Antiplasmodial Potential and Phytochemical Screening of Ten Plants Used as Antimalarial in Mali

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Authors' contributions

This work was carried out in collaboration between all authors. Author MW designed the study, performed the antiplasmodial activity statistical analyses while authors ND, YG and MD collected samples and performed extractions and chemical screening. Author LK supervised drug assay experiments at Well Cornell Medical School at NY. Author KT managed the literature searches and proofreading of the manuscript. Author DD supervised the entire work. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2017/34523

Editor(s):

(1) Elena Maria Varoni, Dipartimento di Scienze Biomediche, Chirurgiche ed Odontoiatriche, University of Milan, Italy.

(2) Marcello Iriti, Professor of Plant Biology and Pathology, Department of Agricultural and Environmental Sciences, Milan State University, Italy.

Reviewers:

(1) Aina, Oluwagbemiga. Olanrewaju, Nigerian Institute of Medical Research, Nigeria.

(2) Patrick Valere Tsouh Fokou, University of Yaounde1, Cameroon.

Complete Peer review History: <http://www.sciencedomain.org/review-history/19715>

Original Research Article

Received 30th May 2017

Accepted 21st June 2017

Published 27th June 2017

ABSTRACT

Aim: This study was designed to determine *in vitro* antiplasmodial activities of extracts from ten Malian medicinal plants against *P. falciparum* strains.

Place and Duration of Study: Collection of plant materials and basic phytochemical screening were done in Bamako, Mali and antiplasmodial activity assessment at Department of Microbiology

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and Immunology, Weill Cornell Medicine, New York, United States of America between September 2013 and February 2014.

Methods: We collected leaves from ten commonly used medicinal plants and prepared ethanol and aqueous extracts. Antiplasmodial activities were evaluated against the chloroquine-sensitive 3D7 *P. falciparum* and chloroquine-resistant Dd2 *P. falciparum* strains, using the fluorescence-based SYBR® green I method. The interactions involving the differential extracts were further analyzed using a variable potency ratio drug combination approach. Chemical screening allowed us to identify the major secondary metabolites from those extract that were active against the parasite.

Results: Plant extracts showed a range of antiplasmodial activity. The ethanol extracts of *Annona senegalensis*, *Bauhinia thonningii*, *Maytenus senegalensis*, and *Fluenggea virosa* showed moderate antiplasmodial activity against 3D7 *P. falciparum* ($17.81 \pm 3.43 \leq IC_{50} \leq 37.64 \pm 0.83$ µg/mL) and against Dd₂ *P. falciparum* strains ($19.58 \pm 3.43 \leq IC_{50} \leq 67.55 \pm 1.76$ µg/mL). Among aqueous extracts only extract from *Bauhinia thonningii* demonstrated moderate antiplasmodial activity against both strains. The results showed that the active extracts contained a group of alkaloids, flavonoids, sterols, saponosides, tannins, coumarins and triterpenoids

Conclusion: *Bauhinia thonningii*, *Maytenus senegalensis*, *Annona senegalensis* and *Fluenggea virosa* possess antiplasmodial activity. These data confirm their use in traditional malaria therapy in Mali and provide evidence for further study for antimalarial drug discovery.

Keywords: Antiplasmodial; phytochemistry; malaria; traditional medicinal plants; in vitro.

1. INTRODUCTION

Malaria remains one of the most devastating infectious diseases with approximately 212 million infections and 429,000 deaths each year, primarily children under the age of 5 in Sub-Saharan Africa [1]. *Plasmodium falciparum*, the deadliest form of the malaria parasite, is responsible for the vast majority of the mortality and morbidity associated with malaria infection. Artemisinin Combination Therapies (ACTs) are currently the frontline treatments against *P. falciparum* malaria. Although these treatments continue to be effective in many parts of the world, the emergence of the malaria parasite resistance to ACTs is an urgent public health concern [1].

The use of medicinal plants in the treatment of diseases has a long history worldwide. Indeed, people from developing countries often do not have access to modern therapeutics such as ACT to treat malaria because of financial, geographical and/or cultural obstacles. The WHO estimates that up to 80% of the world's population relies on traditional medicinal products for some aspects of primary health care. Better knowledge of plants from traditional pharmacopoeias and validated traditional remedies in ITM (*Improved Traditional Medicine*) could lead to access to effective, standardized, available and affordable therapeutics for management of malaria by local populations [2].

In Africa and other countries where malaria is endemic, traditional medicinal plants are frequently used to treat or cure malaria [3]. It is a fact that conventional antimalarials such as quinine and artemisinin derivatives originated from plants. It is therefore important to investigate the antimalarial activity of medicinal plants in order to determine their potential as sources of new antimalarial agents [4].

Annona senegalensis (*Annonaceae*) is used for treating guinea worms and other worms, diarrhoea, gastroenteritis, snakebite, toothache and respiratory infections. The leaves are used for treating pneumonia and as a tonic to promote general well-being [5]. *Bambusa vulgaris* (*Poaceae*): Different extracts of the leaves are used against *Mycobacterium tuberculosis*, to treat infantile epilepsy, fever and hematuria and kidney troubles [6]. *Bauhinia thonningii* (*Cesalpiniaceae*) is rich in tannins and may be used for tanning skins, decoctions of the leaves have fever-repelling and expectoral properties, infusions of leaves and bark are used against worms, dysentery, diarrhoea and malaria [7].

Cerathotheca sesamoides (*Pedaliaceae*): aqueous leaf extracts are used in the treatment of diarrhea, due to the alkaloids, phenolics, flavonoids and saponins found in the extract [8]. The leaf may be an effective oxidant, anti-inflammatory and anti-hypertensive agent while the mucilage can be used as an emollient and lubricant [9].

Euphorbia hirta (Euphorbiaceae): It has many medicinal properties like Hypotensive, anticancer, tonic, exhibits anxiolytic, analgesic, antimalarial, antiasthmatic, and antidiarrheal, antioxidant, antiamoebic, anti-inflammatory, antifungal, antibacterial, antiamoebic and antispasmodic [10]. A methanol extract of the dried fruit pulp and the ethanolic root extract of *Fluenggea virosa* (Phyllanthaceae) have shown significant antifungal activities against *Trychophyton mentagrophytes* and *Candida albicans* while methanol and water extracts of the leaves have shown strong antimalarial activity, significantly inhibiting the growth of *Plasmodium falciparum* *in vitro* in a dose-dependent manner [11].

Ficus plastyphyla (Moraceae) a decoction of the bark is sometimes used in the treatment of leprosy and stomach pains and decoction of the leafy branch tips is said to be an antidote to arrow poisoning [12] *Gardenia aqualla* (Rubiaceae): Its fruits are used as a yellow dye, as well as in traditional medicine for their clearing, calming and cooling properties [13].

Maytenus senegalensis: (Celastraceae) The decoction of the stem bark and root is used traditionally in the folk medicine in Africa for the treatment of a number of diseases and health conditions, including malaria, fever, chest pains, rheumatism, dysmenorrhoea, diarrhoea, dyspepsia eye infection, wounds and snakebites [14]. Stem bark extracts of *Vitex cuneata* (Lamiaceae) can inhibit the growth of clinical isolates of *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli*, suggesting that they may be valuable in the treatment of dysentery and other gastroenteric infections [15].

This study aimed at evaluating the *in vitro* antiplasmodial activities and characterizing the main chemical groups present in extracts from ten plants commonly used as antimalarials in Mali.

2. MATERIALS AND METHODS

2.1 Reagents and Chemicals

Chemicals including RPMI (Roswell Park Memorial Institute) 1640, hypoxanthine, N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid (HEPES), glucose, albumax II, SYBR® green I-lysis buffer, DMEM-F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12),

streptomycin, albumax, Trypsin, Ethylenediaminetetraacetic acid (EDTA) were obtained from GIBCO/Invitrogen life Technologies, USA.

2.2 Selection, Collection and Preparation of Plant Materials

The leaves of *Annona senegalensis*, *Bambusa vulgaris*, *Bauhinia thonningii*, *Cerathotheca sesamoides*, *Euphorbia hirta*, *Fluenggea virosa*, *Ficus plastyphyla*, *Gardenia aqualla*, *Maytenus senegalensis* and *Vitex cuneata* (Table 1) were harvested from the forest around Bamako and Koulikoro Districts, Mali in July 2013. They were selected because high percentage of herbalists uses them in the traditional treatment of malaria in different parts of the country [16]. The plant materials were authenticated by the Department of Traditional Medicine, Public health Research National Institute, Bamako, Mali and herbarium specimen with Voucher numbers (Table 1) were deposited in the same Department. The harvested plant materials were air dried under shade at ambient temperature (28-35°C) for 7 days. They were then coarsely powdered using an electric mill and stored in airtight containers at room temperature until required for use.

2.3 Extraction of Plant Materials

For each plant material, 100 g of powder was extracted by 70% ethanol for 24 hours and then boiled in water in accordance with traditional preparations for 30 minutes. The decoction was then strained through double-layered white cotton material and subsequently through filter paper. The filtrate was dried and kept at 4°C until needed for analysis.

2.4 Antiplasmodial Activity

In vitro susceptibility assays of plant extracts were performed on cultured 3D7 (CQ sensitive) and Dd2 (CQ resistant) strains of *Plasmodium falciparum* [17]. They were maintained in continuous culture under microaerophilic conditions using the method described by Trager and Jensen [18] with the following modifications: both parasite strains were maintained at 3% hematocrit in human red blood cells (type A+) in media comprising RPMI 1640, 25 mM HEPES buffer (pH 7.4), 100 µM hypoxanthine, 16 µM thymidine, 20 µg/mL gentamycin and 0.5% Albumax.

Table 1. Collected plants for antiplasmodial assessment

Plant name	Family	Local name (Bambara)	Voucher specimen number	Place of collection
<i>Annona senegalensis</i>	Annonaceae	Mandé sounzou	MLM/001/13	Bamako
<i>Bauhinia thonningii</i>	Caesalpiniaceae	Niama Ba	MLM/002/13	Koulikoro
<i>Bambusa vulgaris</i>	Poaceae	Boh	MLM/003/13	Bamako
<i>Cerathotheca sesamoides</i>	Pedaliaceae	N'tékou	MLM/004/13	Koulikoro
<i>Euphorbia hirta</i>	Euphorbiaceae	Demba sindji	MLM/005/13	Bamako
<i>Fluenggea virosa</i>	Phyllanthaceae	Souroukou gnègnen	MLM/006/13	Koulikoro
<i>Ficus plastyphyla</i>	Moraceae	N'gaba blé	MLM/007/13	Koulikoro
<i>Gardenia aqualla</i>	Rubiaceae	Bouré tié	MLM/008/13	Bamako
<i>Maytenus senegalensis</i>	Celastraceae	Gnikélé	MLM/009/13	Koulikoro
<i>Vitex Cuneata</i>	Lamiaceae	Konoba	MLM/010/13	Koulikoro

Cultures were grown at 37°C in 75-cm² flasks after gassing with a mixture of 5% CO₂, 1% O₂, and 94% N₂. Parasites were synchronized by 0.3 M alanine-treatment at the ring-stage prior to the assays [19]. Parasite survival while exposed to different concentrations of our extracts was determined by the SYBR-Green I fluorescence-based method [20]. We used for the assay extract final concentration in range of 1000 to 7.8 µg/mL. SYBR-Green I assay was carried out following the adapted procedures described elsewhere [20,21,22].

Briefly, 25 µL of lysis/SYBRGreen I solution was added directly to each 50 µL culture; the plates were wrapped with aluminum foil and incubated at room temperature for 1 h prior to fluorescence reading using a microtiter plate reader (Ex/Em: 485 nm/530 nm). Fluorescence counts were plotted against the drug concentration and the 50% inhibitory concentration (IC₅₀) was determined by analysis of dose-response curves.

The Interaction studies were performed using a modification of the fixed ratios method [23,24, 25], extracts were diluted with culture medium to initial concentrations of 10 times the predetermined IC₅₀ and the solutions combined in ratios of 1:3, 1:1, and 3:1. The IC₅₀ of the extracts alone and in combination were determined. For data interpretation, the IC₅₀ of the extracts in combination were expressed as fractions of the IC₅₀ of the individual extracts. Fractional inhibitory concentrations (FIC) for extract A and for extract B, respectively.

To obtain numeric values for the interactions, results were expressed as the sum FICs (PFICs) of the FICA and FICB. Cutoff ranges were determined by mixing the same extract at

various ratios and accounting for experimental variation.

PFIC values indicate the nature of the interactions as follows: PFIC<1 is synergistic, PFIC 1 to 1.4 is additive, PFIC>1.4 is antagonistic [24].

2.5 Preliminary Phytochemical Analysis of Materials

Plant materials were subjected to qualitative phytochemical screening to determine the presence of the major phytochemical constituents: alkaloids, anthocyanins, flavonoids, phenols, saponins, tannins, triterpenes, and sterols according to standard methods [26,27].

2.6 Statistical Analysis

For calculations and statistical analysis the Probit method was used, to classify the activity levels of each extract the Rasoanaivo table was used [28]. Accordingly the term “very active” means an IC₅₀ less than 5 µg/mL; “active” from >5-50 µg/mL; “weakly active” from >50-100 µg/mL and “inactive” more than 100 µg/mL.

Percent suppression of parasite growth of the treated and control groups were compared using one-way ANOVA and two-tailed Student's t test (GraphPad Prism 4.0, GraphPad Software), with P<0.05 being considered significant.

3. RESULTS AND DISCUSSION

3.1 Collected Plants

Leaves from a total of 10 different plants were collected from June to August 2013 in forest around Bamako and Koulikoro Districts. The

local names, family, place of collection and voucher specimen number are shown in Table 1.

3.2 *In vitro* Antiplasmodial Activity of Extracts

The plants extracts were assessed at concentrations up to 1000 µg/mL against 3D7 and Dd2 strains of *P. falciparum*. The ethanol extracts from six plants have shown moderate antiplasmodial activity. The ethanol extracts of *A. senegalensis*, *B. thonningii*, *F. virosa* and *M. senegalensis* were active against both strain, while water extracts of *V. cuneata* and *E. hirta* were weakly active (Table 2). Two of the ethanol extracts (*C. sesamoides* and *F. plastyphyla*) were more potent against the Dd2 parasite strain. These results demonstrate a potential difference in the target of the active components of the plant extract amongst two parasites of diverse origin and genetic background.

The ethanol extracts of *A. senegalensis*, *B. thonningii*, *F. virosa* and *M. senegalensis* were active against both strain, while water extracts of *V. cuneata* and *E. hirta* were weakly active (Table 2). The ethanol extract of *F. plastyphyla* had shown moderate activity on resistant strain Dd2 but was no active against sensitive strain 3D7, suggesting the use of plants as a source of alternative malaria treatment to curb resistance development against current drug [29,30].

Table 2. *In vitro* antiplasmodial activity of ethanol extracts against 3D7 and Dd2 strains of *P. falciparum*

Plant name	Antiplasmodial activity, µg/mL	
	IC ₅₀ : 3D7	IC ₅₀ : Dd2
<i>A. senegalensis</i>	23.93±0.79	29.47± 2.42
<i>B. thonningii</i>	37.64 ± 0.83	67.51± 1.76
<i>B. vulgaris</i>	>100	≤100
<i>C. sesamoides</i>	>100	63.72± 2.04
<i>E. hirta</i>	71.21± 1.53	73.99± 0.98
<i>F. virosa</i>	23.12± 3.81	44.06±3.21
<i>F.plastyphyla</i>	≤100	34.24±1.65
<i>G. aqualla</i>	ND	ND
<i>M. senegalensis</i>	37.01±4.33	62.35±3.21
<i>V. cuneata</i>	55.85±3.79	63.18±2. 77

Keys 3D7: Chloroquine-sensitive *P. falciparum* strain; Dd2 chloroquine-resistant *P. falciparum* strain;

ND: Not determined; IC₅₀ is expressed in µg/mL ± SD.

*Data was obtained from three independent experiments

In the previous study ethanolic crude extracts of *Annona senegalensis* growing in Democratic Republic of the Congo were had shown the same

moderate activity like in this tudy (IC₅₀ = 32, 52±6, 97 µg/mL) [31].

These findings provide some evidence underlying the traditional use of these plants as antimalarial but not sufficient to confirm the use of plants in traditional medicine, particularly in the field of malaria, a disease which involves many different symptoms and which has a complex physiopathology [32,33].

Among water extracts only the one from *B. thonningii* was active against both strains, the rest of extracts were inactive against these strains (Data are not shown). That could be explained by the extraction procedure we used; the water extract was performed subsequent to the initial ethanol extraction. The active compounds from plants may be more soluble in ethanol than in water as it was shown that artemisinin tea does not have antimalarial activity *in vitro* [34].

For the inactive extracts, several elements might explain the absence of activity such as the fact that our *in vitro* test model reproduced the erythrocytic development stage of the parasite but some plants could also be active against the liver phase of *Plasmodium* development [35].

Our interaction studies with various pairs of active extracts revealed the presence of synergistic, additive and antagonistic interactions. They were mostly additive, but we observed one case of antagonism between extract of *F. virosa* etoh and of *A.senegalensis* etoh. The results have shown also two cases of promising synergetic interaction: *B. thonningii* etoh/ *V. cuneate* etoh and *B. thonningii* etoh/ *V. cuneata* etah (Table 3). In all cases IC₅₀ of extract alone were more than that in mixture with second extract.

In fact, the traditional healers use these plants in different combinations, so our data support this way in their use as they mostly positively interact. These combinations may be suggested to the traditional healers in order to improve efficacy of their remedies for the populations suffering from malaria. These results are consistent with previous observations in different studies conducted in Mali and Kenya on different solvent plant extracts [36,37] and would assist in developing combinations with optimum efficacy for further *in vivo* analyses and exploitation towards a rational antimalarial drug discovery [38].

Table 3. *In vitro* antiplasmodial activity of paired extracts exhibiting different interactions

Combinations	Mean FIC
<i>B thoningii</i> etoh/ <i>B thoningii</i> H ₂ O	1.205±0.05
<i>B thoningii</i> etoh / <i>F virosa</i> etoh	1.145±0.116
<i>B thoningii</i> etoh / <i>V cuneate</i> etoh	0.849±0.252
<i>B thoningii</i> etoh / <i>A senegalensis</i> etoh	1.297±0.06
<i>B thoningii</i> etoh / <i>M senegalensis</i> etoh	1.186±0.081
<i>B thoningii</i> H ₂ O/ <i>F virosa</i> etoh	0.864±1.870
<i>B thoningii</i> H ₂ O/ <i>V cuneate</i> etoh	1.08±0.046
<i>B thoningii</i> H ₂ O/ <i>A senegalensis</i> etoh	1.19±0.033
<i>B thoningii</i> H ₂ O/ <i>M senegalensis</i> etoh	1.194±0.176
<i>F virosa</i> etoh / <i>V cuneate</i> etoh	1.275±0.223
<i>F virosa</i> etoh / <i>A senegalensis</i> etoh	1.524±0.477
<i>F virosa</i> etoh / <i>M senegalensis</i> etoh	1.325±0.271
<i>V cuneate</i> etoh / <i>A senegalensis</i> etoh	1.191±0.345
<i>V cuneate</i> etoh / <i>M senegalensis</i> etoh	1.147±0.146
<i>A senegalensis</i> etoh / <i>M senegalensis</i> etoh	1.044±0.856

*Data was obtained from three independent experiments.

* The ratio were: 1:3; 1:1 and 3:1

* mean FICs was used to classify the overall nature of the interaction which appears to be additive in this study

Table 4. Phytochemical screening of active extracts

Extracts	Alcaloids	Flavonoids	Saponosids	Tanins	steroids	Coumarins	Triterpenoids
<i>A. senegalensis</i>	+	+	-	+	+	+	+
<i>B. thoningii</i>	+	+	-	+	+	+	+
<i>F. virosa</i>	+	+	-	+	+	-	+
<i>M. senegalensis</i>	-	+	+	+	+	-	+

Key: +: presence - : absence

3.3 Phytochemical Screening

All active extracts were screened for phytochemical analysis. Mostly they were positive for alkaloids, flavonoids, tannins, steroids and triterpenoids (Table 4). Only *M. senegalensis* had shown the presence of saponosids and coumarins were found in *A. senegalensis* and *B. thoningii*.

These results are consistent with previous results from a review of the biological activity and chemical analyses of extracts of the component plants from different regions [39,40]. Alkaloids are one of the major antimalarial natural products and various classes have been reported to exhibit promising activities [41].

Antiplasmodial activity may be due to one or more than one group of constituents. Several phytoconstituents have been described in the field of antiplasmodials such as terpenoids, alkaloids, phenolic compounds including flavonoids and quinones [41].

Some nonalkaloidal natural products such as terpenes, flavonoids, and their related

compounds have also been reported to exhibit promising antiplasmodial activities [42].

4. CONCLUSION

The present study allowed us to identify the antiplasmodial activity of ethanol and water extracts obtained from 10 plants against *P. falciparum* and determine some main chemical groups presents in active extracts.

Four plants could be selected as the best candidates for further investigations in the field of new antimalarial drug discovery in traditional medicine.

In the future studies, we plan to focus on the evaluation of the *in vivo* activity of these plants against a mouse model of *Plasmodium berghei*, on the isolation and identification of active compounds through a bioguided fractionation and on the study of the activity and toxicity of these active isolated compounds.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

The authors are thankful to US Fulbright program which support the corresponding author during this at Well Cornell Medical College, New York.

Special thanks to all lab members at the Department of Microbiology and Immunology, Weill Medical College of Cornell University; especially to Kirk W. Deitsch. We are grateful to Department of Traditional Medicine at Bamako helping in identification of selected plants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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