In-vitro Antimalarial Activity of Thelgate *(Aristolochia bracteolata Lam)* Plant Isolated from Juba, South Sudan

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Abstract

Background: *Aristolochia bracteolata Lam* is an important medicinal plant used by herbalists in South Sudan to treat various ailments that include fever, worms, headaches, body aches and diarrhoea. A recent study found that petroleum ether/ chloroform leaves and roots extracts were able to inhibit growth of *P. falciparum invitro*. However, the antimalarial activity of boiled leaves or roots prepared traditionally which is still commonly used has not been evaluated. This study therefore sort to examine the antimalarial activity of *Aristolochia bracteolata lam* against *Plasmodium falcipurum in vitro*

Methods: Leaves, stem, fruit, roots were extracted using various solvents (petroleum ether, methanol and water) and a traditional preparation of leaves and roots was also included. These were then tested for antimalaria activity against chloroquine sensitive *Plasmodium falciparum* 3D7 parasites cultured *in vitro* in human blood group O+ erythrocytes.

Results: All tested plant extracts were found to exhibit antiplasmodic activity with petroleum ether and traditional water extracts showing the highest inhibitory concentration against *in vitro* cultured *P. falciparum* with an IC50 <0.5µg/ml.

Conclusion: The results from this study, further provide scientific evidence for the traditional use of *A. braceoteolata* in the treatment of malaria in South Sudan. Further bioassay guided chromatographic fractionation of extracts is required for the isolation and purification of antimalarial compounds.

Keywords: Malaria, *Aristolochia Bracteolata Lam,* South Sudan

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1. Introduction

Malaria, a leading infectious disease globally, is caused by parasitic protozoans of the genus Plasmodium (Wassmer et al., 2015) . Among the genus p*lasmodium, six* species; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale curtisi*, *Plasmodium ovale wallikeri* and *Plasmodium knowlesi*-a zoonotic parasite in regions of South East Asia, are known to cause malaria disease in humans (Lalremruata et al., 2017). Malaria is a composite infection, whose spectrum of symptoms vary between children and adults (Miller et al, 2002). These symptoms range from none, in persons with asymptomatic parasitemia, to mild, in those with unexplained fever, to severe, in patients with severe anemia, metabolic acidosis, cerebral malaria, and multiorgan system involvement (Beales et al., 2000). Nevertheless, it is worth noting only less than 1–2% of infections lead to life-threatening malaria. Antimalarial drugs and supportive care are the primary ways of treating malaria since the mechanisms used by plasmodium parasites causation malaria are complex and poorly understood (Wassmer et al., 2015).

Malaria persists as a destructive infectious disease that cause major health challenges in Africa and other malaria-endemic countries worldwide (Muluye et al., 2015). Roughly 50% of the world's population reside in malaria risk areas, even so, the most affected regions are sub-Saharan Africa, Asia and Central and Latin America (Muluye et al., 2015). In the year 2017, roughly 219 million incident cases and 435,000 malaria deaths were reported globally with the highest disease burden being in World Health Organization (WHO) African Region (200 million or 92%), followed by the WHO South-East Asia Region with 5% and WHO Eastern Mediterranean Region with 2% (WHO, 2018b).

Mixed methods have been used in the control of malaria including: prophylaxis, Insecticide-treated nets (ITNs) impregnated with inexpensive and long-lasting pyrethroids, employing personal protection measures such as repellents, vector control using insecticides and residual spray program and using environmental and biological vector control (Rugemalila et al., 2007). On the other hand, the emergence and proliferation of mosquitoes that are resistant to insecticides is a growing threat to the efficacy of vector control and management, this combined with the emergence and proliferation of strains of plasmodium that are resistant to commonly used antimalarial drugs has led to curtailing the value of monotherapy in managing malaria thereby necessitating the introduction of more costly Artemisinin Combination Therapy (ACT) in malaria endemic areas both inside and outside Africa. Hence the availability of effective and more stronger antimalarial drugs and management of insecticide resistance are important challenges to be addressed (Rugemalila et al., 2007).

Since antiquity, herbalists in Sudan and the other parts of Africa have been treating febrile fevers typical of malaria with plant extracts. Despite the documentation of these medicinal plants, few studies have been done to ascertain the antimalarial activity of plants used to manage febrile illnesses including malaria. Due to the high resistance of plasmodium parasites to current treatment medication, it is important that new compounds be developed to provide a larger pool of compounds that can be used in treating it hence reduce chances of resistance or treatment failure. This is the reason for this study which seeks to determine the antimalarial activity of *Thelgate* plant (*Aristolochia bracteolata Lam*) extracts used by herbalists in South Sudan in treatment of febrile illnesses.

2. MATERIALS AND METHODS

2.1 Study site

This experimental study was conducted in three different laboratories namely: the Botanical Survey laboratory of Kenya-herbarium at the National Museums of Kenya, Nairobi where plant identification was conducted and a voucher specimen of the plant was deposited; Mount Kenya University, Thika Campus where plant extraction and phytochemical analysis were undertaken at the Pharmacognosy Laboratory; and the Institute of Primate Research (IPR) Malaria Laboratory, Karen, Nairobi, Kenya where both *in-vivo* and *in-vitro* assays were used to determine the antimalarial activity of the plant extracts.

2.2 Plant material used

Aristolochia bracteolata Lam was collected from Juba, South Sudan. The plant name, *Aristolochia bracteolata Lam* was confirmed via the following website http://www.theplantlist.org. The plant was obtained from the location at coordinate: 4.8594°N, 31.5713°E.

2.3 Extraction of plant extracts

Sequential extraction of *Aristolochia bracteolata Lam* (*A. bracteolata*) was undertaken using the following solvents: petroleum ether, methanol and finally water, while a traditional preparation involved plant leaves or roots in boiling water. At each stage, plant parts were soaked in solvents for 48 hours with agitation after which the extract was filtered and concentrated in a rotary vacuum evaporator to obtain various crude extracts. The dried extracts were weighed and stored in aliquots at -20°C.

Following extraction of various plant parts, the subsequent extractions were done sequentially with drying at each stage at room temperature. The yield was weighed and refrigerated at 4°C for further phytochemical analysis.

2.4 Phytochemical screening

Phytochemical analysis of plant extracts was undertaken to screen plants for selected

phytoconstituents that included alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, saponins, steroids, tannins, terpenoids and triterpenoids using standard techniques (Ambala, 2009); Ahmad et al., 2013;)Savithramma et al., 2012). Ethyl acetate, chloroform, methanol, petroleum ether and water, were used as solvents to assay these phytoconstituents.

2.5 In-vitro plasmodia studies

2.5.1 *Plasmodium falciparum* culture and maintenance

Plasmodium falciparum 3D7 parasites were cultured *in vitro* in enriched RPMI 1640 medium containing human blood group O+ erythrocytes using traditional methods (Poindexter, 1976). The culture was gassed and maintained at 37°C in an incubator.

2.5.2 Antiplasmodial activity in invitro cultures

The antimalarial activity of *A. bracteolata* plant extracts at different concentrations was then evaluated in a 96 well plate where parasites were cultured in these wells and incubated for 48 hours using the traditional candle jar method (Trager and Jensen, 1976) in an incubator. Following on from this, parasitemia was determined using the traditional giemsa stain and preparation of thin smears. Percentage parasitaemia was calculated as a percentage of the number of parasitized erythrocytes over the total number of erythrocytes.

2.6 *In vitro* Anti-Plasmodial Assay

Plant extracts were assessed for *in vitro* antiplasmodial activity using the SYBR Green I-Based Fluorescence Assay set up as described by (Smilkstein et al., 2004). Crude extracts were prepared at 25mg/mL in dimethyl sulfoxide (DMSO), while the positive control, chloroquine (Sigma-Aldrich, New Delhi, India) stock solution, was used as standard drug and prepared in water (Milli-Q grade) at 1mM. All stock solutions were then diluted in 96-well round-bottom, tissue culture-grade plates (Corning, New York, USA) with fresh RPMI 1640 culture medium to achieve the required concentrations for testing. In all cases, except for chloroquine (positive control), the final solution contained 0.4 % DMSO, which was found to be non-toxic to the parasite. Extracts were tested at concentrations ranging from 0.10 to 100 μg/mL, and chloroquine was used at 10mg/ml. All tests were performed in triplicate and 100 μL of sorbitol-synchronized parasites incubated under normal culture conditions (37˚C, 5% CO_2, 5% O_2, 90% N_2) at 1% parasitemia and 2% hematocrit for 48 hours. 0.4% (v/v) DMSO was used as the negative control for the experiment. After incubation, 100μL of SYBR green, I lysis buffer (Tris (20 mM, pH 7.5), EDTA (5 mM), saponin (0.008%, w/v), and Triton X-100 (0.08%, v/v) was added to each well, mixed gently, and incubated in dark at 37°C for 1 h. Fluorescence was then measured with a Victor fluorescence multi-well plate reader (Perkin Elmer, Waltham, MA, USA) with excitation and emission wavelength bands centered at 485 and 530 nm, respectively. The fluorescence counts were plotted against drug concentration and 50% inhibitory concentration (IC_{50}) determined by analysis of dose–response curves using the IC Estimator-version 1.2 software, Free Software Foundation, Boston, MA, USA). Resistance indices (RIs) were calculated as IC50PfINDO/IC50Pf 3D7. Results were then validated microscopically by examination of Giemsa-stained smears of extract-treated/untreated parasite culture.

3. RESULTS

3.1 *Aristolochia bracteolata Lam* plant extraction

Leaves, fruits, roots and plant stem parts of *A. bracteolata* were exposed to various solvents for extraction namely: petroleum ether, methanol, water and traditional water extraction (boiling) as shown in Table 1 below. The highest yields of extracts were obtained with petroleum ether for all plant parts other than leaves. Water extraction yielded similar weights of extracts for all the four plant parts, while traditional water extract (boiled water) gave the lowest yield for all plant parts, with stem parts specifically giving the lowest yield (7.4g)

3.2 Comparative analysis of phytochemical constituents of three different parts of *A. bracteolata*

Phytochemical analysis of the plant extracts revealed that the various extracts of *A. bracteolate* contained alkaloids, cardiac glycosides, flavonoids, glycosides, phenols, saponins, steroids, tannins, terpenoids and triterpenoids (Table 2).

Table 1: Variation in weight of plant extracts obtained under different solvent conditions

	Water (g)	Methanol (g)	Petroleum ether (g)	Boiled water (g)
Leaves	27.36	30.874	18.56	18.9
Fruit	26.92	21.44	39.42	18.8
Root	26.28	20.124	36.75	20.4
Stem	27.76	30.26	35.96	7.4

However, phenols were detected only in methanolic extracts o and the cardiac glycosides were found in root extracts of the solvents chloroform, ethyl acetate and methanol. Ethyl acetate extracts showed the presence of rich variety of secondary metabolites. Petroleum ether, chloroform and water extracts showed the least variety of these secondary metabolites. Compared to all other solvent extracts, methanolic leaf and root extracts had higher numbers of secondary metabolites with a high degree of precipitation (+++). Triterpenoids and resins were determined to be present but in a lesser amount, (+) only, in all extracts.

3.3 In vitro antiplasmodial activity of *A. bracteolata* leaves, stem, fruit and roots.

The in vitro antiplasmodic activity of crude extracts of *A. bracteolata* were assessed against *P. falciparum* infected blood group O+ red blood cells. The results are graphically presented in Figure 1. The four solvents (water, traditional boiled water, methanol and petroleum ether) from the four plant parts (stem, fruit, root and leaves) showed antiparasitic activity against *P. falciparum* parasites in vitro. Methanol leaves and fruit extracts did not show a change in antiparasitic activity as the drug concentration decreased. However, stem and root methanol extracts showed higher antiparasitic activity at higher concentration. Similarly, root petroleum extracts showed higher antiparasitic activity with increase in plant concentration as opposed to stem extracts. Leaves and fruit extracts had the same level of inhibition irrespective of plant concentration. Similarly, in traditional water extract, root extracts had higher antiparasitic activity against *P. falciparum* growth at higher concentrations. There was no change in parasite inhibition for stem, leaves and fruit extract irrespective of plant concentration. On the other hand, stem water extracts showed reduced antiparasitic activity with concurrent reduction in extract concentration

Table 2: Phytochemical analysis results

Phytochemical constituents	Chemical tests	Leaves	Stems	Roots	Fruits
Alkaloids	Meyer's reagent test	+++	+++	+++	+++
	Dragendoff's reagent test	+++	+++	+++	+++
Carbohydrates	Benedict's test	+ (Deep green)	+ (Deep green)	++ (yellow)	+ (Deep green)
	Fehling's test	+ (Deep green)	++ (brown)	+++ (brick red)	+++ (brick red)
Flavonoids					
Phenolic compounds	Lead acetate test	+++	+++	+	+
Tannins	Lead acetate test	+++	+++	+++	+++

	Ferric chloride test	+++	+++	+++	+++
Saponin	Froth test	++	+++	++	++
	Haemolytic test	+++	+++	+++	+++
Cardiac glycosides	Keller-killiani test	+++	+++	+++	+++
	Kedde test	+++	+++	+++	+++
Anthracine glycosides	Borntrager test 1	+++	++	++	+++
	Modified borntrager test 2	+++	+++	+++	+++
Phlobatannin		-	-	-	-
Terpenoids/terpenes	Salkowski test				
Coumarins		+++	+++	-	-
Proteins	Xanthopoetic test	+++	+++	+++	+++
Gum and mucilage		+	++	+++	+++
Volatile oil		-	-	-	+++
Fixed oils		-	-	-	++

Key: Present + **,** Absent **-,** Highly present +++, Moderate amount ++, traces amount +

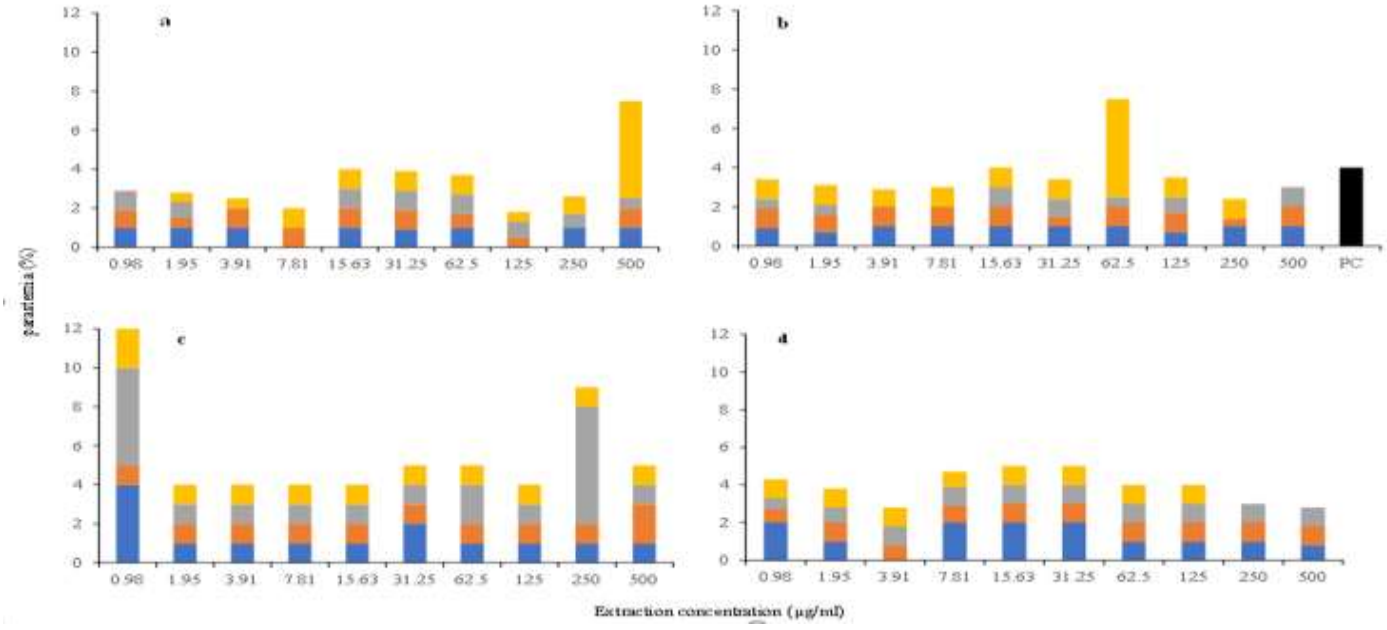

Figure 1: *In vitro* antiparasitic activity of Methanol, Petroleum ether, Water and Traditional water extracts of A. b*racteolata* plant parts against *P. falciparum* infected erythrocytes.

(a) Methanol extract, (b) Petroleum ether extract, (c) water extract, (d) water traditional extract
Leaves, Flowers Root Stem PC- positive control, infected erythrocytes

3.4 IC50 of plant extract evaluated in vitro

The Inhibitory Concentration 50% (IC_{50}) for the different extracts was determined to quantify the *in vitro* antiplasmodic activity of the plant extracts. Mean percentages of parasitemia and the dose–response curves-IC Estimator-version 1.2 software was used to obtain the IC_{50} values. The extract with $IC_{50} < 0.5$ µg/mL was considered highly active, while those with an IC_{50} between 0.5-5µg/mL was considered to be moderately active. Extracts showing an IC_{50} of 5–10 µg/mL indicated low activity, while those with $IC_{50} > 10$ µg/mL were considered inactive. The mean IC_{50} values obtained from the plot graphs are indicated in Table 4. The methanol extracts of leaves, stem and fruit of *A. bracteolata* had moderate activity against *in vitro* cultured *P. falciparum* parasites with IC_{50} ranging from 1.15 to 1.37 µg/mL. Similar results were obtained from petroleum and traditional water extracts of leaves. Petroleum and traditional water extracts of fruit and root of *A. bracteolata* were highly active against *in vitro* cultured *P. falciparum* parasites as they displayed IC_{50} that were less than 0.5 µg/mL.

Table 3: The IC_{50} of plant extract evaluated *invitro*

	Leaves (µg/mL)	Fruit (µg/mL)	Stem (µg/mL)	Root (µg/mL)
Methanol extract	1.37	1.36	1.15	ND
Petroleum extract	0.78	0.39	ND	0.32
Water extract	ND	ND	ND	ND
Traditional extract	0.81	0.9	ND	0.26

ND- no data

4. DISCUSSION

Teklehaymanot in 2017, in a survey of medicinal and edible plants, reported that different parts of *Aristolochia bracteolata Lam* could be used in the treatment of different ailments among the study population. Our study incorporated different parts of the plant to examine the antiplasmodic activity. From the *in vitro* experiments in this study, traditionally prepared (boiled) leaves extracts of *A. bracteolata* had the highest antiplasmodic activity against *P. berghei* infected mice compared to root and stem extracts. The low percentage parasitemia with corresponding high percentage chemosuppression and longer survival time noted when leave extracts were administered, suggests that leaf extracts are more superior in the treatment of malaria compared to stem and root extracts. Our findings agree with those of (Ahmed et al., 2010), who also observed that leaves extracts of *A. bracteolata* had high antiplasmodic activity at low concentrations. Even so, our study only used traditional water extracts in testing the *in vitro* activity of *A. bracteolate*. Das et al., 2016 had earlier reported higher antiplasmodic activity from chloroform, ethyl acetate and n-butane extracts of *Aristolochia griffithii* in comparison to water extracts.

Moreover, *A. bracteolata* tested in our study showed superior antiparasitic activity at lower drug concentrations of 125 µg/ml compared to antiparasitic activity of 93% obtained by (Berthi et al., 2018) while using drug concentrations of 1000 mg/kg of *Picrolemma huberi* plant.

5. Conclusion

From the results, this study indicates that *A. bracteolata* has antiplasmodial activity against *P. falciparum*. This justifies the traditional use of the plant in treatment of various symptoms traditionally and therefore, this study has added to the literature that can be used in further studies as there is potential for development of antimalarial compounds from this plant.

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