

Crude Ethanolic Stem Bark Extract of *Zanthoxylum zanthoxyloides* and Its Fractions: *In-Vitro* and *In-Vivo* Antiplasmodial Activity

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ABSTRACT

Malaria is a global problem, as treatment failure has hampered the efficacy of most anti-malarial medications. The goal of this study was to see if stem bark extract from *Zanthoxylum zanthoxyloides* had antiplasmodial properties that could be used to treat both susceptible and resistant parasites. The stem bark of *Z. zanthoxyloides* (500g) was crushed and extracted with ethanol. The extract was tested for antiplasmodial activity *in vitro* against the chloroquine-sensitive (CQS) strain NF54 and chloroquine-resistant strains (CQR) K1 of *P. falciparum*, as well as *in vivo* against the CQS(NK65) strain of *P. berghei* at 100, 200, and 400 mg/kg bw. Bioassay-guided fractionation of the extract was performed. The crude extract had an *in vitro* activity of 1076.4 56.4 and 1315.1 121.6 ng/ml against chloroquine sensitive and resistant parasites, respectively while standard drugs (chloroquine and artesunate) were 10.94 nM (3478.92 ng/ml) and 9.24 nM (3215.52ng/ml) for CQS and 310.68 nM (98796 ng/ml) and 10.94 nM (3650.52 ng/ml) for CQR respectively. At Day 7, mice treated with 100, 200, and 400 mg/kg bw crude extract had parasite densities of 1159, 928, and 869 parasites/ μ l, respectively (compared to positive control that had 123 parasites/ μ l). *In vitro* antiplasmodial activity was best in the K2, K4, and K6 fractions (IC₅₀ were 6670, 6890, and 6480 ng/ml), but *in vivo* antiplasmodial activity was best in the K4 fraction (1183 parasites/ μ l). The stem bark extract of *Z. zanthoxyloides* have remarkable antiplasmodial activity against both chloroquine sensitive and drug resistant *P. falciparum* supporting its ethnomedicinal use in malaria treatment. The extract of *Z. zanthoxyloides* has promising antiplasmodial activity and could be used to generate therapeutic leads against the multidrug-resistant K1 strain of *P. falciparum*, in addition to providing an alternative allopathic antiplasmodial medication.

Keywords: Malaria, drug-resistant parasite, treatment, zanthoxyloides

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Introduction

Malaria is a global problem, as treatment failure has harmed the efficacy of most anti-malarial medications. Malaria is a deadly protozoan disease caused by plasmodium parasites that spread through the bites of infected female Anopheles mosquitos. (Addisu *et al.*, 2020) Of the 172 of Plasmodium species, six species can infect humans, *P. malariae*, *P. falciparum*, *P. vivax*, *P. ovale*, *P. knowlesi* and *P. simium*. (Grigg and Snounou, 2017). Malaria infection is particularly dangerous for pregnant women, children, splenectomized and immunocompromised patients, as well as healthy persons who have never been exposed to Plasmodium (Talapko *et al.*, 2019). If left untreated, severe malaria complications can result in death. Africa have the highest incidence of malaria (94%, i.e. 213 million) and higher death cases (93% i.e. 380 000 out of 405 000 worldwide) as a result of the disease (Forkuo *et al.*, 2020, WHO, 2021). The disease burden is increasing rapidly, not just as a result of vector resistance to existing pesticides, but also as a result of parasite resistance to conventional medications and their negative effects (Adesina *et al.*, 2020). Thus there is a need for new antiplasmodial drug from natural source that will be safe and also cost effective. Thus, a new antiplasmodial medication derived from natural sources that is both safe and cost effective is required.

Zanthoxylum zanthoxyloides is one of several plant extracts from the Rutaceae family that are currently utilized in the management and treatment of illnesses. *Z. zanthoxyloides* is a shrub of 6-12m in height. Its leaves are globrous and alternate. It smells like pepper when crushed. *Zanthoxylum zanthoxyloides* Lam also known as Fagara, orinata, is a West Africa native plant that is widely used (Image *et al.*, 2011 and Ynalvez *et al.*, 2012). Malaria, toothache, sickle cell anemia, elephantiasis, sexual dysfunction, paralysis, oedema, venereal illnesses, and overall bodily weakness are all treated using root or stem bark,

decoctions, infusions or macerations (Guendehou *et al.*, 2018). Because of its pungent and numbing impact on the palate when chewed, it is commonly employed in the treatment of dental caries, sore gums, and toothache.

Furthermore, some of these assertions have been pharmacologically validated. The leaves extract exhibited antidiabetic and hypolipidaemic effects (Aloke *et al.*, 2012). Free radical scavenging and metal ion chelating properties have been observed/discovered in *Z. zanthoxyloides* stem extract (Adekunle *et al.*, 2012). A broad-spectrum antimicrobial activity has been demonstrated for *Z. zanthoxyloides* extract (Ynalvez *et al.*, 2012). The ethanolic extract of *Z. zanthoxyloides* has been found to have antibacterial action. (Orafidiya *et al.*, 2010). Its antiparasitic activity has been found against *Ascaris lumbricoides* and *Leishmania major* (Maximin *et al.*, 2007 and Barnabas *et al.*, 2011). The antiprotease and membrane stabilizing properties of *Zanthoxylum zanthoxyloides* extract have been proven (Image *et al.*, 2011). *Z. zanthoxyloides* has also been reported to exhibit antisickling, free radical quenching and antifungal potentials (Nurain *et al.*, 2017, Pauline *et al.*, 2013 and Chaaiba *et al.*, 2003). Recent investigations have also confirmed its anticancer and anti-inflammatory properties (Kassim *et al.*, 2015, Diatta *et al.*, 2014 and Larsen *et al.*, 2015). It has been suggested that all part of *Z. zanthoxyloides* extracts could be used as a bio-pesticide for food storage (Udo, 2011). Denloye *et al.* (2010) also shown *Z. zanthoxyloides'* ability to protect grains from insect infestation. Some of the bioactive compounds isolated from *Zanthoxylum zanthoxyloides* include burkinabins (Queiroz *et al.*, 2006), geraniol, citronellol, limonene, α -pinene, myrcene (Fogang *et al.*, 2012), and alkaloids belonging to the class of acridone (Wouatsa *et al.*, 2013). This work was designed to obtain bioactive compounds/fraction with antiplasmodial potential from *Z. zanthoxyloides* that could be used to cure both sensitive and resistant parasite infections.

Materials and Methods

Crude extract preparation

Fresh *Z. zanthoxyloides* stem bark was collected and air dried at a temperature of 35-38 °C. It was pulverized and 0.5 kg of pulverised plant sample was exhaustively extracted by using ethyl alcohol under reflux at 60°C. Rotary evaporator was used to concentrate the filtrate and the concentrate was placed in a fume cupboard for complete evaporation of solvent. The extract was kept refrigerated at -8 °C in an in air tight container.

Antiplasmodial activities

In vitro antiplasmodial assay

The antiplasmodial potential of the extract was tested against NF54 (Chloroquine sensitive) and K1 (Chloroquine resistant) strains of *Plasmodium falciparum* and was cultured in sealed flasks at 37 °C, in a 91, 5 and 3 percent atmospheric N₂ CO₂ and O₂, in RPMI 1640, 25mM HEPES, pH 7.4, augmented with heat inactivated 10 % human serum and red blood cells to attain a packed cell volume of 2 percent. Parasites at their ring stage were synchronized with 5 % sorbitol by serial dilution and 1% parasitemia was used for the research work. 1mg/ml stock solution of plant extract in dimethyl sulphoxide (DMSO) was prepared and diluted as required for each experiments, in triplicate. The stock solutions were diluted in augmented RPMI 1640 medium to yield final reaction medium containing 0.2% DMSO. An equal volume of 1% parasitemia, 4% hematocrit culture were added and gently mixed thoroughly. Equal amount of DMSO were used in the negative controls. 1µM Artesunate and 1µM Chloroquine were used as positive controls. Cultures were incubation of culture was at 37 °C for 2 days (one parasite erythrocytic life cycle). The ring stage parasites were fixed by substituting 1 percent formaldehyde in phosphate buffer solution for the serum medium. The parasitemia of treated and control cultures were compared using aliquots (50 l) of each culture added to 5 ml round-bottom

polystyrene tubes containing 0.5 ml 0.1 percent Triton X- 100 and 1 nM YOYO nuclear dye (Molecular Probes) in phosphate buffer solution (PBS) (Singh *et al.*, 2001). The IC₅₀ values were calculated using linear interpolation from parasite-extract interaction schizont growth inhibition curves (Log of concentration versus percent inhibition) (Mustofa *et al.*, 2007).

In vivo antiplasmodial screening

Fidock *et al.* (2004) four-day curative test was employed in this study. Thin and thick tail tip blood smears of mice were pre-screened for rodent parasites using microscopy.

Eighteen Swiss albino mice in the range 15-19 g were selected and shared into five groups of three mice each. Groups I, II and III served as the test groups while groups IV and V were positive and negative control groups and were treated with chloroquine and untreated respectively. *Plasmodium berghei* (approximately 1 x 10⁷ infected red cells) was inoculated via intraperitoneal route into all the mice. The infection was proven in animals at 72hours post parasite inoculation. After the establishment of infection, mice in groups I – III were orally treated with the crude extract at 100, 200 and 400 mg/kg bw once daily for 4 days while group IV was treated with 5 mg/kg bw of Chloroquine phosphate to serve as the positive control group and group V was given normal saline at 0.9 w/v to serve as negative control.

Blood smears (both thin and thick) from the tail blood were prepared for each animal on a single slide on days 4, 7, 14, 28 and 42. Methanol was used to fix the thin films, and all of them were stained for 30 minutes with a 3 percent Giemsa stain with a pH of 7.0. Microscopic examination of the blood films were done using oil immersion (100X) objectives according to WHO (2010). Parasite densities were determined with the thick films while the stages and parasite species were identified using the thin films. Parasite density per microliter of blood

(parasitemia) was estimated from the thick film, taking the number of leucocytes per microliter of blood as 8,000 and expressed as follows

$$\text{Parasite density}/\mu\text{l} = \frac{\text{parasite count} \times 8,000}{\text{No of WBC counted}} \quad (\text{WHO, 2010}).$$

Bioassay guided fractionation of crude extract

The crude *Z. zanthoxyloides* extract was subjected to column chromatography using silica gel and eluted with solvents of rising polarity (n-hexane, chloroform, ethylacetate and methanol). This afforded /resulted/gave numerous fractions that were grouped according to of their TLC profile. The combined fractions were then dried under a fume-hood. The antiplasmodial effects of the fractions were evaluated as described earlier.

Results

Percentage Yield

The extract yield was 2.5%.

***In vitro* Antiplasmodial Activity of Crude *Z. zanthoxyloides* Extract**

The *in vitro* antiplasmodial activity of crude *Z. zanthoxyloides* extract was higher compared to the standard drugs used while its resistant index was comparable to that of artesunate.

***In vivo* Antiplasmodial Activity of Crude *Z. zanthoxyloides* Extract**

A dose dependent effect was observed in the *in vivo* antiplasmodial activity of crude *Z. zanthoxyloides* extract. Only the positive control group survived beyond day 28. Only the survival time of the positive control was beyond 28 days.

***In vitro* Antiplasmodial Activity of Fractions *Z. zanthoxyloides* Extract**

The antiplasmodial activities K2, K4 and K6 were higher/better compared to other fractions.

Table 1: *In vitro* Antiplasmodial Activity of Crude *Z. zanthoxyloides* Extract against NF54 and K1

Extracts/standards	IC ₅₀ (ng/ml)CQS(NF54)	IC ₅₀ (ng/ml)CQR(K1)	Resistance Index (RI) IC ₅₀ K1/ IC ₅₀ NF
<i>Z. zanthoxyloides</i>	1076.4 ±56.4 ^a	1315.1 ± 121.6 ^a	1.222
CQ (nM)	10.94/3478.92	310.68/98796.24	28.39
ART (nM)	9.24/3215.52	10.94/3650.52	1.184

Key: CQ=chloroquine, ART=artesunate, CQS= chloroquine sensitive strain and CQR= chloroquine resistant strain

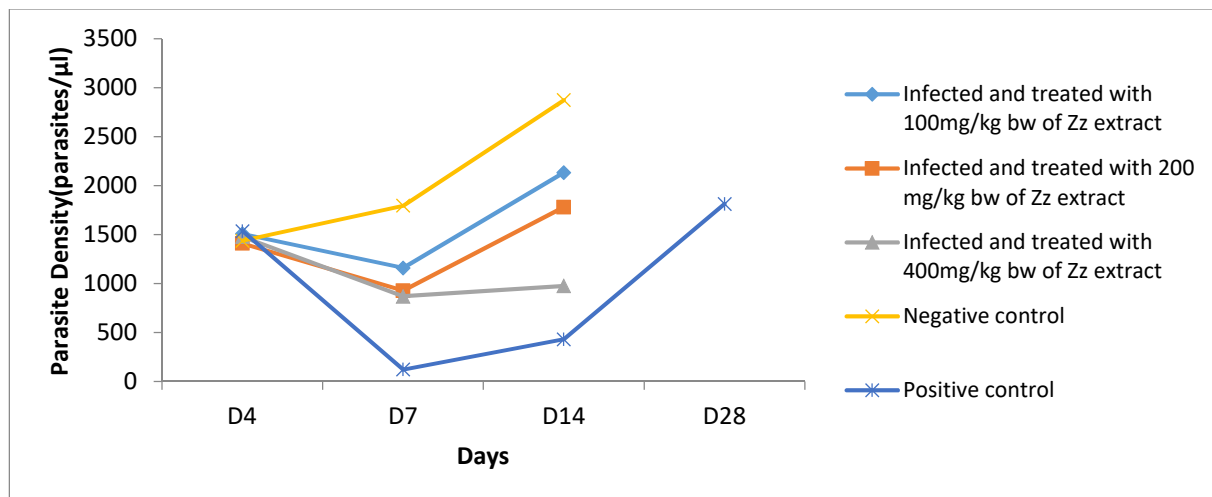


Figure 1: *In vivo* antiplasmodial activity of *P. berghei* infected mice treated with ethanolic stem bark extract of *Zanthoxylum zanthoxyloides*.

Table 2: *In vitro* Antiplasmodial Activity of *Z. zanthoxyloides* fractions against NF54

Fractions/Standards	IC ₅₀ (ng/ml)CQS(NF54)
K1	>10
K2	6.67
K2	8.53
K4	6.89
K5	8.07
K6	6.48
K7	>10
K8	>10
CQ(nM)	10.94/3478.92
ART(nM)	9.24/3215.52

NB: K1 – K8 are the fractions, while CQ is the chloroquine phosphate and ART is the artesunate

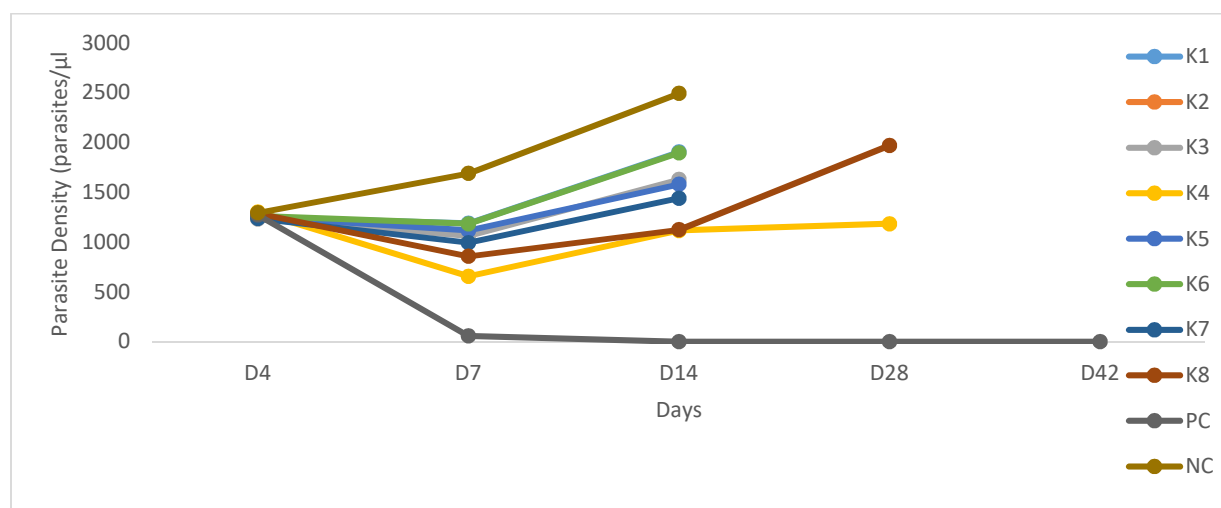


Figure 2: *In vivo* antiplasmodial activity of *P. berghei* infected mice treated with column fractions of *Zanthoxylum zanthoxyloides* stem bark extract.

NB: K1 – K8 are groups treated the fractions, while PC is the standard control (chloroquine phosphate) and NC is the negative control (Normal saline treated).

Discussion

Generally, the benchmarks for measuring antiplasmodial effect against *P. falciparum* according to World Health Organization are: pronounced activity for $IC_{50} < 5 \mu\text{g/ml}$, good activity for $5 < IC_{50} < 10 \mu\text{g/ml}$, moderate activity for $10 < IC_{50} < 20$, low activity for $20 < IC_{50} < 40 \mu\text{g/ml}$ and inactive for $IC_{50} > 40 \mu\text{g/ml}$ (Nyongbela *et al.*, 2016). As a result, antiplasmodial activity was found pronounced in *Z. zanthoxyloides* fractions. This is because, based on the results of the *in vitro* antiplasmodial test, *Z. zanthoxyloides* extract could be a viable alternative to artesunate as an antiplasmodial drug due to similarity in resistance index. Resistance Index (DRI) is a measure of drug effectiveness in relation to drug resistance, which is caused by misuse and overuse of medications. A corner-stone in the fight against drug resistance is the restrictive use of medications (Vandenbroucke-Grauls *et al.*, 2019). It has been previously documented that the lower the resistance index the better the antiplasmodial agent against resistant strain of the parasite (Joseph *et al.*, 2017). The *in vitro* antiplasmodial assay had also shown this extract to be a superior antiplasmodial agent compared to chloroquine. Presently, *Z. zanthoxyloides* extract could provide substitute antiplasmodial agent in Africa, where *P. falciparum* resistance is prevalent/predominant and in Asia where multidrug resistance K1 strain emanate (Saifi *et al.*, 2013). The extraordinary *in vitro* action could be ascribed to the fact that only the non-protein-bound fraction of a medication is pharmacologically active *in vivo*, which makes the serum effect to be considered amid treatment administration (Li *et al.*, 2017). The outcomes of *in vitro* assay constantly require to be guaranteed in *in vivo* settings, since living systems are more complex and dynamic in nature/framework.

The *in vivo* antiplasmodial effect was in a concentration dependent manner. As the dose of extract increases the parasite density decreases. The antiplasmodial property of this extract could

be linked to its ability either inhibit plasmodium parasite growth or destruction of parasite infected erythrocytes (Tajuddeen and Van Heerden, 2019). The distinction recorded/perceived in antiplasmodial action during the *in vivo* test may well be accredited to the rise in the concentration of antiplasmodial agent in the extract as the dosage increases (Nyandwaro *et al.*, 2020).

The *in vitro* antiplasmodial testing of the fractions resulted in diminished antiplasmodial property of the crude extract compared to the fractions. The bioactive component responsible for the effect could have been chemically altered during the fractionation process, which could explain the observed decrease in antiplasmodial activity. Another reason could be more than one active principle in the crude extract working synergistically to elicit the antiplasmodial activity separation of which had resulted in a diminished antiplasmodial activity. Lopes *et al.* (2013) reported a related observation with crude extract having higher activity compared to the fractions. Moreover, halogen containing solvents are identified to affect/modify alkaloids basically and consequently may adjust/alter their action. Chloroform utilized amid the course of fractionation may be accountable for the recorded changes. Elution with chloroform on silica gel columns containing quaternary alkaloids such as berberine and palmatine are known to create adduct chloroform compounds (Maltese *et al.*, 2009), which could be accountable for a decline or complete lost in activity of compound. In this research there was a decrease in activity of the fractions compared to the crude extract.

Furthermore, previous studies have shown *Z. zanthoxyloides* extract contain berberine and benzophenanthridine, chelerythrine (Orafidiya *et al.*, 2010, Adesina, 2005). These two substances are potent antiplasmodial agents (Bekono *et al.*, 2020). Berberine's antimalarial activity has been studied *in vitro* against the mefloquine-resistant D-6 clone of *Plasmodium falciparum*, as well as

sulfadoxine, pyrimethamine, chloroquine and quinine-resistant W-2 clone (Chandel *et al.*, 2015). Similarly, *Z. zanthoxyloides* has produced nitidine, an antimalarial chemical (Patel *et al.*, 2010). Instead of directly affecting the biocrystallization process in the vacuole, nitidine is thought to disrupt the interaction between glutathione (a biological antioxidant) and haem in the parasite's cytoplasm (Bouquet *et al.*, 2012). As a result, the parasite suffers from heme poisoning.

Other species from *Zanthoxylum* family also contain antiplasmodial agent nitidine (Muganga *et al.*, 2014). In addition, Perala and Ciddi, (2018) reported the occurrence of chelerythrine in the extract of *Zanthoxylum rhetsa*. This plant belongs to the same family with *Z. zanthoxyloides*. Additionally, antimalarial viability has pharmacologically been proven/demonstrated (Gansane *et al.*, 2010). Kassim *et al.*, (2005) illustrated the root extract of *Z. zanthoxyloides* possess *in vitro* anti-malarial property against 3D7 strain of *P. falciparum* and have attributed its activity to benzophenanthridine alkaloid, fagaronine. However, in this study the antiplasmodial efficacy of the stem bark extract was tested *in vitro* and *in vivo*, and also against K1 of *P. falciparum*. K1 is a multidrug resistant of *P. falciparum* parasite (Pan *et al.*, 2018). The K1 strain used in this study is known to be resistant to Chloroquine and sulfadoxine (antimalarial drugs). *Z. zanthoxyloides* could provide a good candidate against resistant strains of *P. falciparum*.

Because the extract exhibited a decrease in IC₅₀ of the K1 compared to the NF54 strain of *P. falciparum*, it will be useful lead for the creation of novel medicine, especially in Africa where chloroquine and sulfadoxine resistance has been widely reported/documentated. While *Z. zanthoxyloides* extract exhibited an index that was comparable to Artsunate, it was significantly below the resistant index for chloroquine (standard antimalarial drug). The lower the

resistance index in contrast to chloroquine, the better the antiplasmodial agent, it has been claimed (Joseph *et al.*, 2017).

Conclusion

Finally, this research has established that ethanolic extracts of *Z. zanthoxyloides* stem bark exhibit exceptional antiplasmodial efficacy against both chloroquine susceptible and drug resistant *P. falciparum*, supporting its ethnomedicinal use in malaria treatment. Antiplasmodial activity of *Z. zanthoxyloides* against the multidrug-resistant K1 strain of *Plasmodium falciparum* seems promising. *Z. zanthoxyloides* extract has promising antiplasmodial activity and could be used to create/generate therapeutic leads against the multidrug-resistant K1 strain of *P. falciparum*, in addition to providing an alternative to allopathic antiplasmodial medicines

Competing Interest

The authors declare no competing interests

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