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Antiplasmodial Activity of Leaf Extracts of *Zanthoxylum chalybeum Engl.*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors GSB and NM were involved in the design of the study. All Authors were involved in execution of the study, statistical analysis, writing the protocol and the first draft of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Background: Medicinal herbs have long been used in the treatment of malaria in the endemic tropical and subtropical regions of the world, especially in sub-Saharan Africa where malaria has remained as a top killer disease to children under five years and expectant mothers. Among the herbs commonly used in Uganda to treat malaria is *Zanthoxylum chalybeum (ZC)* root-bark and to a lesser extent its leaves. However, the continued use of the root-bark has led to plant extinction due to the destructive method of harvesting the herb as opposed to the leaves.
Aim: The study investigated the *antiplasmodial* activity of the crude ether and methanol leaf extracts of *ZC* with chloroquine diphosphate as a positive control.
Materials and Methods: The *in vitro* Micro-Test (MARK III) kit was used to determine the *antiplasmodial* activity of *Z. chalybeum Engl.* ether and methanol extracts. The effective

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concentration that caused a 50% *P. falciparum* schizonts suppression (EC_{50}) values were determined. The *antiplasmodial* activities of the ether and methanol extracts were expressed as 50% effective concentration (EC_{50}) that was determined from dose-response curve by non-linear regression analysis (curve-fit) using GraphPad Prism (version 6) software at 95% confidence intervals.

Results: The results showed that ether extract of *ZC* had EC_{50} value of 13.39 (95% CI: 10.82 – 16.59) µg/ml and methanol extract had EC_{50} value of 8.10 (95% CI: 5.89 – 11.12) µg/ml. The chloroquine diphosphate, standard had EC_{50} value of 25.33 (95% CI: 17.07 – 37.60) µg/ml.

Conclusion: The ether and methanol extracts of *Z. chalybeum Engl.* contains compounds that caused *Plasmodium falciparum* schizonts suppression at a lower concentration, hence the continued use of the herb by the traditional herbalist and local communities in Uganda, in the treatment of malaria.

Keywords: Plasmodium falciparum; EC50; Zanthoxylum chalybeum Engl; leaf extract malaria.

1. INTRODUCTION

Medicinal herbs have long been used in the treatment of malaria in endemic tropical and subtropical regions of the world especially in sub-Saharan Africa where it has remained as a top killer disease in children under five years and expectant mothers. It is a major cause of morbidity and mortality globally, accounting for about 216 million cases and about 655 000 deaths in 2010 [1]. Of these, about 80% of the cases and 90% of the deaths occurred in sub-Saharan Africa [1]. Malaria is caused by different protozoan species of *Plasmodia* including *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*. The *P. falciparum* is the most deadly specie, accounting for the highest malaria cases globally [1,2]. In Uganda, malaria is a major public health problem, contributing to 30% to 50% of outpatient care, 15% to 50% of hospital admissions and 9% to 14% of inpatient deaths [1,3,4]. However, the high cost of the effective antimalarial drugs, the poor quality drugs and the increased emergence of *Plasmodial* resistance has forced many poor individuals especially in rural areas to use medicinal herbs as alternative source of medicine in malaria treatment and prevention. Among the herbs commonly used includes the different species of *Zanthoxylum* genus [5,6]. There are different species of the genus used globally especially in Asia, Africa and America to treat a number of diseases in humans and animals [7,8]. The genus is reported to contain many secondary metabolites (phytochemicals) with medicinal properties including alkaloids, benzophenanthridines such as isoquinoline alkaloids, benzylisoquinoline alkaloids, berberine and protoberberine alkaloids, aporphine alkaloids, quinoline alkaloids, bishoderninyl terpene, indolopyridoquinazoline, canthin-6-one, quinazoline and carbazole alkaloids, lignans, coumarins, amides, flavonoids, terpenes and sterols [8]. The herbs in the genus are reported to be used in relief of dental problems, treatment of malaria, gastrointestinal disorders, gonorrhea and lung diseases, diarrhea in animals and humans, emmenagogue action, rheumatism, helminthes in animals and humans, aphrodisiac, analgesic, skin diseases, febrifuge, hemorrhages, genito-urinary diseases, cancer, diuretic, stomachache and convulsions [7,8]. In addition to the medicinal properties, some species are also used as pesticides, provides building materials and textile dyes [7,8]. Among the species of *Zanthoxylum* genus commonly used in treatment and prevention of malaria is *Zanthoxylum chalybeum Engl.* [7,8]. The root-bark of *Z. chalybeum Engl.* is the most commonly used part of the plant by both the local communities and the traditional herbalist globally in the

treatment of malaria [9,10]. The leaves are also used at a lesser extent. However, most of the antiplasmodial studies on the herb have been done on the root-bark and hence a need to investigate the activity on the leaves and document if it has comparable activity and hence use as an alternative to the root-bark. Routinely, the root-bark of the herb is commonly used as a decoction where the plant materials are mashed and boiled in water at varying temperatures and the dissolved active chemical substances are administered to the individual orally [7,9,24]. Also it's used as concoction where it is combined with various ingredients, usually herbs, spices, condiments, powdery substances, or minerals, mixed up together, minced, dissolved or macerated into a liquid so as they can be ingested or drunk [7,9,24]. The root-bark has been reported to contain various compounds with antiplasmodial activity including alkaloids, flavonoids, sesquiterpene lactones and saponins [9]. However, despite of the wide use of the *Z. chalybeum Engl* herb in malaria treatment by the local communities and traditional herbalists, the methods of harvesting the root-bark of the herb as opposed to the leaves is not sustainable since it destroys the plant and hence eventually leading to its extinction [5,6,9,10]. The aim of the study was to investigate the antiplasmodial activity of the ether and methanol extracts of *Z. chalybeum Engl*. against *P. falciparum* and it also determined the EC_{50} values of the extracts using chloroquine diphosphate as positive control.

2. MATERIALS AND METHODS

2.1 Study Design

An experimental study investigated the activities of the ether and methanol extracts of *Z. chalybeum Engl.* on the *P. falciparum* parasites. The root-bark of the herb has been reported to be used in various communities of Uganda to treat malaria [7,8,11,12]. However the method of harvesting the root-bark of the herb is destructive and may lead to the extinction of the plant as opposed. Hence the use of the leaves for antimalarial activity was suggested.

2.2 Collection, Identification, Processing and Extraction of the Herb

The *Z. chalybeum Engl.* leaves were collected from Wakiso district in Central Uganda. The leaves were used in the study, to determine if they have similar antiplasmodial activity against the wild strains of *P. falciparum* as compared to the root-bark. The leaves were collected in October 2007. Botanical identification was carried out at Makerere University herbarium by a taxonomist. The voucher number of *Z. chalybeum Engl.* was retrieved from the achieves at the herbarium as JRST 563. The leaves were cleaned using clean water and then air-dried in a shade until constant weight was obtained at the Department of Pharmacology and Therapeutics laboratory, Makerere University College of Health Sciences. The dry leaves were then pounded in a wooden mortar using a pestle to obtain a powder. A total of 500g of herbal powder was soaked in 1500 ml of ether in Erlenmeyer flasks for 3 days. It was then filtered using a Whatman No.1 filter paper in a Buchner funnel. The filtrate was collected in a flat bottomed flask with a stopper. The process of ether extraction was repeated twice to ensure maximum yield of the extracts. The residue was then air dried for about 4 hours and then soaked in 1500 ml of methanol and the process was repeated as for the ether extraction. The dry ether and methanol extracts were obtained from the filtrate by recovering the solvents using a Heidolph model rotary evaporator (BUCHI Rotavapor R-205 model). To obtain complete dry ether and methanol extracts, they were exposed to room temperature for 24 hours in dark sample bottles to prevent direct exposure

to light that would cause oxidation of the compounds in the extracts that are sensitive to light.

2.3 Antiplasmodial Activity Study

The antiplasmodial activity of the *Z. chalybeum Engl.* ether and methanol extracts were evaluated at the Department of Microbiology and Parasitology, Makerere University College of Veterinary Medicine, Animal Resources and Biosecurity, using standard methods [13-16].

2.3.1 Preparation of culture media and the *P. falciparum* organisms

The culture medium was prepared by dissolving 10.4g of powdered RPMI 1640 (Sigma-Aldrich Chemical Company, Munich, Germany) and 5.94 g of HEPES (N-2 hydroxyethyl piperazine-N-2'-ethanesulphonic acid) (Sigma-Aldrich Chemical Company, Munich, Germany) in 1 litre of sterile distilled water. The medium was filtered using 0.22 mm Millipore filter and then stored at 4ºC ready for use. The wash medium was prepared by adding 1.6 ml of 7.5 % (w/v) of $NaHCO_3$ (Sigma-Aldrich Chemical Company, Munich, Germany) to 43.4 ml of the medium above (RPMI 1640 with HEPES) in 50 ml centrifuge tubes [13,14,17,18]. The wild strains of *P. falciparum* organisms were isolated from blood samples with mono-infection in the Microbiology Laboratory at Mulago hospital complex, National referral hospital assessment center. The blood samples were obtained during the screening of patients with uncomplicated malaria prior to treatment. The *P. falciparum* in infected red blood cells were isolated and cultured with the freshly prepared wash culture medium [14,17,18]. The *in vitro* cultivation of *P. falciparum* isolates followed a modification of the standard culture techniques [15]. The culture medium consisted of RPMI 1640 (Sigma Aldrich), 2 g glucose and 40 µg/mL gentamycin sulphate with supplemented 10% AB^+ serum. Culture medium was sterilized by filtration through a Millipore filter of 0.22 µm porosity and the pH was adjusted to 7.4 by the addition of 4.2 mL of sterile 5% sodium bicarbonate. Blood culture of *P. falciparum* was continuously cultured according to the method described by Trager and Jensen [15]. The parasites were maintained in continuous culture on human erythrocytes (O^+ blood group), in RPMI 1640 medium supplemented with 10% of human AB^+serum, 25 mM HEPES, 19 mM sodium carbonate and 45 µg/mL gentamicin sulfate, at pH 7.2 in an atmosphere of 91% nitrogen, 6% carbon dioxide and 3% oxygen. The medium was changed each day. The plates with the parasite culture were put in a candle jar and placed in the incubator set at 37.5ºC for 24–30 h, depending on the developmental stage of the parasite. After 24-hour incubation, a thin smear was prepared from the control well to observe the mature schizonts and if more than 10% schizonts were seen, it was considered to be valid. The thick smears were prepared from each well by discarding the excess media with a micropipette [14,17,18].

2.3.2 Preparation of different concentrations of the ether and methanol extracts and chloroquine diphosphate solutions

The methanol and ether extract of *Z. chalybeum Engl.*, stock solutions were each made by dissolving 100 µg of the each extract with 0.2 ml of dimethylsulfoxide (DMSO) to facilitate the dissolution and then topped up with distilled water to give a stock solution of concentration of 100 µg/ml of ether and methanol extract respectively. Serial dilutions of the stock solutions were made using distilled water to produce 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml and 1.0 µg/ml of ether and methanol extracts respectively. The chloroquine diphosphate used as positive control was obtained from Sigma-Aldrich Chemical Company, Munich, Germany. The stock solution of chloroquine diphosphate was obtained by dissolving

100 µg in 1% ethanol and topped up with culture medium to produce a concentration of 100 µg/ml and this was then diluted subsequently with culture medium to achieve the required concentrations for the bioassay similar to the extracts. The culture medium was used as negative control. The varying concentrations were used in the in *vitro* bioassay to determine the antiplasmodial activity of *Z. chalybeum Engl.* extracts.

2.3.3 In vitro bioassay to determine the antiplasmodial activity of *Zanthoxylum chalybeum Engl.* extracts

The *in vitro* Micro-Test (MARK III) kit was used to determine the antiplasmodial activity of *Z. chalybeum Engl.* ether and methanol extracts [14,17,18]. The preparation of ether, methanol extracts and chloroquine diphosphate concentrations and the design of the test plates were based on the standard methods [14,17,18]. In a 96 well tissue culture microtitre plates, 5 µl of parasite suspension were transferred in each well and then each was inoculated with 25 µl of the varying concentration of the extracts and controls to produce a final volume of 30 µl per well. The first well served as the control with only the culture medium, the second well contained only the *P. falciparum* infected medium and served as the negative control and the third well served as the positive control with chloroquine diphosphate solution. The culture plates with *P. falciparum* parasites and the ether and methanol extracts and chloroquine diphosphate were incubated at 37ºC in a candle jar (5% CO_2, 17% O_2, 78% N_2) according to the method of Trager & Jensen [15], for 18-48 hours depending on the time taken by the parasite to develop into schizonts [14,17,18]. After incubation, blood smears using glass slides from each well were fixed in methanol, stained with Giemsa stain and microscopically observed for the presence of parasites. The number of infected red blood cells (RBCs) per 200 white blood cells was counted. The parasite density was estimated as the number of mature schizonts per 200 white blood cells (WBC) from which the inhibitory concentrations of 50% of *P. falciparum* schizonts (EC50) were determined using a $\log_{10}$ (concentration) - response curve.

2.3.4 Data analysis and determination of In Vitro effective Concentration (EC50) values of the ether and methanol extracts of *Z. chalybeum Engl*

The effective concentration (EC_{50}), defined as the drug concentration required to suppress 50% of schizonts development were determined. The antiplasmodial activities of the ether and methanol leaf extracts were expressed as 50% effective concentration (EC_{50}) determined from $\log_{10}$ [concentration] - response curve by non-linear regression analysis (curve-fit) using GraphPad Prism (version 6) software at 95% confidence intervals (CI). All experiments were performed in triplicates and the results were expressed as mean percentage of *antiplasmodial* schizonts suppression. Crude extracts with EC_{50} values > 50 µg/ml were considered to be inactive [13].

3. RESULTS AND DISCUSSION

Antiplasmodial activity of the ether and methanol extracts of *Z. chalybeum Engl.* were determined using chloroquine diphosphate as positive control and the culture medium as negative control. The results show that ether extract had EC_{50} value of 13.39 (10.82 - 16.59) µg/ml at 95% CI; methanol extract had EC50 value of 8.10 (5.89 – 11.12) µg/ml and choloroquine diphosphate had EC_{50} value of 25.33 (17.07 – 37.60) µg/ml (Table 1). There was no suppression of *P. falciparum* schizonts in the culture medium. The results also show that methanol extract had the lowest EC_{50} value as compared to the ether extract and chloroquine diphosphate used as a control drug. The $\log_{10}$ (concentration) of the extracts

and control drug versus the % schizonts suppression per 200 WBC curve, show that methanol extract curve was more on the left followed by the ether extract and finally the chloroquine diphosphate curve (Fig. 1).

Table 1. Antiplasmodial activity values with 50% schizonts suppression per 200 WBC (EC_{50}) of ether and methanol extracts of *Z. chalybeum Engl.*

Medicinal herb and drug	Solvent extract	EC_{50} (95% CI) µg/ml
Z. chalybeum Engl.	Ether	13.39 (10.82 – 16.59)
	Methanol	8.10 (5.89 – 11.12)
Chloroquine disphosphate (Positive control)		25.33 (17.07 – 37.60)
Culture medium (Negative control)		No schizont suppression

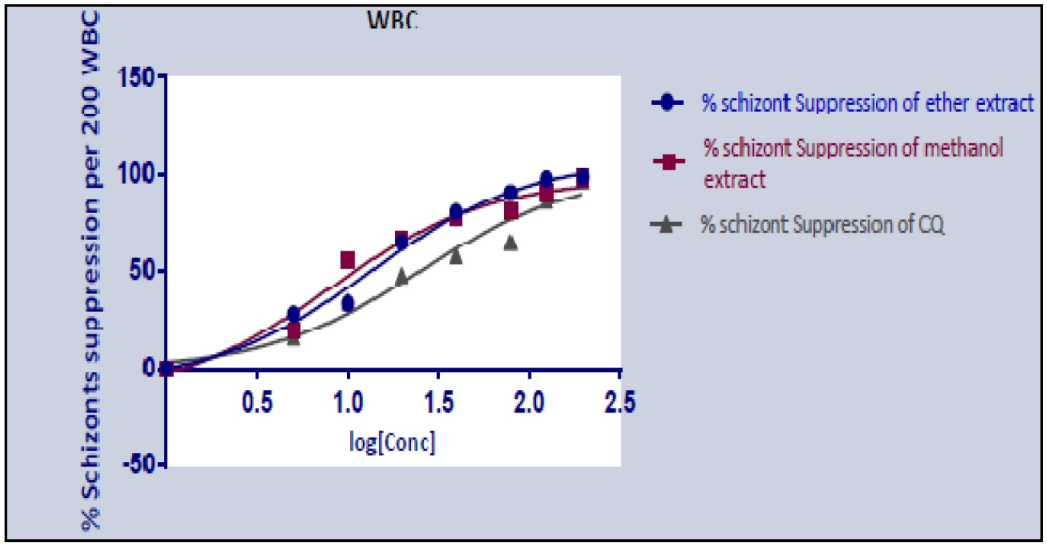

Fig. 1. Log_{10} Concentration-Response of ether and methanol extracts of *Z. chalybeum Engl.* on % *P. falciparum* schizonts suppression per 200 WBC

Key: CQ – chloroquine diphosphate; log (Conc) – log_{10} [concentration], concentration in µg/ml; WBC – white cell blood count

The observed low effective concentration (EC_{50}) of methanol leaf extract of *Z. chalybeum Engl.* required to suppress 50% of *P. falciparum* schizonts per 200 WBC cell count as compared to the ether extract and chloroquine disphosphate as control, could have been due to the various secondary phytochemical compounds that have been isolated from the leaf extract and the results show similar activity to previous studies done on the root-bark of the herb [7,8-10]. These compounds include the flavonoids, alkaloids, coumarins, amides, flavonoids, terpenes and sterols, sesquiterpene lactones and saponins [7-9]. The most notable secondary metabolites from various species of plants that have been reported to have antiplasmodial activity are alkaloids, steroids and triterpenoids and sesquiterpene lactones [7,8,19]. Even the currently used antimalarial drugs in the conventional medicine are derived from medicinal herbs such as quinine, a major alkaloid of cinchona from Cinchona tree [19] and artemisinin, a sesquiterpene lactone endoperoxides derived from *Qing hao* (*Artemisia annua*) [19-21]. The observed low EC_{50} value of methanol extract as compared to the ether extract could possibly be due to the presence of high quantities of

alkaloids, steroids and triterpenoids such as sesquiterpene that were extracted by the methanol solvent in large quantities [9,19,20]. Previous studies on the in vitro antiplasmodial activities of the root-bark extracts of *Z. chalybeum Engl.* have showed strong activities of the herb against *Plasmodium falciparum* organisms [10,23]. Although however, the continued use of the root-bark of the herb by the local communities and traditional herbalist in treatment of malaria does not conserve the plant since this method destroys the plant and hence leading to eventual extinction. As a result of this problem, the study utilized the leaves that also showed to have antiplasmodial activity though the EC_{50} values were slightly higher as compared to the previous studies that utilized the root-bark of the plant [20,21,23]. And the difference could possibly be due to the accumulation of the active secondary metabolites in the root-bark as compared to the leaves. However, the high EC_{50} value of chloroquine diphosphate as compared to the methanol and ether extracts of *Z. chalybeum Engl.* may be due to the resistance wild of *P. falciparum* protozoal organisms to chloroquine diphosphate that would require a high concentration of the drug to cause the suppression of 50% schizonts development, poor quality, expired drug or it had undergone degradation thus reducing its activity. Though however the EC_{50} value of chloroquine diphosphate obtained from the study correlates with the values obtained from previous studies [20, 21,22]. The culture medium used as negative control did not suppress the *P. falciparum* schizonts development and this was due to lack of active compound with antiplasmodial activity in the culture medium. The results therefore showed that the ether and methanol leaf extracts of *Z. chalybeum Engl.* herb contains various secondary metabolites with antiplasmodial activity and hence the reason for its use as a decoction and concoction [7,9] by the local communities and traditional herbalist in the treatment of malaria in Uganda.

4. CONCLUSION

The ether and methanol leaf extracts of *Z. chalybeum Engl.* contains secondary metabolites compounds with antiplasmodial activity and this could be the reason why they are used by traditional herbalists and local communities in Uganda in the management and treatment of malaria. The leaves of the herb also contains active secondary metabolites against *P. falciparum* and hence they can be utilized as an alternative in the treatment of malaria as opposed to the root-bark whose method of harvesting does not favor the sustainability and survival of the plant hence predisposing it to extinction.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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