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Journal of Saudi Chemical Society (2016) **20**, 2–6

King Saud University

Journal of Saudi Chemical Society

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ORIGINAL ARTICLE

Phytochemical composition, cytotoxicity and *in vitro* antiplasmodial activity of fractions from *Alafia barteri* olive (Hook F. Icon)-Apocynaceae

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Received 29 February 2012; accepted 12 May 2012
Available online 19 May 2012

KEYWORDS

Alafia barteri;
Apocynaceae;
Antiplasmodial activity;
Brine shrimp lethality;
Phytochemical composition;
Ethanolic extract

Abstract The ethanolic extract of *Alafia barteri* (Hook F. Icon) was dissolved in distilled water and successively partitioned in *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The fractions were evaluated for phytochemical composition, lethality against brine shrimp larvae and *in vitro* antiplasmodial activity against *Plasmodium falciparum* strain. The obtained results revealed that the roots and leaf extracts of *A. barteri* exhibited broad spectrum of antiplasmodial activity (IC_{50} 1.5 ± 0.7–6.2 ± 0.80 μg/mL). The aqueous leaf fractions displayed the most potent antiplasmodial activity with an IC_{50} value of 1.5 ± 0.7 μg/mL, which is comparable to reference antimalarial drug (IC_{50} value of 1.3 ± 0.2 μg/mL). The leaf fractions displayed higher activity than the root extracts. The highest minimum lethal concentration (105.2 ± 0.8 ppm) was exhibited by the aqueous leaf extract followed closely by the root extract (120.2 ± 1.1 ppm). The leaf extracts contained higher polyphenols (45.3 ± 0.85 mgGAE/g) and flavonoids (18.10 ± 0.2 mgCTE/g) than the root extracts. The *n*-hexane and EtOAc extracts/fractions displayed lower activity on brine shrimp larvae.
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1. Introduction

In developing countries, medicinal plants have been an integral part of the ethno botanical aspects of people. Modern medicine has been obtained from folk medicine and traditional system through chemical and pharmaceutical screening. Plants commonly used in traditional medicine are sources of active antimalarial compounds such as quinine from *Cinchona succruba* (Rubiaceae) tree bark and artemisinin from *Artemisia annua* plant (Ancolio et al., 2002).

Human malaria caused by *Plasmodium falciparum* is an important disease in tropical and subtropical regions. *Plasmodium*, transmitted by female *Anopheles* mosquito, is reported for millions of death in developing countries (Farnsworth et al., 1985; Shearly, 1996; Muller et al., 2002). Mortality, estimated at over one million per year, has risen in recent years as a result of resistance to antimalarial medicines and drug resistant strains to malaria-induced *Anophele* parasites (WHO,

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.jscs.2012.05.003

1970, 2006; Severeni et al., 1993; WHO, 2006). There are reported cases of alternative therapy for the management of malaria in developing country (Abayomi, 1993; Ancolio et al., 2002; Muregi et al., 2003; Bidla et al., 2004). The climatic conditions of tropical and subtropical countries favour high breeding rate of mosquito implicated for the spread of *Anophele*-induced malaria in Nigeria and other tropical regions. Medicinal plants have demonstrated antimalarial activity in previous reports (Vantadoosi and Vaziri 2001; Joseph et al., 2004; Kaur et al., 2009). In South-Western Nigeria, many people rely on traditional medicine because of its affordability, availability and ethno botanical information/traditional practices.

In South-Western Nigeria (Lagos), A. barteri has been used for the treatment of malaria (Olowokudejo et al., 2008). The morphology of A. barteri has been described (Irvine, 1961). Apocynaceae is quite a large family with about 200 genera and 2000 species known, including genus like Alafia, Catharanthus, Alstonia, etc (Irvine, 1961). Plants in the Apocynaceae are poisonous, rich in alkaloids, glycerides and flavonoids obtained from the leaves, seeds, stems, roots and latex and are known source of anti-malarial activities (Leeuwenberg, 1997; Siu Kuin et al., 2011).*Alafia barteri* Oliver (Hook F. Icon) is a tropical rainforest plant, native to the West and Central Africa, stretching from Guinea Bissau to Cameroon, Congo and Nigeria (Irvine, 1961). *Alafia barteri* is called *agbari-etu* by the natives of South-Western Nigeria (Lagos), meaning instant fever remedy. Leaf infusion and root decoctions from *A. barteri* are used in Nigeria and other African countries as a remedy for malaria (Dalziel, 1937). In Nigerian traditional medicine, the stem and root decoctions of *A. barteri* are used for treating rheumatic pains, toothache, eye infection and sickle-cell anaemia (Leeuwenberg, 1997; Olowokudejo et al., 2008). Previous pharmacological studies revealed intrinsic antimicrobial activities of the roots and stem extracts of *A. barteri* grown in Nigeria (Adekunle and Okoli, 2002; Hamid and Aiyelaagbe, 2011). Preliminary phytochemical report on the stem extracts of *A. barteri* showed the presence of reducing sugars, steroids, flavonoids and anthraquinones (Hamid and Aiyelaagbe, 2011).

To the best of our knowledge, there is scanty report on the antiplasmodial, cytotoxic activity and phytochemical composition of *A. barteri*. In continuation of our search for antimalarial phytochemicals from natural source, *A. barteri* was selected on the basis of its ethno botanical uses. In the present study, extracts from the roots and leaves of *A. barteri* are evaluated for phytochemical composition, antitumor activity against crustacean *Artemia salina* larvae Leach and *in vitro* antiplasmodial activity on *P. falciparum* strain in order to establish a scientific basis for its ethnomedicinal use.

2. Materials and methods

2.1. Plant material

2.1.1. Collection of plant material

The roots and leaves of *A. barteri* were collected in May 2009 at Olokemeji forest, Eruwa, Ibarapa Local Government, Oyo State, Nigeria. The plant was authenticated by Mr. T.K. Odewo (taxonomist) of the Forest Research Institute of Nigeria (FRIN). A voucher specimen of the plant was compared with similar sample in FRIN herbarium, under the accession number FHI 108800.

2.1.2. Preparation and extraction of plant material

Plant materials were washed with water, cut into smaller particle size, dried under shade for 10 days, and grounded into coarse powder form. Air-dried, powdered roots (1.2 k g) and leaves (700 g) of *A. barteri* were successively and exhaustively extracted separately with distilled ethanol (EtOH, 7.5 L) each by means of aspirator bottle fitted with extraction gadgets. Extracts were evaporated in vacuo, yielding 42 and 65 g of the ethanolic leaf and root extracts respectively. Twenty grams of the leaf ethanolic extract was separately dissolved in 2 L distilled water and exhaustively partitioned thrice with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol (BuOH) in succession. Each fraction was evaporated in vacuo to afford semi-dried hexane (10 g), ethyl acetate (7.2 g) and butanol (5.6 g) leaf fraction. 30 g of the root ethanolic extract of *A. barteri* was dissolved in 2 L of distilled water and successively partitioned in succession with *n*-hexane, ethyl acetate and *n*-butanol. Concentrations of each fraction are: roots hexane (6.5 g), root ethyl acetate (4.3 g) and root butanol extract (3.6 g).

2.2. Phytochemical analysis of extracts

2.2.1. TLC analysis of extracts/fractions

3 mg of each fraction was separately dissolved in 5 mL EtOH. Identification of various phytochemicals was carried out by TLC on silica gel 60 F_{254} Merck (0.25 mm thick).

Flavonoids: *n*-butanol/acetic acid/water (4:1:5) was used as solvent system while 1% $FeCl_3$ and 5% $AlCl_3$ (in EtOH solution) serve as reagents. Anthraquinones were revealed with NaOH (25%), using EtOAc/MeOH/H_2O (8:1:1) as mobile phase. Terpenes and steroids were identified with Libermann–Buchard reagents, using *n*-hexane/CH_2Cl_2 (1:2) as mobile phase. Aqueous solution was used to detect saponnins by the froth test (Harbone, 1973). Alkaloids were tested for with Dragendoff's reagent, while EtOAc/isopropanol/NH_3 (16:3:1) was used as mobile phase.

2.2.2. Phytochemical composition of the ethanolic fraction of A. barteri

Total polyphenolic content was assayed by the Folin–Ciocalteau colorimetric method (using gallic acid as standard) with slight modification (Bao et al., 2005). Total phenolic concentrations were expressed as gallic acid equivalents (GAE) per gram dry matter (Bao et al., 2005). Tannin content was determined using the rhodanine assay as described by Makkar (2000). The concentration of tannins in the fractions was expressed as GAE per gram dry matter. The flavonoid content was determined by procedure of Jia et al. (1999), with slight modification and expressed as mg catechin equivalent per 100 g dry matter. Briefly, 50 μL of each ethanolic fraction was diluted with 950 μL glacial acetic acid, followed by the addition of 2.5 mL of 4% HCl in methanol (v/v) and 2.5 mL vanillin reagent (4% vanillin in glacial acetic acid, w/v), after which the reaction mixture was incubated for 20 min at room temperature. After incubation, absorbance at 500 nm was measured using a UV–Vis spectrophotometer against water blank. Saponnins were analysed as described by Harbone (1973). The presence of alkaloids was determined quantitatively using the method of

Makkar and Goodchild (1996). Terpenoids were quantified by a method described by Harbone (1973). Each analysis was carried out in triplicate.

2.3. Biological assay

2.3.1. Brine shrimp lethality bioassay

The crude extracts were tested against *Artemia salina* larvae according to methodology described by Mc Laughlin (Mc Laughlin et al., 1982). The extracts were tested using initial concentration of 10, 100 and 1000 μg/mL in vials containing 5 mL of brine and 10 shrimps, carried out in triplicate. The data obtained were subjected to analysis (using Finney computer programme, Finey, 1971) to determine the LC_{50}. The lethal concentration at 50% (LC_{50} value) and the standard error were calculated by Probit analysis (Finey, 1971).

2.3.2. In vitro test for antiplasmodial activity

The *in vitro* test for the antiplasmodial activity of fractions was evaluated following the method described by Tona et al. (1999). Ten milligrams of each fraction was dissolved in 10 mL dimethyl sulphoxide (DMSO) and diluted with culture medium to give series of test concentrations ranging from 0.5 to 500 μg/mL. Each assay was carried out in triplicate against *P. falciparum* chloroquine-sensitive infected human blood. Quinine-2HCl obtained from Pharmaceutical Research Unit, University College Hospital, Ibadan, Nigeria was used as antimalarial reference drug.

2.4. Statistical analysis

All data were expressed as the mean ± S.E.M., data was subjected to two-way ANOVA followed by Student's *t*-test, using Microsoft Excel* and Statistical* computer software packages. Differences in mean were considered significant when $P \leqslant 0.05$.

3. Results and discussion

Total polyphenols, flavonoids, tannins, alkaloids and saponnins in the ethanolic leaf and root extracts of *A. barteri* are presented in Table 1. The result revealed higher concentration of phytochemicals in the leaves. The phytochemicals were dominated by total polyphenolic constituent (45.3 ± 0.85 mg GAE/g), followed by tannins (30.1 ± 0.08 μg GAE/g), flavonoids (18.10 ± 0.02 mg CTE/g dry matter) and saponnins (7.7 mg/100 dry matter). The concentration level of alkaloid was low in the leaves and root extracts of *A. bateri* (3.0 ± 0.03 and 3.5 ± 0.02 mg/100 g dry matter). The brine shrimp lethality bioassay of fractions from *A. barteri* against *A. salina* larvae is presented in Table 2. The result indicated various activities of fractions to the brine shrimp larva at 100 ppm. The leaves and roots ethanol fractions were moderately toxic to *A. salina* larvae (LC_{50} = 105.2 ± 0.8–1000 ppm). Highest toxicity to *A. salina* larvae was observed in the leaves compared to the root fractions of *A. barteri* (Table 2). Hexane fractions exhibited very low toxicity and bioactivity on *A. salina* larvae. The brine shrimp larvae (*A. salina*) has been used as target organism to detect bioactive compounds in plant extracts and toxicity to this crustacean has a good correlation with antitumor activity (Silva et al., 2007; Shoeb et al., 2007; Subhan et al., 2008). The result of *in vitro* antiplasmodial activity of fractions from *A. barteri* on chloroquine-sensitive strain of *P. falciparum* is shown in Table 3. The fractions displayed intrinsic antiplasmodial activity on the tested strain (IC_{50} 1.5 ± 0.7–6.2 ± 0.8 μg/mL), except the *n*-hexane fraction which exhibited very low activity (> 100 μg/mL). The leaves aqueous and *n*-butanol fractions of *A. barteri* exhibited the highest antiplasmodial activity (IC_{50} < 4 μg/mL). The roots displayed lower antiplasmodial activity than the leaf fractions on *P. falciparum* tested strains (Table 3). A relatively weak antiplasmodial activity is displayed by the *n*-hexane and EtOAc fractions. In each case, the reference antimalarial drug demonstrated higher activity than the extracts/fractions. Previous preliminary phytochemical report on *A. barteri* led to the detection of some metabolites in the stem extracts, though not quantified (Hamid and Aiyelaagbe, 2011). Results obtained in this study confirmed similar metabolite, in addition with alkaloids. The TLC and phytochemical contents described in this report revealed the presence of high levels of some secondary metabolites in the root and leaf extracts of *A. barteri*. Secondary metabolites such as total polyphenols, flavonoids, triterpenoids and saponnins are displayed at various concentration levels (Table 1). Polyphenols, flavonoids and alkaloids have been reported for wide varieties of pharmacological activities, including antiplasmodial activity (Joseph et al., 2004; Makker et al., 2007; Kaur et al., 2009). High levels of polyphenols and flavonoids reported in the roots and leaves fractions of *A. barteri* could be responsible for its antiplasmodial activity. The exact mechanism of antimalarial actions of polyphenols and flavonoids is unclear, but some are shown to inhibit the influx of L-glutamine and myoinositol into infected erythrocytes. The ethanolic leaf and root extracts of *A. barteri* showed

Table 1 Phytochemical content in the ethanol extracts of *A. barteri*.

Plant secondary metabolites	Concentration	
	Leaves extract	Roots extract
Total polyphenolic content (mg GAE/g dry matter)	45.3 ± 0.85	30.2 ± 0.60
Flavonoids (mg CTE/g dry matter)	18.10 ± 0.02	16.8 ± 0.01
Tannins (μg GAE/g dry matter)	30.1 ± 0.08	10.10 ± 0.02
Saponnins (mg/100 g dry matter)	7.7 ± 0.11	5. 2 ± 0.02
Alkaloids (mg/100 g dry matter)	3.5 ± 0.03	3.0 ± 0.02
Terpenoids (mg/100 g dry matter)	35.5 ± 1.20	30.2 ± 1.1

Values represent the mean ± SD, $n = 3$; $P \leqslant 0.05$. GAE = gallic acid equivalent; LCE = leucocyanidin equivalent; CTE = catechin equivalent.

Table 2 Result of brine shrimp lethality assay of *A. barteri* on *A. salina* larvae.

Plant parts	Extracts	LC_{50} (ppm)
Leaves	Water	105.2 ± 0.8
	n-Butanol	320.3 ± 7.3
	Ethyl acetate	300.5 ± 6.9
	n-Hexane	1000.2 ± 18.1
Roots	Water	120.2 ± 1.1
	n-Butanol	350.2 ± 7.5
	Ethyl acetate	600. 2 ± 15.3
	n-Hexane	1000.1 ± 20.2

LC_{50} = lethal concentration (50%), SD, $n = 3$; $P \leqslant 0.05$.

Table 3 In vitro antiplasmodial activity of extracts from *Alafia barteri*.

Plant parts	Extracts	IC_{50} (μg/mL)
Leaves	Water	1.5 ± 0.7
	n-Butanol	2.8 ± 0.5
	Ethyl acetate	7.2 ± 0.6
	n-Hexane	> 100
Roots	Water	3.0 ± 0.4
	n-Butanol	4.1 ± 0.8
	Ethyl acetate	10.2 ± 0.8
	n-Hexane	> 100
Reference drug	Quinine 2HCl	0.95 ± 0.25

Average ± standard deviation.

promising antiplasmodial activity with the highest inhibition on the chloroquine-resistant strain (1.5 ± 0.7 and 3.0 ± 0.4 μg/mL respectively). Previous reports had implicated representatives of polyphenols and flavonoids, alkaloids, triterpenes, saponnins and tannins as phytochemicals that inhibit *P. falciparum* growth *in vitro* and *in vivo* (Wright and Phillipson, 1990; Christenson and Kharazni, 2001; Joseph et al., 2004; Kaur et al., 2009). Terpenoids such as betulinic acid have been reported as antiplasmodial compound (Bringmann et al., 1997). In this study, antiplasmodial activities of the extracts can be ascribed to the phytochemical constituents present in them, thus justifying the use of *A. barteri* for the treatment of malaria in traditional medicine. In each case assayed, standard antimalarial drug was more active than the extracts. It is assumed that isolation and purification would potentiate activity. The brine shrimp lethality assay reported in this study supports bioactivity of the extracts of *A. bateri*. The extracts show moderate toxicity to the crustacean *A. salina* larvae assayed (Table 2).

4. Conclusion

The results obtained in this study suggest that the leaf and root extracts of *A. barteri* possess good antiplasmodial activity, ascribable to high level of polyphenols and flavonoids in the extracts. Extracts/fractions obtained from *A. barteri* displayed intrinsic activity against brine shrimp larvae, suggestive of bioactivity and its potential antitumor activity. The result of antiplasmodial assay supports folkloric application of extracts of *A. barteri* in traditional medicine for the management of malaria. Further study should be conducted on the isolation and purification of bioactive extracts with a view to isolate active antiplasmodial compounds.

Acknowledgements

We thank Mr. T.K. Odewo (taxonomist) of the Forest Research Institute of Nigeria (FRIN Herbarium section), Jericho, Ibadan, Oyo State, Nigeria for the collection and authentication of plant material used in this study. The Pharmaceutical Research Unit, University College Hospital, Ibadan, Nigeria is thanked for the reference antimalarial drug provided.

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