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In vitro antiplasmodial activity and cytotoxicity of nine plants traditionally used in Gabon

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ABSTRACT

Aim of the study: As part of a project to identify new compounds active on malarial parasites, we tested the *in vitro* antiplasmodial activity of nine plants traditionally used to treat malaria symptoms in Haut-Ogooué Province, South-East Gabon.
Materials and methods: Dichloromethane and methanolic extracts of each plant were tested for their antiplasmodial activity on two chloroquine-resistant strains of *Plasmodium falciparum* (FCB and W2), based on lactate dehydrogenase activity. Cytotoxicity was assessed with the MTT test on MRC-5 human diploid embryonic lung cells.
Results: The methanolic extract of *Staudtia gabonensis* and the dichloromethane extract of *Adhatoda latibracteata* showed high antiplasmodial activity ($IC_{50} < 1$ μg/ml) and low cytotoxicity, with selectivity indexes of about 58.25 and 16.43, respectively. The methanolic extract of *Monodora myristica* and the dichloromethane extract of *Afromomum giganteum* also showed promising activity ($1 < IC_{50} < 10$ μg/ml) and low cytotoxicity, with selectivity indexes about 15.70 and 12.48, respectively. Dichloromethane extracts of *Monodora myristica* and *Leonotis Africana* showed moderate activity ($10 < IC_{50} < 40$ μg/ml), with selectivity indexes about 6.07 and 28.89, respectively. Both extracts of *Culcasia lancifolia* had IC_{50} values of 10–40 μg/ml but high cytotoxicity (selectivity indexes <2.77). The methanolic extract of *Dorstenia klaineana* had moderate antiplasmodial activity (IC_{50} around 17 μg/ml) but strong cytotoxicity (0.43 μg/ml), giving a selectivity index of about 0.03.
Conclusions: Most extracts of nine selected plants traditionally used to treat malaria in Gabon had interesting antiplasmodial activity *in vitro*. This supports continued investigations of traditional medicines in the search for new antimalarial agents. The compounds responsible for the observed antiplasmodial effects are under investigation.

1. Introduction

Malaria remains a major public health problem, especially in tropical and subtropical regions, despite new funds for research and control programs to roll back malaria. Two of the main reasons of malaria persistence are increasing insecticide resistance of mosquito vectors and *Plasmodium falciparum* drug resistance (Wellems and Plowe, 2001). Morbidity and mortality due to malaria have fallen in recent years with the advent of artemisinin-based combination therapy (ACT) and widespread use of impregnated bed nets (World Health Organisation, 2008). However, ACT treatment failures have been reported in some countries (Alker et al., 2007; Holmgren et al., 2007) and artemisinin-resistant malaria parasites

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Table 1
Studied plants from Haut-Ogooué, families and traditional medicinal uses.

Botanic name	Family	Voucher number Gabon*	Site of plants collection	Traditional medicinal uses
Adhatoda latibracteata	Acanthaceae	Bongui, No. 001	Epila	Fever, Headache, Vomiting
Aframomum giganteum	Zingiberaceae	Bourobou, No. 225	Omoi	Fever, Headache, Deworming, Tooth pains
Culcasia lancifolia	Araceae	Bongui, No. 002	Kele	Fever, Headache, Vomiting
Dorstenia klaineana	Moraceae	Bongui, No. 003	Kele	Fever, Headache, Vomiting
Leonotis africana	Lamiaceae	Bongui, No. 004	Mvouna	Headache, Vomiting, Tummyache, Healing, Diarrhea
Monodora myristica	Annonaceae	Sosef M, No. 2022	Epila	Fever, Headache, Vomiting, Deworming, Constipation
Rinorea subintegrifolia	Violaceae	Sosef M, No. 1357	Onkoua	Headache, Rheumatism, Tummyache
Staudtia gabonensis	Myristicaceae	ReitsmaB, No. 1531	Mvouna	Fever, Headache, Healing, Gonorrhea, Rheumatism, Eye's pains
Nauclea latifolia	Rubiaceae	Rei, No. 1648	Maboukou	Fever, Headache, Tummyache

Voucher number Gabon*: name of author who collected the specimen and number of collection order.

were recently detected in Cambodia (Maude et al., 2009), justifying the search for new antimalarial drugs. In addition, the use of these drugs is very limited in some rural areas, where the populations prefer less expensive traditional plant remedies (Newman et al., 2002). Several plants have been shown to possess strong antiplasmodial activity (for review see Kaur et al., 2009). Indeed, the best two current antimalarials (quinine and artemisinin) are both derived from plants.

Several families of phytochemical compounds have antimalarial activity, including alkaloids, terpenes, quassinoids, flavonoids, limonoids, chalcones, xanthones and quinoleines (Chhabra et al., 1987; Schwikkard and van Heerden, 2002; Batista et al., 2009).

Malaria transmission is hyperendemic and perennial in Gabon, a country with a population of about 1.5 million people. Up to 80% of Gabon is covered by forest, representing a potential source of natural compounds of medical value. Some medicinal plants used in northern and southern Gabon were listed in the 1990s (Akendengue, 1992; Akendengue and Louis, 1994), of which *Uvaria klaineana* has been shown to have antiplasmodial activity (Akendengue et al., 2002).

In this paper, using ethnobotanical information obtained from traditional healers living in Haut-Ogooué Province, south-eastern Gabon, we selected nine Gabonese medicinal plants used to treat malaria symptoms and tested their extracts for *in vitro* antiplasmodial activity and cytotoxicity.

2. Materials and methods

2.1. Plants and extraction procedures

The plants were collected in April 2008 in Haut-Ogooué Province, south-eastern Gabon, where malaria is endemic and the vegetation is a savanna–forest mosaic. The plants were collected in the Plateaux Batéké region composed mainly of shrubland. The families and traditional uses of the nine selected plants are indicated in Table 1.

The protocol of traditional preparation and of use of each tested plant were obtained from notices of traditional healers (unpublished information).

Each species was identified by comparison with voucher specimens deposited at the Gabonese herbarium in Libreville. Plant stems (100 g) were dried for 5 days then crushed and macerated in solvents at room temperature for about 24 h. The material was then extracted first with dichloromethane and then with methanol. The amount of solvent was at least 10 times the volume of plant material. Filtrates were prepared and evaporated to dryness under reduced pressure with a rotary evaporator (Rotavapor®) at 30 °C. Extracts of *Nauclea latifolia* were used as positive controls (Zirihi et al., 2005).

2.2. Parasite culture

Plasmodium falciparum strains FCB and W2 (both chloroquine-resistant) from MR4® (Malaria Research and Reference Reagent Resource Center) were grown under standard conditions as previously described (Trager and Jensen, 1976). The parasites were synchronized by repeated 5% sorbitol treatment. The plant extracts were dissolved in 100 μL of DMSO at an initial concentration of 200 mg/ml and then serially diluted with culture medium before being added to synchronous parasite cultures. The concentration range was 500–0.05 μg/ml. Two hundred microliters of synchronized trophozoite suspension (1.5% final hematocrit in RPMI 1640 + 0.5% Albumax®) was incubated in triplicate with the different concentrations of plant extracts in 96-well flat-bottom plates (NUNC, VWR International, Strasbourg, France). The plates were then placed at 37 °C as previously described (Douki et al., 2003) for 42 h, before being frozen at −20 °C for 3 h to stop parasite growth.

Dihydroartemisinin (Sigma–Aldrich) and chloroquine were used as positive and negative controls of parasite growth inhibition in all experiments.

2.3. Antiplasmodial activity

Antiplasmodial activity was analyzed by measuring *Plasmodium* lactate dehydrogenase (pLDH) activity with a commercial ELISA method as recommended by the manufacturer (ELISA-Malaria antigen test; DiaMed AG) (Kaddouri et al., 2006). The ELISA plates are coated with a monoclonal antibody (MAb) against pan-*Plasmodium* LDH. The absorbance of each well of plate was read with a microplate spectrophotometer (LP400; Bio-Rad) at 450 nm, with a reference wavelength of 620 nm. All experiments were performed at least in triplicate.

The results were expressed as the mean IC_{50} (the drug concentration that reduced parasitaemia to 50%).

2.4. Cytotoxicity assay

The cytotoxicity of the extracts was assessed with MRC-5 human diploid embryonic lung cells, using a tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma®) colorimetric method based on reagent cleavage by mitochondrial dehydrogenase in viable cells (Mosmann, 1983). Briefly, 5000 cells per well were seeded in 96-well microplates in culture medium (DMEM + 10% inactivated SVF + 2 mM L-glutamine + penicillin/streptomycin/neomycin (0.5/0.5/1 μg/ml)). After 24 h, the cells were washed and incubated with eight concentrations (from 500 to 0.05 μg/ml) of each extract for 7 days at 37 °C in 5% CO_2-air. Cytotoxicity was scored as the percentage reduction in absorbance at 540 nm versus untreated

Table 2
In vitro antiplasmodial activity, cytotoxicity and selectivity index of the selected plant extracts.

Plants species	Extract	Antiplasmodial activity (IC_{50}, μg/ml)*		Cytotoxicity MRC-5	Selectivity index[a]	
		FCB	W2	(LD_{50}, μg/ml)*	FCB	W2
Adhatoda latibracteata	CH_2CL_2	0.7 ± 0.2	1.6 ± 0.1	11.5 ± 2.4	16.43	7.19
	CH_3OH	NT	NT	NT	ND	ND
Aframomum giganteum	CH_2CL_2	8.3 ± 2.7	13.5 ± 1.9	168.5 ± 31.8	20.30	12.48
	CH_3OH	410 ± 25	460 ± 75	47.2 ± 9.3	0.12	0.10
Culcasia lancifolia	CH_2CL_2	8.9 ± 1.6	10.0 ± 2.0	10.25 ± 3.1	1.15	1.3
	CH_3OH	25.0 ± 0.9	16.0 ± 2.5	44.3 ± 10.3	1.77	2.77
Dorstenia klaineana	CH_2CL_2	16.7 ± 5.3	17.0 ± 3.8	0.43 ± 0.2	0.03	0.03
	CH_3OH	30.9 ± 2.8	33.7 ± 4.1	29.3 ± 3.7	0.95	0.87
Leonotis africana	CH_2CL_2	27.1 ± 7.2	15.2 ± 4.0	439.1 ± 7.5	16.20	28.89
	CH_3OH	NT	NT	NT	ND	ND
Monodora myristica	CH_2CL_2	25.0 ± 4.3	28.1 ± 1.5	170.5 ± 30.6	6.82	6.07
	CH_3OH	5.5 ± 2.0	6.1 ± 1.5	95.7 ± 12.3	17.4	15.7
Rinorea subintegrifolia	CH_2 CL_2	NT	NT	NT	ND	ND
	CH_3OH	360.7 ± 46.9	408.0 ± 64.0	520 ± 21.7	1.44	1.27
Staudtia gabonensis	CH_2CL_2	8.1 ± 1.5	15.5 ± 2.6	123.2 ± 10.6	15.20	7.95
	CH_3OH	0.8 ± 0.4	0.8 ± 0.4	46.6 ± 9.5	58.25	58.25
Nauclea latifolia	CH_2CL_2	6.6 ± 2.4	8.0 ± 2.7	75.5 ± 12.9	11.44	9.44
	CH_3OH	17.1 ± 2.8	18.4 ± 1.9	56.5 ± 23.5	3.30	3.07
Chloroquine		0.6 ± 0.4	0.4 ± 0.2	ND	ND	ND
Artemisinin		0.04 ± 0.01	0.002	ND	ND	ND

NT: not tested; ND: not determined. (IC_{50}, μg/ml)* = Inhibition concentration 50% ± standard deviation. (LD_{50}, μg/ml)** = Lethal dose 50% ± standard deviation.
[a] Selectivity index = LD_{50}/IC_{50}.

control cultures. All experiments were performed at least in triplicate.

The results were expressed as the mean lethal dose 50 (LD_{50} = the drug concentration that reduced the number of viable cells by 50%).

A selectivity index (SI), corresponding to the ratio between the cytotoxic and antiparasitic activities of each plant extract, was calculated as follows:

$$\text{SI Plasmodium} = \frac{LD_{50\,\text{MRC-5}}}{IC_{50\,\text{Plasmodium}}}.$$

3. Results and discussion

We investigated the *in vitro* activity of extracts of nine plants on two *Plasmodium falciparum* strains (FCB and W2). The plants were selected because their antiplasmodial activities had not previously been described. We have not tested leaf extracts because of excessive chlorophyll levels, which hamper the isolation of other compounds. Based on WHO guidelines and previous data (Jonville et al., 2008) antiplasmodial activity was classified as follows: high (IC_{50} < 5 μg/ml), promising (5 < IC_{50} < 15 μg/ml), moderate (15 < IC_{50} < 50 μg/ml) and inactive (IC_{50} > 50 μg/ml). Only two extracts showed high antiplasmodial activity, while seven showed promising activity. Although methanolic extracts of some plants were more active than the corresponding dichloromethane extracts, the latter tended to be more active overall, and also more selective (Table 2).

Although the traditional healers used some plants to treat malaria symptoms these had no antiplasmodial activity *in vitro*. It should be noted that we only tested antiplasmodial activity on the asexual erythrocytic stage of *Plasmodium falciparum*, and the extracts that we found inactive might possibly inhibit other parasite stages. Alternatively, some plants without *in vitro* antiplasmodial activity may stimulate the immune response; this is the case of *Markhamia lutea*, which inhibits *Plasmodium berghei* growth in mice but is inactive *in vitro* (Hakizamungu and Weri, 1988).

3.1. *Adhatoda latibracteata*

People in Haut-Ogooué use this plant to treat fever, headache and vomiting; the stems and leaves are boiled and then drunk, while crushed leaves are applied to the head and stomach.

The dichloromethane extract of *Adhatoda latibracteata* had very high antimalarial activity, with IC_{50} values of 0.7 ± 0.2 and 1.6 ± 0.1 μg/ml on strains FCB and W2, respectively. It was weakly cytotoxic, yielding selectivity indexes of 16.43 and 7.19. *Adhatoda vasica*, a specie of the same genus containing certain alkaloids, has been shown to be active against fever, cestodal disease, respiratory tract infections, and airway inflammation (Claeson et al., 2000; Sharafkhaneh et al., 2007; Yadav and Tangpu, 2008). It was recently reported that *Adhatoda justicia*, other member of the *Adhatoda* genus, is used to treat bronchitis in India (Rajakumar and Shivanna, 2009).

Other members of the Acanthaceae family, such as *Andrographis paniculata*, *Hypoestes rosea* and *Clinacanths siamensis*, contain terpenes and xanthones with antiplasmodial activity (Tuntiwachwuttikul et al., 2003; Ojo-Amaize et al., 2007; Mishra et al., 2009). Thus, compounds with antiplasmodial activity found in Acanthaceae might also be present in *Adhatoda latibracteata.*

3.2. *Aframomum giganteum*

This plant is used as a boiled leaf decoction by traditional healers in Haut-Ogooué to treat headache and fever. It is used as a deworming and purgative agent. It is also used to treat tooth pains. The dichloromethane extract of *Aframomum giganteum* exhibited promising and selective antiplasmodial activity, with IC_{50} values of 8.3 ± 2.7 and 13.5 ± 1.9 μg/ml on strains FCB and W2, respectively, and selectivity indexes of about 20.30 and 12.48. In contrast, methanolic extracts had no antimalarial activity (IC_{50} = 410 ± 25 and 460 ± 75 μg/ml) and were poorly selective (SI 0.12 and 0.10), suggesting that most of the compounds responsible for the antiplasmodial activity of *Aframomum giganteum* are extracted in dichloromethane.

Aframomum giganteum is an aromatic plant containing several terpenes (De Bernardi et al., 1981) that are very active on asexual blood stages of *Plasmodium falciparum*. This plant is a potential

source of a new antimalarial drug. The genus *Aframomum* includes several species with strong antiplasmodial activity (Duker-Eshun et al., 2002; Kenmogne et al., 2006) mediated by flavonoids, diaryl heptanoids and sesquiterpenes (De Bernardi et al., 1981; Kamnaing et al., 2003). Our results are consistent with the use of this plant for traditional medicine in Haut-Ogooué.

3.3. Culcasia lancifolia

This is a highly perfumed plant used by women as a comestics. It is also used by traditional healers to treat malaria symptoms (headache, fever and vomiting) consistently with our *in vitro* data. The dichloromethane extract of *Culcasia lancifolia* had promising antimalarial activity, with IC_{50} values of 8.9 ± 1.6 and 10.0 ± 2.0 μg/ml on strains FCB and W2, respectively, although its selectivity was limited (1.15 and 1.03, respectively). The methanolic extract of *Culcasia lancifolia* had moderate antimalarial activity. Several sesquiterpenes have been isolated from *Culcasia scandens*, another member of the *Culcasia* genus which has analgesic and anti-inflammatory properties (Okoli and Akah, 2000, 2004; Okoli et al., 2006), but has not previously been studied for its antiplasmodial activity.

3.4. Dorstenia klaineana

Dorstenia klaineana (synonymous *Dorstenia klainei*) is used to treat malaria symptoms in Gabon. Some traditional healers make an infusion containing *Dorstenia klaineana* and *Leonotis africana* to treat malarial symptoms. This plant is also used in aromatic perfume in spiritual ceremonies. We found that its extracts had no antiplasmodial activity and were strongly toxic for cultured human cells: the dichloromethane extract had IC_{50} values of 16.7 ± 5.3 and 17.0 ± 3.8 μg/ml on strains FCB and W2, respectively, and selectivity indexes of about 0.03, while the methanolic extract had IC_{50} values of 30.9 ± 2.8 and 33.7 ± 4.1 μg/ml FCB and W2, respectively, and poor selectivity indexes (0.95 and 0.87). This is consistent with a recent report that methanolic extracts of *Dorstenia klaineana* have no antiplasmodial activity, although such activity was found after fractionation with hexane–ethyl acetate (Boyom et al., 2009). Other members of the Moraceae family (*Artocarpus rigidus* BLUME subsp. *Rigidus* and *Ficus fistulosa* Reinw.) exhibit antiplasmodial activity driven by the flavonoid artonin F, the flavonoid 7-demethylartonol E, and the flavonoid cycloartobiloxanthone, Verrucarin L acetate (Zhang et al., 2002; Namdaung et al., 2006).

3.5. Leonotis africana

Rural populations in Haut-Ogooué Province use this plant as a healing agent after circumcision. Another use of this plant is in the contraction of loving pact between lovers. The leaves of this plant are used against malaria symptoms, in the form of infusions and baths. The same plant is used to treat diarrhea too. We found that the dichloromethane extract of *Leonotis africana* had moderate antimalarial activity (IC_{50} 27.1 ± 7.2 and 15.2 ± 4.0 μg/ml on strains FCB and W2, respectively), and weak cytotoxicity (LD_{50} 439.1 ± 7.5 μg/ml), giving selectivity indexes of 16.20 and 28.89. This plant is a member of the Lamiaceae family. It was recently reported that other Lamiaceae are used in traditional medicine in the Yanesha region of Peru. We found that *Leonotis Africana* exhibited moderate but selective antiplasmodial activity, in keeping with the reported activity of Lamiaceae from Yanesha (Valadeau et al., 2009). The Lamiaceae family may thus be a future source of antiplasmodial compounds, as several antiplasmodial terpenes and flavonoids have been isolated from these species (van Baren et al., 2006; Agnihotri et al., 2008; Henchiri et al., 2009). One, *Plectranthus amboinicus*, was found to have antiplasmodial activity in albino mice (Periyanayagam et al., 2008).

3.6. Monodora myristica

In Haut-Ogooué region, this member of the Annonaceae is used in an infusion containing *Nauclea latifolia* too, to treat malaria symptoms. Some people used this against constipation. To treat headache leaves of *Monodora myristica* are applied on the face. It is also used in Western Africa as a deworming agent. We found that the dichloromethane extract exhibited moderate antimalarial activity (IC_{50} of 25.0 ± 4.3 and 28.1 ± 1.5 μg/ml on strains FCB and W2, respectively) in keeping with previously published data (Okpekon et al., 2004), and also weak cytotoxicity (LD_{50} of 170.5 ± 30.6 μg/ml), giving selectivity indexes of 6.82 and 6.07. In contrast, the methanolic extract of *Monodora myristica* had high antimalarial activity (IC_{50} of 5.5 ± 2.0 and 6.1 ± 1.5 μg/ml on strains FCB and W2, respectively) and weak cytotoxicity (LD_{50} 95.7 ± 12.3 μg/ml), giving good selectivity indexes (17.40 and 15.69). Terpenes, alkaloids and other families of compounds with antiplasmodial activity have been isolated from several members of the Annonaceae (Mahiou et al., 2000; Boyom et al., 2003; Kanokmedhakul et al., 2007). The presence of such compounds in *Monodora myristica* could explain the antiplasmodial activity observed here.

3.7. Staudtia gabonensis

The methanolic extract of *Staudtia gabonensis* showed high antimalarial activity (IC_{50} of 0.8 ± 0.4 and 0.8 ± 0.6 μg/ml on strains FCB and W2, respectively) and the best selectivity indexes (58.25 for both strains). The dichloromethane extract showed promising antimalarial activity (IC_{50} of 8.1 ± 1.5 and 15.5 ± 2.6 μg/ml on strains FCB and W2, respectively) and high selectivity indexes of 15.20 and 7.95. *Staudtia gabonensis* is also used in other regions of Gabon, and notably in Libreville region, where it is combined with *Enantia chlorantha* Oliv or *Combretodendron africanum* Excell. in an infusion used to treat malaria (Lamidi et al., 2002). This plant is also used as healing agent because of its red sap. Eye pain, gonorrhea and articular rheumatism are also treated by the decoction of leaves of this plant. This plant is a member of the Myristicaceae family, other members of which also exhibit antiplasmodial activity. Several compounds with antiplasmodial properties have been isolated from Myristicaceae (Ancolio et al., 2002; Abrantes et al., 2008; Rangkaew et al., 2009). Whereas we found that the methanolic extract was most active, dichloromethane extracts and lignans from *Pycnanthus angolensis* have been reported to have strong antiplasmodial activity (Abrantes et al., 2008).

3.8. Rinorea subintegrifolia

This plant is used by traditional healer in decoction with lemons to treat malaria symptoms. But, it is also used as an aroma agent during ancestral cults because of its smell. We only tested a methanolic extract of *Rinorea subintegrifolia* because insufficient material was collected. This extract was not active against *Plasmodium falciparum in vitro*. The activity of this plant has not previously been tested, although *Viola websteri* Hemsl, another member of the Violaceae family, has antiplasmodial activity and active compounds have been isolated from it (Chung et al., 2009; Lee et al., 2009).

3.9. Nauclea latifolia

Some previous data have showed the antiplasmodial activity of this plant. The dichloromethane extract of *Nauclea latifolia* showed promising antimalarial activity (IC_{50} 6.6 ± 2.4 and 8.0 ± 2.7 μg/ml

on strains FCB and W2, respectively) with selectivity indexes of 11.44 and 9.44. The methanolic extract showed moderate antimalarial activity (IC_{50} 17.1 ± 2.8 and 18.4 ± 1.9 μg/ml on strains FCB and W2, respectively) but was poorly selective (selectivity indexes both around 3.3). These results are consistent with other published data from central West Africa (Traore-Keita et al., 2000; Zirihi et al., 2005). Although the active components have yet to be identified, this plant shows hypoglycemic, anticonvulsant, anxiolytic, antinociceptive, anti-inflammatory and anti-pyretic properties that justify its use to treat malaria symptoms (Gidado et al., 2008; Ngo Bum et al., 2009; Abbah et al., 2010). Compounds with antifungal properties have also been isolated from *Nauclea latifolia* (Ata et al., 2009).

4. Conclusion

Overall, the *in vitro* activities of these Gabonese plant extracts are compatible with their use as traditional remedies for malaria. We are currently attempting to isolate compounds with antiplasmodial activity from the most promising of these plants, prior to testing in animal models of malaria.

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