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ORIGINAL ARTICLE

Phytochemical screening and antiplasmodial activity of *Mundulea antanossarum* seeds from Madagascar

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ABSTRACT

Malaria is one of the three deadly dangerous infectious diseases and is successfully treated with medicinal plants in endemic regions. This work was carried out with the aim to investigate the phytochemical screening and antiplasmodial activities of Mundulea antanossarum seeds extract using two standardized parasites strains (Plasmodium falciparum FcM29-Cameroon and P. yoelii subsp nigeriensis) as model systems. The in vitro antiplasmodial activity of the plant extracts was evaluated by isotopic micro-test, while the in vivo anti-malarial activity was determined by the classical 4-day suppressive test. Qualitative chemical screening was carried out using standard methods of analysis. The ethanolic crude extract and Dichloromethane fraction of the seeds of Mundulea antanossarum showed interesting in vitro antiplasmodial activity against Plasmodium falciparum with the IC50 values of 1.08 ± 0.072 µg/mL and 0.215 ± 0.008 µg/mL respectively. The in vivo bioassay revealed also that ethanol and dichloromethane soluble extracts have interesting bioactivity with the ED50 values of 5.020 ± 0.563 and 2.500 ± 0.462 mg/kg.bw/day, respectively. The phytochemical screening analysis of Mundulea antanossarum seeds extract revealed the presence of flavonoids, terpenoids, saponins, tannins, and total phenols. However, alkaloids were absent. The present findings validated the ethno-medical claim that the extract of Mundulea antanossarum could be useful in treating malaria and fever. For the best of our knowledge, this is the first report on the antimalarial activity of this plant species in the literature.

Keywords: *Traditional medicine, Mundulea antanossarum, Plasmodium spp., malaria, Madagascar*

Introduction

Malaria is considered as one of the three deadly dangerous infectious diseases including tuberculosis and HIV. Africa is the most affected continent with about 90% of all malaria deaths cases. Plasmodium falciparum resistance to artemisinin has been detected in four countries in South East Asia including Cambodia, Myanmar, Thailand and Vietnam.[1] Traditional medicines have been used to treat malaria for thousands of years and are the source of the two main groups of modern antimalarial drugs (artemisinin and quinine). Recent findings indicate that over 1200 plant species from 160 families are used to treat malaria and fever in malaria endemic areas and on average, a fifth of patients use traditional herbal remedies.[2]

The discovery and development of new antimalarial drugs from medicinal plants is mostly conducted by researchers in the world in recent decades. A better example is the discovery of new antimalarial artemisinin and its derivatives from Artemisia annua that has been used in China for centuries traditionally. This proves that the medicinal plants are a natural source of new antimalarials that still need to be explored.[3] Other herbs that are proven to have antimalarial activities are Nigella sativa[4], Vernonia staehelinoides[5], Acalypha fruticosa, Azadirachta indica and Dendrosicyos socotrana[6], Arcangelisia flava, Fibraurea tinctoria, Harrisonia perforated, Irvingia malayana, Elaeocarpus kontumensis and Anneslea fragrans[7], Andrographis paniculata, Hedyotis corymbosa8, Enicostemma littorale[9] and Quassia amara.[10]

In Madagascar it is estimated that about 80% of the population is still dependent on traditional medicine, which essentially involves the use of plants.[11] Despite their wide use in the traditional health care system, the work that has been done to evaluate the safety and efficacy of Malagasy traditional medicine plants is not extensive. During an ethno-botanical survey conducted in the South of Madagascar, it was reported that Mundulea antanossarum (Leguminosae) endemic to Madagascar and known under the vernacular name of "Malaignarety" (Malagasy name) is traditionally used by the local

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communities to treat malaria, hypertension, fever, and for wound-healing.

The aims of this study were to evaluate the *in vitro* and *in vivo* antimalarial activities of *M. antanossarum* in order to validate scientifically the traditional use of this plant species as antimalarial.

Materials and Methods

Plant identification and preparation of extracts

The plant species of *Mundulea antanossarum* Baill. (Syn. Mundulea anceps var. mangokyensis "R.Vig., p.p.A, Leguminosae) was collected in the south of Madagascar based on its ethnomedical use in the islands. The seeds of Mundulea antanossarum was collected in Ambolisaka commune Andranohinaly at approximately 49 km of Toliara city. The plant sample was identified by comparison with reference specimens available at the Department of Botany, Tsimbazaza Zoological and Botanical Park, Antananarivo. Voucher specimens with assigned sample number RBFMT-12 was deposited at the Herbarium of the Laboratory of Applied Chemistry, Layflaylle Street, University of Toliara, Madagascar.

Test extract preparation

The plant material (6 Kg of *M. antanossarum*) was kept at room temperature (25 to 30 °C) for air drying (two weeks). The air-dried and powdered material (1 kg) was extracted by repeated maceration with ethanol 90° (3×4 hrs, 3 L) at room temperature. After filtering the mixture, the aqueous-ethanol filtrates were pooled, dried over Na_2SO_4 and evaporated to dryness under reduced pressure using a rotary evaporator to yield crude ethanolic extract (33.27 g). Thirty grams (30 g) of the crude ethanolic extract were suspended in water and sequentially partitioned with n-hexane, dichloromethane, ethyl acetate and n-butanol (1:1, v/v) to yield the corresponding extract fractions. The different extracts were evaporated to dryness on an evaporator apparatus and were evaluated for their anti-malarial properties. All extracts were stored at +4 °C.

Phytochemical screening

The dried and powdered plant material (10 g) was repeatedly extracted by cold percolation with 95% ethanol and water (100 mL × 2) for 48 hours. Chemical screening was done as below.[12]

Detection of phenols (Ferric Chloride Test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

Detection of flavonoids

The ethanol extract (5 mL) was added to a concentrated sulphuric acid (1 mL) and 500 mg of magnesium. A pink or red coloration that disappear on standing (3 min) indicates the presence of flavonoids.

Detection of tannins

Two methods were used to test for tannins. First, about 1 mL of the ethanol extract was added in 2 mL of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration. Second, 2 mL of the aqueous extract was added to 2 ml of water, a 1 to 2 drops of diluted ferric chloride solution was added . A dark green or blue green coloration indicates the presence of tannins.

Detection of saponins

To 1 ml of aqueous extract was added 0.3 mL of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth for 20 min.

Detection of alkaloids

Five mL of the aqueous extract was added to 2 mL of HCl. To this acidic medium, 1 mL of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

Detection of triterpenoids

Ten (10) mg of the aqueous extract was dissolved in 1 mL of chloroform; 1 mL of acetic anhydride was added following the addition of 2 mL of concentrated sulphuric acid. Formation of reddish violet color indicates the presence of triterpenoids

Anti-malarial bioassays

Parasites strain and in vitro culture conditions

The asexual erythrocytes' stages of Plasmodium falciparum FcM29-Cameroon, a highly chloroquine-resistant strain were grown continuously in stock cultures by a modification of the methods of Trager and Jensen using glucose-enriched RPMI 1640 medium, supplemented with 10% human serum at 37 °C as previously reported.[13-17]

In vitro antiplasmodial activity

The antiplasmodial activity of the plant extracts was evaluated by an isotopic micro-test which determines the inhibition of radio labeled

hypoxanthine up take by malaria parasite as an indicator of growth.

Test extract preparation

Methanol (MeOH, 200 µL) or dimethylsulfoxide (DMSO) was added to 1 gm sample of extracts and further diluted as required in water. The MeOH or DMSO concentration for tested dilutions was not greater than 1%. Initial concentration of the plant extracts was 50 µg/mL diluted with five-fold dilutions to make five concentrations, the lowest being 0.08 µg/mL.

Each test included an untreated control with the solvent and a positive control: chloroquine sulphate (Sigma, France) and ethanolic crude extract of Cinchona stem bark.

Isotopic micro-test

Two hundred micro liters (200 µL) of total culture medium with the diluted extract (20 µL) and the suspension (180 µL) of Plasmodium falciparum-infected human red blood cells in medium (O+ group, 1% haematocrit) with 1% asynchronous parasitaemia was placed into the wells of 96-well micro titre plates. After 18 h incubation of the parasites with the extracts at 37 °C, [^{3}H] hypoxanthine (Amersham, UK) was added to each well and the incubation was continued for another 24 h at the same conditions. The mean values for uptake of ^{3}H-hypoxanthine (disintegration per minute, dpm) in control (untreated) and tested parasitized erythrocytes, expressed as the percentage of inhibition, were calculated. The anti-malarial activity of extracts was expressed by the inhibitory concentrations 50% (IC_{50}), representing the concentration of drug that induced 50% parasitemia decrease compared to control culture. The extract concentration at which the parasite growth (ie [^{3}H] hypoxanthine uptake) is inhibited by 50% (IC_{50}) was calculated by a non-linear regression analysis processed on dose–response curves with the help of Mikro Win Hidex 2000 software. Liquid scintillation counting was operated on CHAMELEONTM V multilabel counter plate.

In vivo antiplasmodial activity

The *in vivo* anti-malarial activity of plant extracts was determined by the classical 4-day suppressive test against *Plasmodium yoelii* subsp nigeriensis strain. [13,14,16,17]

Preparation of parasite inoculum

Five albino Swiss mice of 6 to 8 weeks old and weight ranging between 19 and 30 g were inoculated by intravenous route with 200 µL of the parasite suspension. On day 3, a blood smear was carried out for each mouse. Only the blood of the mice having presented a parasitemia between 30 and 50% was used as inoculum.

In vivo antiplasmodial assay

To realize the test, 25 male Swiss albino mice were inoculated by intravenous route with 10^7 *Plasmodium yoelii* infected red blood cells (parasite inoculum) and randomly divided into five groups of five mice per cage. Chloroquine (CQ) and 3% Tween 80 were used as positive and negative control respectively.

The test extracts were prepared in three different doses (200 mg/kg, 400 mg/kg, and 600 mg/kg of body weight) and CQ at 25 mg/kg in a volume of 0.2 mL and vehicles at 0.5 mL/mouse. Each extract was administered as a single dose per day. All the extracts and the drug were given through intragastric route by using standard intragastric tube to insure safe ingestion of the extracts and the drug. Treatment was started after 3 hrs of infection on day 0 and was then continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D4) blood sample was collected from tail snip of each mouse. Thin smears were prepared and stained with 10% Giemsa solution. Then, each stained slide was examined under the microscope with an oil immersion objective of 100× magnification power to evaluate the percent suppression of each extract with respect to the control groups.

All experimental procedures were approved by the Malagasy Institute of Applied Research (IMRA) Avarabohitra- Itaosy lot AVB 77, P.O. Box 3833, 102 Antananarivo-Madagascar.

Statistical analysis

The results of in vitro study are given as Mean ±S tandard Deviation obtained from three independent experiments. The results of in vivo study were expressed also as Mean ± Standard Deviation and analyzed with Student's t-test for paired data using Origin version 8.5 Pro package software. All data were analyzed at a 95% confidence interval (α= 0.05).

Results and Discussion

Extract yields and chemical screening

The extract yields of the aerial part of Mundulea antanossarum obtained with different solvents are in the order: ethanol (33.27 g) > dichloromethane (12.85 g) > ethyl acetate (5.72 g) > aqueous (5.20 g) > n-hexane (4.15 g) > n-

butanol (3.08 g). Ethanol (EtOH) and dichloromethane extracts displayed interesting yield and were submitted to *in vivo* test. The increase in ethanolic extract yield clearly indicates that polar substances are being mobilized. This also shows that most of the phytochemicals present in *Mundulea antanossarum* are polar compounds and would be more soluble in polar solvents.

The results of phytochemical screening of *Mundulea antanossarum* revealed the presence of flavonoids, terpenoids, saponins, tannins, and total phenols in the seeds extract. However, alkaloids were absent.

Anti-malarial activities of plant extracts

The plant Mundulea antanossarum is used in the traditional medicine of Madagascar to treat malaria, fever and data were retrieved from computerized compilation of medicinal plants.[18] Results of antiplasmodial activity of *M. antanossarum* seed extracts are given in Table 1.).

Table 1. *In vitro* and *in vivo* antiplasmodial activities of extracts from the seeds of *Mundulea antanossarum*

Tested extracts	Antiplasmodial activities (n = 6)	
	In vitro test (IC_{50} in µg/mL)	*In vivo* test (ED_{50} in mg/kg)
Ethanol	1.080 ± 0.072	5.02±0.563
n-Hexane	13. 560 ± 1.713	ND
Dichloromethane	0.215 ± 0.008	2.50 ±0.462
Ethyl acetate	18.631 ± 2.194	ND
n-Butanol	23.462 ± 1.748	ND
Water	20.086 ± 2.872	ND

(Legend: ND no determined, IC_{50} inhibitory concentrations 50%, ED_{50} extract dose 50%, Positive control: Chloroquine (IC_{50}=265.480±45.130 nM); Cinchona sp ethanolic crude extract 100 mg/kg (%Chemo-suppression of *P. yoelii*=33.000±2.630).

The ethanolic crude extract and methylene chloride (Dichloromethane) fraction of the seeds of Mundulea antanossarum showed interesting *in vitro* antiplasmodial activity against Plasmodium falciparum and the rest of the extracts had weak antiplasmodial activity.

The IC_{50} values obtained for the ethanolic and dichloromethane extracts are interesting when compared to previous studies. [19, 20] Indeed, *Artemisia annua* (the source of artemisinin) and *Azadirachta indica* (Neem) have an IC_{50} of 3.9 µg/mL and 2.3 to 12.5 µg/ mL, respectively.[19, 20] These plant species are considered as reference medicinal plants by numerous authors due to their wide use in the treatment of malaria.[20] Gessler *et al.*[21] recommended that if the extract displayed an IC_{50} less than 10 µg/mL, the antiplasmodial activity is interesting, if $10<IC_{50}<50$ µg/mL the antiplasmodial activity is moderate and over 50 µg/mL, the bio-activity is very weak. Based on this criterion, the ethanol and dichloromethane extracts with the IC_{50} values of 1.08 ± 0.072 µg/mL and 0.215 ± 0.008 µg/mL respectively, can be concluded as having interesting antiplasmodial activity *in vitro*. These results suggest that more active compounds may be extracted with an organic polar solvent like ethanol and should be evaluated for in vivo antiplasmodial activity.

For this purpose, a 4-day suppressive test was performed on male Swiss mice using P. yoeli N67. The results summarized in Table 1 show that ethanol and dichloromethane soluble extracts displayed interesting bioactivity in vivo with the ED_{50} values of 5.020 ± 0.563 and 2.500 ± 0.462 mg/kg.bw/day, respectively. These results are consistent with the values obtained from the *in vitro* antiplasmodial study.

The antiplasmodial property of the plant extracts may be attributed to the presence of some phytochemicals which might have conferred some protective effect against oxidative stress induced in the host parasitized red blood cells (RBCs) by the malaria parasite.[22, 23] In malarial infection, oxidative damage to the erythrocyte membrane has been reported as causal relationship between haemichrome production and band 3 aggregations in oxidatively stressed RBCs. This relationship could account for the deposition of band 3-specific autologous IgG antibody and consequent deposition of fragments of complement C3c leading thus to the alteration of the surface of the infected RBCs and subsequent phagocytosis by macrophages.[24] In this case, plant extracts may

function as antioxidant by reversing these changes due to their bioactive principles content.[25] Besides, phytochemical screening revealed that crude extract of *Mundulea antanossarum* could contain a mixture of polar (total phenols, tannins, flavonoids, saponins) and no polar (triterpenoids) compounds with antioxidant and/or antimalarial activities.[26-32]

Conclusions

In the present study, we evaluated the chemical composition and the antimalarial of *Mundulea antanossarum* seeds extract. Results revealed that extracts from this plant species could be a valuable source of naturally occurring antimalarial agents. This scientific based evidence supports the possibility of using *M. antanossarum* extracts as an affordable medicine for managing malaria and allied complications. It is therefore necessary to evaluate the *in vitro* and *in vivo* toxicity of plant in order to guarantee their harmlessness.

Conflict of Interest

None

Acknowledgements

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