1.71 [a]	-8.09	0.017
T-80 (NC)	0.2ml	58.0±1.51	52.4±0.92 [a]	-9.65	0.004
CQ	25	56.8±0.96	56.8±0.89*	0.00	0.674

Keys: Values for the packed cell volume (PCV) and body weight (BW) are presented as mean(M) ± standard error of the mean(SEM); n=5 (number of mice/group in a single experiment); D0: day 0 (day-of-extract-inoculation); D4=day 4 (the fifth day pos-extract-inoculation; mg/kg/day: 1milligram of extract per one kilogram of mice for one day; NC: negative control; T-80= Tween-80 (NC for chloroform extract); dH_2O: distilled water (NC for aqueous and methanol extract); CQ: chloroquine phosphate; *: significant compared with that of NC; [a]: significant difference between D0 and D4. **the first value is for the comparison between D0 and D4, the second is with the NC.

Table 3. Effect of crude aqueous, methanol and chloroform leaf extracts of *O. quadripartita* on BW of *P. berghei*-infected mice

Description	Dose (mg/kg/day)	BW(g)			
		D0	D4	% change	*p*-value**
Aqueous	200	31.2±0.37	26.5±0.76 [a]	-15.06	0.000, 1.000
	400	25.2±0.37	21.8±0.58 [a]	-13.49	0.000, 0.005
	600	28.6±0.40	25.1±0.47 [a]	-12.23	0.000, 0.000
Methanol	200	30.6±0.40	26.4±0.24 [a]	-13.72	0.000, 1.000
	400	28.2±0.37	24.6±0.40 [a]	-12.76	0.000, 0.522
	600	25.6±0.40	23.2±0.37[a]	-09.37	0.000, 0.112
Chloroform	200	27.6±0.24	26.2±0.37*	-05.07	0.027, 0.000
	400	28.6±0.50	30.8±0.58*	+07.69	0.001, 0.000
	600	27.0±0.31	29.6±0.40*	+09.62	0.000, 0.000
dH_2O (NC)	0.2ml	29.0±0.31	23.6±0.24 [a]	-18.62	0.000
T-80 (NC)	0.2ml	28.8±0.48	23.0±0.44 [a]	-20.13	0.000
CQ	25	29.8±0.48	33.2±0.37*[a]	+11.40	0.000

Keys: Values for the packed cell volume (PCV) and body weight (BW) are presented as mean(M) ± standard error of the mean(SEM); n=5 (number of mice/group in a single experiment); D0: day 0 (day-of-extract-inoculation); D4=day 4 (the fifth day pos-extract-inoculation; mg/kg/day: 1milligram of extract per one kilogram of mice for one day; NC: negative control; T-80= Tween-80 (NC for chloroform extract); dH_2O: distilled water (NC for aqueous and methanol extract); CQ: chloroquine phosphate; *: significant compared with that of NC; [a]: significant difference between D0 and D4. **the first value is for the comparison between D0 and D4, the second is with the NC.

4.3.2 Effect of crude leaf extracts on parasitaemia

Although there was clear regression of parasitaemia among aqueous or methanol extract-treated mice, compared to the NC, the difference was statistically significant only for the methanol extract at 600mg/kg dose. With regard to the chloroform extract, however, there was significant decline in % parasitaemia at all the three doses in a dose-dependent manner. The maximum parasitaemia reduction was 38.26% (for the 600mg) compared to the value of the negative control group (65.4%). The evidence showed that the chloroform extract, was though significantly associated with lowered parasitaemia compared to the NC as well as the other two concurrently tested extracts, the mice had still microscopically detectable parasitaemia.

Correspondingly, in all groups of extract-treated-mice parasitaemia were increasingly reduced in a dose-dependent manner with the highest suppression being for the chloroform extract - the highest (41.26%, at 600mg/kg dose), mild (37.5%, at 400mg/kg), and the least (31.7%, at 200mg/kg).

Similarly, for the aqueous and methanol extracts all the three doses, except aqueous at 200mg/kg, were correlated with significantly increased mice survival compared to the NC (dH_20). The MST of chloroform extract-treated mice was also significantly higher compared to the NC (3% T-80) groups and it was prolonged in a dose-dependent manner (Table 3).

Table 4. Suppressive effect of crude aqueous, chloroform and methanol of the leaves extracts of *O. quadripartita in P. berghei*-infected mice

Description	Dose (mg/kg/day)	% parasitaemia	*p*-value	% suppression	MST	*p*-value
Aqueous	200	63.02±3.91	0.740	0.37	6.2±0.37	0.418
	400	54.62±2.20	0.142	16.53	7.4±0.51*	0.018
	600	51.26±2.05	0.056	21.67	8.2±0.37*	0.001
Methanol	200	55.92±0.84	0.196	14.5	7.4±0.51*	0.018
	400	50.94±1.24	0.510	22.16	8.2±0.37*	0.001
	600	49.48±2.06*	0.033	24.4	8.4±0.24*	0.000
Chloroform	200	44.48±10.18*	0.006	31.7	8.0±1.14*	0.004
	400	40.7±9.66*	0.001	37.5	9.8±0.58*	0.000
	600	38.26±8.78*	0.001	41.26	11±0.63*	0.000
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	0.000	100	ND	

Keys: Values for the packed cell volume (PCV) and body weight (BW) are presented as mean(M) ± standard error of the mean(SEM); n=5 (number of mice/group in a single experiment); D0: day 0 (day-of-extract-inoculation); D4=day 4 (the fifth day pos-extract-inoculation; mg/kg/day: 1milligram of extract per one kilogram of mice for one day; NC: negative control; T-80= Tween-80 (NC for chloroform extract); dH_2O: distilled water (NC for aqueous and methanol extract); CQ: chloroquine phosphate; *: significant compared with that of NC.

5. Discussion

While methanol yielded significantly higher extraction efficiency, the chloroform extract product was the lowest. The probable reason for this variation could be due to high concentration of polar compounds in the leaf of the plant species that better dissolved in methanol which is a polar solvent. 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). While chloroform is preferred to extract terpenoids and flavonoids water is employed to solubilize a wide range of plant metabolites like anthocyanins, starches, tannins, saponin2s, terpenoids, polypeptides and lectins. Methanol releases more diverse phytochemicals such as anthocyanins, tannins, saponins, terpenoids, xanthoxylines, totarol, quassinoids, lectones, flavones, phenols and polyphenols (Cown 1999).

The finding that the 3% T-80, which was used as a NC (for the chloroform extract) in the present study and in which the extract was dissolved to deliver to the mice, neither suppressed malaria parasitaemia in the infected mice nor prevented weight loss and PCV depletion indicated lack of possible T-related effect on the malaria parasites.

Oral administration of the methanol, aqueous and chloroform extract of *O. quadripartita* did not show changes in the general appearance or behavioral pattern of the experimental mice till the end of 14 days. Further, no death was observed in the animals receiving the extract up to a dose of 2000 mg/kg BW, 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 2000 mg/kg could indicate that the test extracts were safe.

Significant BW increase among *P. berghei*-infected mice four days after ingesting 400 and 600mg chloroform crude leaf extract of *O. quadripartita* 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 murine malaria (Timms et al. 2001). The present result is in agreement with other similar studies that reported mice BW loss using different plant products and extraction solvents (Amelo et al. 2013, Eyasu et al. 2013, Mohammed et al. 2014). Lack of significant reduction in the mean BW among aqueous and methanol extract-fed infected mice four days post-treatment further signifies the positive effect of the plant material on mice BW.

The absence of significant PCV reduction among extract-treated mice at the doses of 200 and 400mg/kg of the aqueous extract may indicate the protective activity of the crude extract. Furthermore, observing a significantly lower PCV reduction among the same groups of mice at the highest dose (600mg/kg) shows the presence of anti-plasmodial chemicals in the dose administered. But it seems that the activity of the methanol extract was not strong enough to significantly prevent PCV reduction among *P. berghei*-infected mice. On the contrary the PCV of chloroform extract-treated mice remained not significantly changed on D4, irrespective of dose underlining the protective role of the extract against malaria.

The influence of malaria on hematological parameters is extensively investigated and PCV reduction is considered a hallmark of both human and rodent malaria (reviewed in Lamikanra et al. 2017). Infected mice may suffer from severe malarial anemia because of rapid erythrocyte destruction, either by parasitaemia and/or spleen reticulo-endothelial cells. 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). Furthermore, *P. berghei* increased erythrocyte fragility and led to subsequent reduction of PCV in infected-mice (Iyawe and Onigbinde 2009).

Multiple other *in vivo* studies on rodent malaria using diverse plant species from 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 could not permit a discussion from comparative

perspective. Although the antimicrobial (Geyid et al. 2005, Tadesse et al. 2003) and antiparasitic activities (Al-Jaber et al. 2010) of *O. quadripartita* were investigated to some extent its plasmodial effect was little explored before.

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 effective (Fidock et al. 2004, Krettli et al. 2009). Accordingly, the chloroform extract of *O. quadripartita* which showed 31.7% suppression at the lowest, 37.5% at the medium and 41.26% at the highest doses can be classified as effective in reduction. The dose-dependent variation in suppression 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 percent suppression of parasitaemia with increased dose was observed by several other studies for different plant species (Muzemil 2008, Eyasu et al. 2013, Mohammed et al. 2014).

However, both the aqueous and methanol extract of *O. quadripartita* didn't display comparable suppressive activity on *P. berghei* even at the highest dose delivered (600mg/kg). This at least undetectable level of anti-malarial activity of aqueous and methanol crude leaf extract of *O. quadripartita* may be an indication that the active ingredients extracted by these solvents might have less potent anti-malarial property. 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 or methanol extracts in the current study were higher in terms of yield but they contained little compounds that were efficacious at least against *P. berghei.*

A prolonged MST with significant difference, compared to the NC, was observed for mice treated with all the three extracts regardless of dose except for 200mg/kg of the aqueous extract implying the role of the plant material in the control of murine malaria. Particularly, the chloroform extract was highly associated with prolonged MST even at the minimum dose implicating the dominant presence of antimalarial bioactive compounds of the plant tissue in this extract.

Nevertheless, the chloroform extract itself was far less effective compared to CQ, the standard drug against *P. berghei*. CQ treatment (25mg/kg) during the infection seemed radically cleared parasitaemia or at least there was no microscopically detectable parasitaemia. Rodent malaria clinical manifestation like diarrhea, lethargy, piloerection, reduced locomotor activity, etc were non-existent among the CQ-treated showing that the parasitological cure was clinical as well. Mice were appearing and acting healthy on day 28. The undetectable level 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.

6. Conclusion and Recommendation

6.1 Conclusion

The methanolic crude extract yield of the dried leaves of *O. quadripartita* was the highest (32.75%) followed by aqueous (6.65%) and chloroform extracts (1.44%) implying that most of the compounds in the dried leaves of the plant have relatively similar chemical property with methanol. When orally administered, no adverse effects were noted for the plant extracts ranging from 1000-2000mg/kg doses signifying the safety of the extracted phytochemicals in mice via the oral route. The aqueous and methanolic extracts showed some parasite suppressive effects on *P. berghei*-infected mice in a dose-related fashion and the chloroform extract was observed to have the strongest activity. The anti-plasmodial activity as well as lack of toxicity of the crude extract found in the present study which, is the first of its kind to report anti-plasmodial effect 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.

6.2. Recommendations

In this study 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 only preliminary; confirmatory studies followed by isolation and characterization 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 prior to its evaluation against human malaria.

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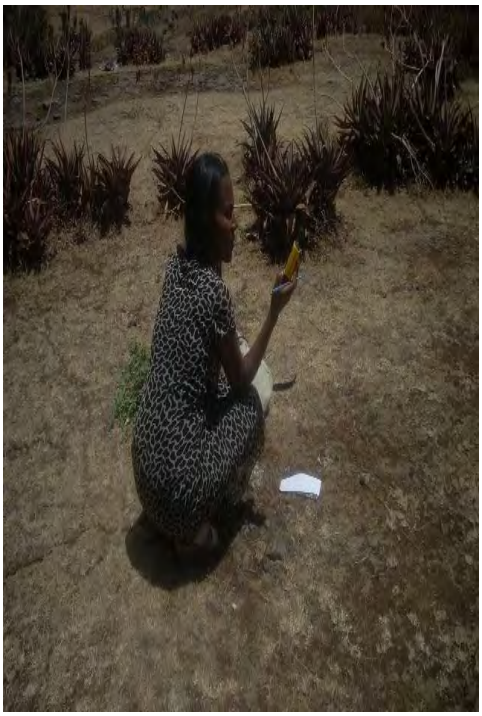
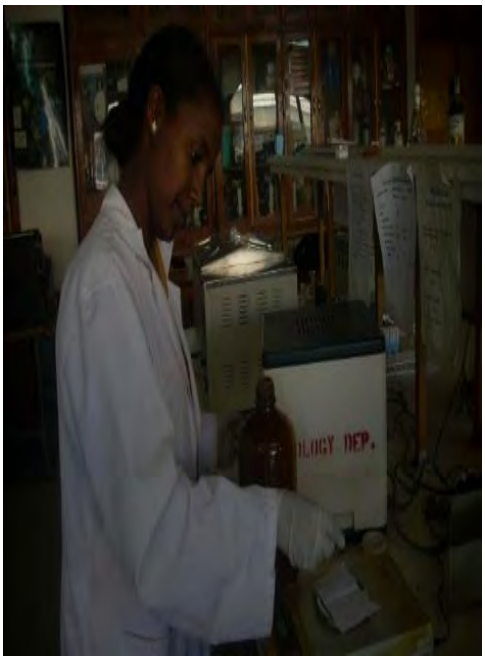
8. Annex

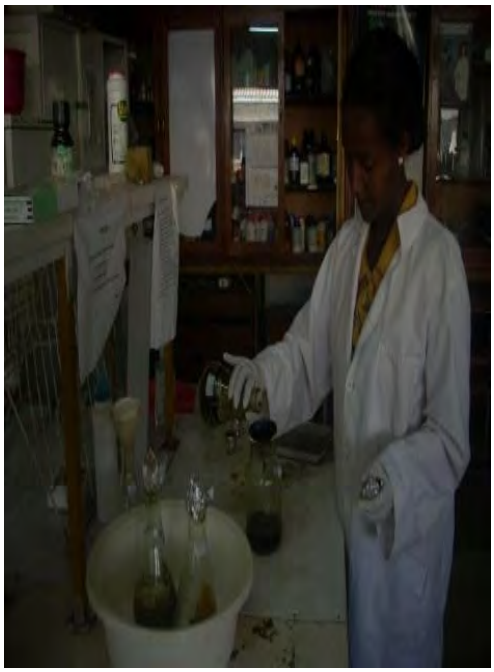
Collection of the plant



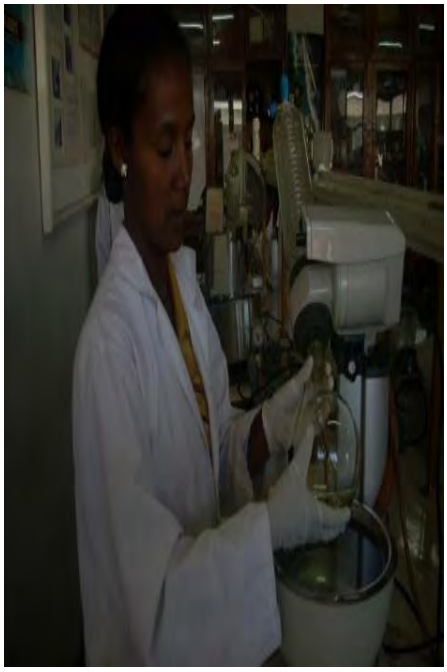
GPS of the place



Weighing the plant



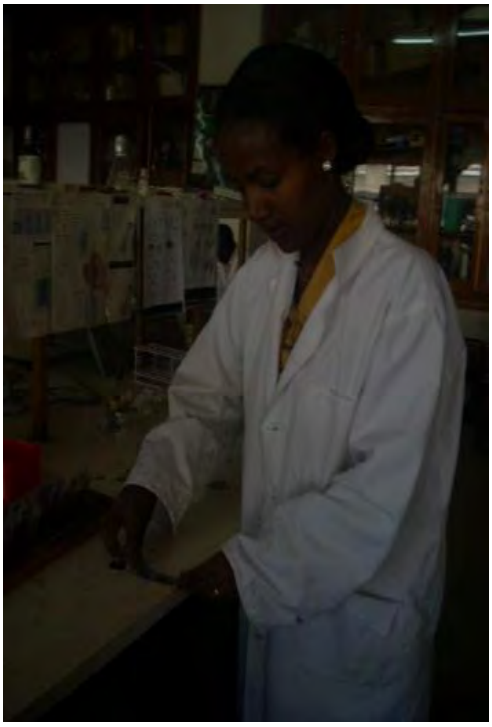
Extraction of the plant



Preparation of the plant

Acclimatized mice

Inoculating parasitemia



Thin-smear preparation

Declaration

I, the undersigned, declare that this Thesis is 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.

Senait Girma

Signature _____Submission date _____

Supervisors' statement

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

1. Name Dr Hassen Mamo Signature _____ Date _____
2. Name Prof Berhanu Erko Signature _____ Date _____
3. Name Dr Mirutse Giday Signature _____ Date _____