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## Evaluation of *In vitro* Antimalarial Activity of *Citrus aurantifolia* (CHRISTM.) and *Ziziphus mauritiana* (LAM) Using Heme Polymerization Inhibition Assay

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### ABSTRACT

*Citrus aurantifolia* and *Ziziphus mauritiana* (Lam) are plants that have numerous medicinal properties and are used extensively in traditional medicine for the treatment of several diseases. Malaria parasites metabolize haemoglobin and detoxify the resulting haem by polymerizing it to form haemozoin (malaria pigment). This study aimed at evaluating the antiplasmodial activity through *in vitro* cell-free heme polymerization inhibition assay (HPIA) of *C. aurantifolia* and *Z. mauritiana*. The analysis was conducted using a 96-well microtiter plate reader at an absorption of 400 nm, at different concentrations (0-2 mg/ml). The IC<sub>50</sub> was determined by non-linear regression analysis using GraphPad Prism. The result indicated that both the root extracts of *C. aurantifolia* and *Z. mauritiana* showed significant difference ( $p < 0.0001$ ) with IC<sub>50</sub> of 0.81 mg/ml and 0.77 mg/ml respectively compared to positive control. Phytochemical screening of the ethanol root extracts showed the presence of saponins, alkaloids, terpenoids, glycosides, steroids, and anthraquinone, while tannins and reducing sugars were not detected in both samples used, flavonoid was detected in *C. aurantifolia*. Both the extracts exhibited potential for inhibiting heme polymerization *in vitro*.

**Keywords:** Heme; Polymerization; Antiplasmodial; *C. aurantifolia*; *Z. mauritiana*

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### INTRODUCTION

Malaria is an infectious disease caused by the intracellular parasite plasmodium, there are five different species of the parasite; *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malarie*, *P. knowlesi* (Lam *et al.*, 2012). The alarming rate at which *P. falciparum* has developed resistance to chloroquine and other synthetic antimalarial drugs makes it necessary to search for more effective antimalarial compounds (Sha'a *et al.*, 2011). Malaria parasites metabolize hemoglobin and detoxify the resulting haem by

polymerization/crystallization to form haemozoin (malaria pigment). This approach is useful for screening drugs belonging to chemical classes known to inhibit the target and it is the mechanism for quinoline class of antimalarials (Basilico *et al.*, 1998).

*Citrus aurantifolia* (family Rutaceae) and *Ziziphus mauritiana* Lam (family Rhamnaceae) are plants that have numerous medicinal properties and are used as traditional medicine for the treatment of several diseases (Etebong *et al.*, 2019).



*Citrus aurantifolia* plant is used in traditional medicine as an astringent, antiseptic, antihelmintic, mosquito repellent, digestive stimulant, stomach diseases, tonic diuretic, headaches, arthritis, sore throats, cough and cold (Enejoh *et al.*, 2015) while *Ziziphus mauritiana* is used for the treatment of various diseases such as healing, hemostatic, antiseptic, stimulant, tonic, anti-diarrhoea, diabetics, sedatives, nausea, vomiting and abdominal pains associated with pregnancy, respiratory ailments, fever, healing of fresh wounds, dysentery etc (Abalaka *et al.*, 2010; Chebouat *et al.*, 2013; Umar and Babalola, 2019).

Nevertheless, this study was designed to determine the *in vitro* assay by haem polymerization inhibition (HPIA). To identify molecules with haem polymerization inhibitory activity (HPIA) and quantify their potency, a simple inexpensive, qualitative *in vitro* spectrophotometric microassay was developed, where beta-haematin, a polymer identical to haemozoin can be obtained from haematin at acidic pH (3). This assay evaluates and identify compounds that have haem polymerization inhibitory activity, where relative amount of polymerized and unpolymerized haematin are determine based on solubility of test compounds and controls, where final product (solid precipitate of polymerized hematin) using infrared spectroscopy at an absorption of 400 nm (Afshar *et al.*, 2011) or 405 nm (Basilico *et al.*, 1998).

Clinical use of antimalarial drugs is hindered by various degree of parasite resistance. Thus, evaluation of potential promising compounds that can serve as molecular targets for synthesis of new antimalarial drugs. Hence, the need to explore and develop new compounds that can hindered mechanism of action of malarial parasites. The aim of the study was to evaluate the *in vitro* cell-free haem polymerization inhibition assay (HPIA) of *C. aurantifolia* and *Z. mauritiana* Lam.

## MATERIALS AND METHODS

### Plant Sample Collection

Plant samples were collected from Kumbotso LGA, Kano State. The Plant samples include, leaves, stem bark and root of both *C. aurantifolia* and *Z. mauritiana*.

### Identification of Plant

The two plants species were identified and authenticated by Malam Bahauddeen at herbarium unit of Plant Biology Bayero University Kano. The plants were *Citrus aurantifolia* (Christm) and *Ziziphus mauritiana* Lam. Voucher (BUKHAN 0113 and BUKHAN 0233) for each specie was deposited in the herbarium for future reference.

### Collection and Extraction of Plant materials:

Three parts of the plant materials (leaves, root and stem bark) were air dried and grounded using mortar and pestle and then extracted as described by Siddiqui and Patil (2015) with little modifications. One hundred and fifty (150 g) of each of the plant materials were weighed and percolated with 96 % ethanol (750 mL) for two weeks. The percolates were then evaporated to dryness using a rotary evaporator (R110) at 40°C. The crude residue from each were weighed and labelled.

### Phytochemical Qualitative Screening

The extracts were subjected to phytochemical screening, to determine the classes of secondary metabolites present in the plant materials following the protocol (Olanlokun *et al.*, 2017). These include alkaloids, saponins, reducing sugar, tannins, terpenoids, flavonoids, glycosides, anthraquinone, phenolic compounds and steroids.

### *In Vitro* Haem Polymerization Inhibition Assay:

The potential antiplasmodial activity of plant extracts were evaluated *in vitro* by the method described by Lam *et al.*, (2014) with some modifications. Different concentrations of the plant extract were prepared (0-2 mg/mL in 10 % DMSO).



One hundred microliter (100  $\mu$ l) of 300  $\mu$ M of hematin freshly dissolved in 0.1 M NaOH, 10 mM oleic acid and 10  $\mu$ M HCl and the volume was adjusted to 1000  $\mu$ L using 500 mM sodium acetate buffer, pH 5. Fifty microliters (500  $\mu$ l) of different doses of extract were added to triplicate test well. Fifty microliters (50  $\mu$ l) of chloroquine diphosphate and artesunate were used as a positive control and DMSO/distilled water as negative control. The samples were incubated for 24 hrs at 37°C with regular shaking. After incubation, samples were centrifuged at 14000 rpm at 21 °C for 10 minutes. The hemozoin pellets were repeatedly washed with sonication for 30 minutes, at 21 °C in 2.5 % (w/v) SDS in phosphate buffered saline and finally washed in 0.1 M sodium bicarbonate, pH 9.0. The pellets were re-suspended in 1 mL of 0.1 M NaOH. One hundred and fifty microliters (150  $\mu$ l) of each fraction were transferred to microtiter plate. The hemozoin content was then determined by measuring the absorbance at 400 nm with spectroscopic microtiter plate reader. The results were recorded as percentage inhibition (I %) of haem polymerization/crystallization compared to negative control using the following formula:

$$I\% = \frac{[AN - AS]}{AN} \times 100$$

Where AN= absorbance of negative, and AS= absorbance of test samples

### **Inhibitory Concentration at 50% (IC<sub>50</sub>)**

The values of IC<sub>50</sub> were measure graphically (concentration versus percentage inhibition) by non-linear regression analysis using GraphPad Prism. Chloroquine diphosphate and artesunate were used as positive control and DMSO/distilled water as negative control.

### **Statistical Analysis**

Data were expressed as Mean  $\pm$  Standard Deviation, and the differences of the mean percentage inhibitions were analysed using ANOVA at 95 % confidence interval using GraphPad Prism software and Bonferroni Post Tests.

## **RESULTS**

The result for the preliminary phytochemical screening of three different plant parts (leaves, root and stem bark) of *C. aurantifolia* and *Z. mauritiana*, were shown in Table 1. The leaves of *C. aurantifolia* had all the secondary metabolites except anthraquinone. Tannins, reducing sugars and anthraquinone were absent in the root extract, tannins, terpenoids, reducing sugars, phenols and anthraquinone were absent in the stem bark. The leaves of *Z. mauritiana* had presence of almost all metabolites except terpenoids and anthraquinone, the stem bark extract showed the presence of almost all with the exception of terpenoid and alkaloids and the root extract have the least of metabolites while tannins, flavonoids, reducing sugars and anthraquinone were not found.



Table 1: Phytochemical Composition of ethanolic extract of *Z. mauritiana* and *C. aurantifolia*

Secondary Metabolites	<i>C. aurantifolia</i>			<i>Z. mauritiana</i>		
	Leaves	Root	Stem bark	Leaves	Root	Stem bark
Saponins	+	+	+	+	+	+
Alkaloids	+	+	+	+	+	-
Flavonoids	+	+	+	+	-	+
Tannins	+	-	-	+	-	+
Terpenoids	+	+	-	-	+	-
Reducing Sugars	+	-	-	+	-	+
Glycosides	+	+	+	+	+	+
Steroids	+	+	+	+	+	+
Phenols	+	+	-	+	+	+
Anthraquinones	-	-	-	-	-	+

Key: (+) = Present, (-) = Absent

Inhibitory concentration at 50% (IC<sub>50</sub>) of ethanol extract of *C. aurantifolia* and *Z. mauritiana* presented in Table 2. The result indicated that root extracts of both plant species had significantly (P<0.05) lowest

IC<sub>50</sub> values of 0.77mg/ml and 0.81mg/ml and respectively comparable to chloroquine with IC<sub>50</sub> value of 0.86mg/ml. Thus, inhibition was higher in both leaves and stem extracts.

Table 2: The IC<sub>50</sub> and P-values of *C. aurantifolia* and *Z. mauritiana* plant parts

Plant Species	Plant Parts	IC <sub>50</sub>	P-value
<i>Citrus aurantifolia</i>	Leaves	0.99±0.040 <sup>b</sup>	>0.05
	Root	0.77±0.050 <sup>a</sup>	<0.001
	Stem bark	1.02±0.041 <sup>b</sup>	>0.05
<i>Ziziphus mauritiana</i>	Leaves	0.95±0.042 <sup>d</sup>	<0.01
	Root	0.81±0.059 <sup>c</sup>	<0.05
	Stem bark	1.34±0.047 <sup>d</sup>	>0.05
Chloroquine	-	0.86±0.090 <sup>b,d</sup>	<0.001
Artesunate	-	1.05±0.041 <sup>b,d</sup>	<0.01

Key: letter a and c were significantly lower than b, d

Haemozoin formation inhibition (I%) of ethanol extract of *C. aurantifolia* and *Z. mauritiana* were shown in figures 1 and 2. The ethanolic root extract of both plant

species showed higher percentage inhibition (32.58% and 32.71%) than leaves and stem bark and comparable to chloroquine and artesunate (41.13% and 47.00%).

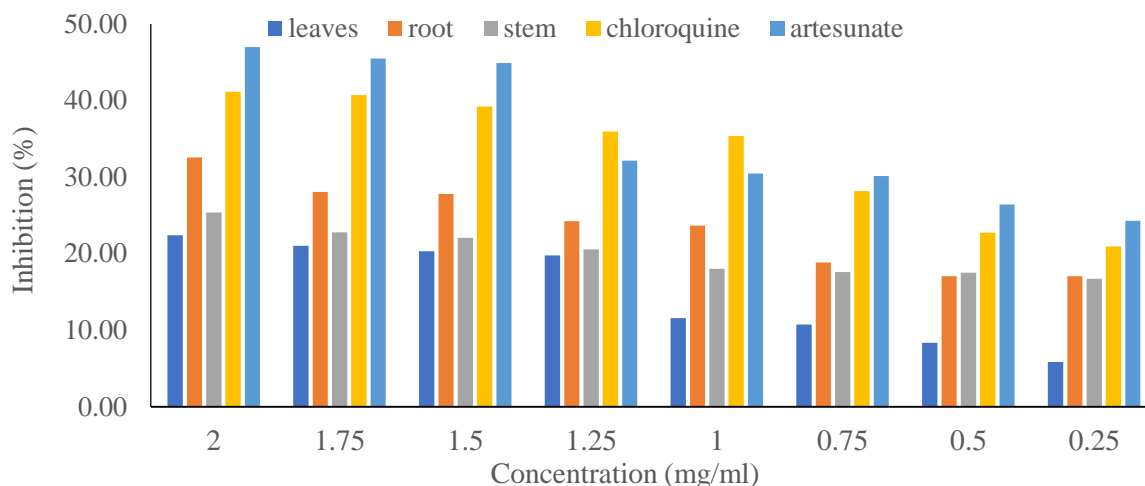


Figure 1: Heme Polymerisation Inhibition Assay (HPIA) of *C. aurantifolia*

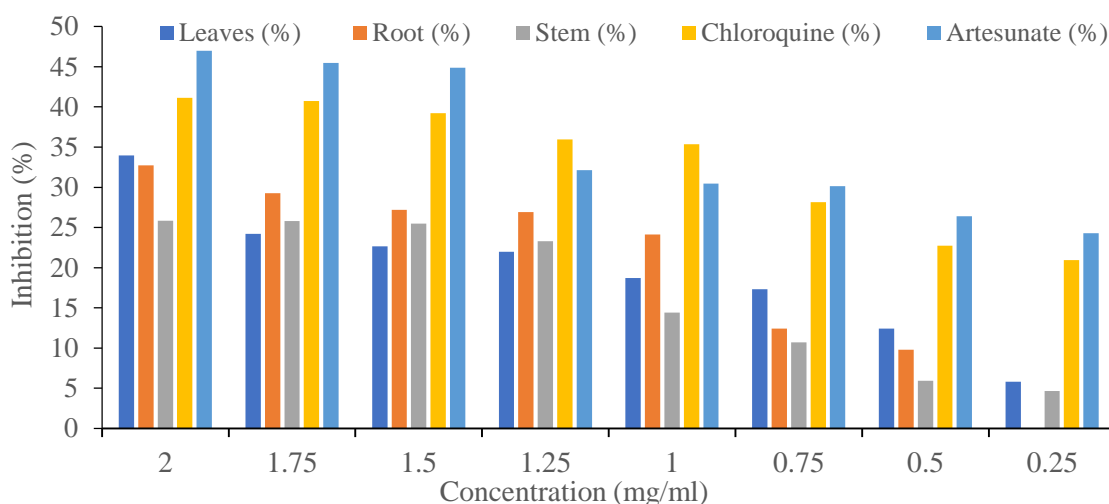


Figure 2: Heme Polymerisation Inhibition Assay (HPIA) of *Z. mauritiana*

## DISCUSSION

The use of plant-based medicines to treat malaria and other diseases is still important and practiced in Nigeria and other parts of African countries, mostly in the rural areas due to high cost and unavailability of patented drugs Olanlokun *et al.* (2017), many plant parts such as leaves, stem-bark, roots, fruits, seeds, flower have bioactive properties to various degrees or concentration which also reflects in their therapeutic efficacy (Fasola and Iyamah, 2014).

Phytochemical constituents are known to possess medicinal properties, and anti-inflammatory, diuretic, anticancer,

antidiabetic, antifungal, antimalarial and antidiarrhea activity (Palejkar *et al.*, 2012), anticholinestrase, radical scavenging, antispasmodic, anticoagulant, antifungal, antihypertension, anti-lipidemia, antioxidant, antiplatelets, and anti-parasitic (Narang and Jiraungkoorskul, 2016). In this study, the result of the secondary phytochemical screening confirms the presence of almost all the metabolites in different plant parts of *Z. mauritiana* and *C. aurantifolia*. The leaves of *Citrus aurantifolia* have all the secondary metabolites except anthraquinone, this is similar to the work of Ugwu *et al.* (2018) and Al-Namani *et al.* (2018) except steroids and saponins respectively.



There was no presence in the root extract in this study of tannins, reducing sugars and anthraquinone which is similar to the work of Elhag *et al.* (2018) but reducing sugars present in the work using different solvent, Fasola and Iyama (2014) reported the root having highest composition of alkaloids, flavonoids, saponin, tannins and terpenoids and this is conformed with the result of this study except with the tannins, reducing sugars and anthraquinone. The stem bark of has the least number of secondary metabolites which is similar to the findings of Nata'ala *et al.* (2018) except for anthraquinone, which is present.

Various studies have established the antimalarial activity of *C. aurantifolia* and *Z. mauritiana* extracts by *in vitro* *P. falciparum* assay (Adegoke *et al.*, 2011; Dabo *et al.*, 2013). A biochemical process that is unique to malaria parasites is formation of haemozoin crystals in the acidic food vacuole. The parasite degrades haemoglobin within the infected erythrocytes and it uses the catabolic product as source of nutrition for its survival and development (Asnaashari *et al.*, 2015). A by-product called haem (ferriprotoporphyrin IX) is released, which is toxic to the parasite. The parasite protect itself by detoxifying the free haem through the process of polymerization, where the haem is crystallized into an insoluble compound (hemozoin) known as malaria pigment (Lam *et al.*, 2014; Asnaashari *et al.*, 2015; Wande & Babatunde, 2017; Abiodun & Oladepo, 2018).

Main target of quinoline antimalarials is haem polymerisation (Olanlokun *et al.*, 2017). Chloroquine acts by forming quinoline-haem complexes which terminates haemozoin chain extension. Artemisinin and its derivatives also act by blocking free haem biocrystallization and haemoglobin degradation (Lam *et al.*, 2014). The absorbance (400nm) of the samples is inversely proportional to the drug efficacy; the lower the absorbance, the more efficient the drug is or the higher the inhibition

(Olanlokun *et al.*, 2017). Although the result showed all the plant parts inhibited haem polymerization to some certain level, that of the root extracts of both the plants indicated high inhibition of the haem polymerization with IC<sub>50</sub> values of 0.81mg/ml and 0.77mg/ml for *C. aurantifolia* and *Z. mauritiana*, which suggests presence of antimalarial compounds in the root extract that exhibit HPIA mechanism of action comparable to the positive control (chloroquine diphosphate and artesunate) with IC<sub>50</sub> values of 0.67mg/ml and 1.05mg/ml for respectively. The result of this study is similar to various works such as, the study of Baelmans *et al.* (2000) where five extracts of *Aloysia virgata*, *Bixa Orellana*, *Caesalpinia pluviosa*, *Mascagnia stannea*, and *Trichilia pleenea* exhibited more than 70% hematin inhibition polymerization at 2.5 mg/ml. In another study also, *Artemisia scoparia* and *A. spicigera* were evaluated for their *in vitro* antimalarial activity using the heme biocrystallization and inhibition assay, and dichloromethane of both extracts exhibited moderate antimalarial potential with IC<sub>50</sub> values of 0.778 mg/mL and 0.999 mg/mL respectively comparable to positive control chloroquine with IC<sub>50</sub> value of 0.043 mg/mL (Afshar *et al.*, 2011). Also, dichloromethane (DCM) extracts of both *Artemisia armeniaca* and *A. aucheri* plants exhibited significant antimalarial activity with IC<sub>50</sub> values of 1.36 mg/mL and 1.83 mg/mL respectively and the most potent fraction belong to DCM extract of *A. armeniaca* with IC<sub>50</sub> values of 0.47 mg/mL (Lam *et al.*, 2014).

## CONCLUSION

Phytochemical screening of the plants extract showed the presences of alkaloids, flavonoids, terpenes, and phenols in all parts of the species which are major classes of natural products that exhibit antimalarial activity.



Saponin, glycosides and steroids were present in all plant parts while anthraquinones is absent across the species. The result indicated that both the root extracts of *C. aurantifolia* and *Z. mauritiana* showed higher activity compared to leaves and stem bark of the same plants. Hence, have the most promising potential as an agent to inhibit beta-haemozoin formation. Further studies on the Heme polymerization inhibition assay of both the root extracts using different solvents should be carryout on the *in vivo* to test efficacy of *C. aurantifolia* and *Z. mauritiana* root extract against malarial parasites.

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