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Research Article

In-vitro Antimalarial and Antileishmanial Studies of *Markhamia platycalyx* Sprague Leaves

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Abstract

Bignoniaceae is rich in active metabolites and includes numerous genera of high economic and therapeutic values. One of these plants is *Markhamia platycalyx*. The petroleum ether fraction of the total ethanol leaf extract of *M. platycalyx* exhibited IC_{50} 26760 ng/mL against *Plasmodium falciparum* D_6 and 22430 ng/mL against *P. falciparum* W_2 in the 2nd phase assay. It did not show any cytotoxic activity against the VERO mammalian cells, indicating the safety of the petroleum ether fraction. Additionally, the dichloromethane fraction of the same extract was the most active fraction against *Leishmania donovani* amastigotes in THP1 with 86% inhibition in the 1ry phase assay, which was higher than Amphotericin B.

Introduction

Natural products have extensive significant attention in recent years due to their various pharmacological activities as cytotoxic [1], hepatoprotective [2], anti-inflammatory [3], anti-pyretic [3], gastroprotective [3], anti-diabetic [3],….etc.

The parasitic diseases (malaria, trypanosomiasis, leishmaniasis, schistosomiasis, lymphatic filariasis and onchorcerciasis) in tropical regions have been a dangerous public health problem especially in middle- and low-income countries. These diseases affect millions of people, resulting in thousands of death annually [4-5].

Family Bignoniaceae is rich in the secondary metabolites and includes numerous genera of high economic and therapeutic values [6,7]. It is found in tropical and subtropical areas with a few species in temperate climates [8]. It is comprised of 104 genera and 860 species [8]. One of these species is *Markhamia platycalyx* Sprague (Syn. *Dolichandrone platycalyx* Baker). *M. platycalyx* tree, known in Uganda under the native name Lusambia, is said to yield "the finest of local timbers" (Mahon) [9].

By reviewing the literature, some researchers considered *M. platycalyx* as a synonym of *M. lutea*, however, a recent study on cultivated plants in Egypt, classified both of them in two different lineages [10].

By reviewing the antimalarial and antileishmanial literature of the genus; *M. lutea* was investigated for many therapeutic activities such as antimalarial [11], antileishmanial [11] and antiviral [12]. Moreover, the stem bark of *M. tomentosa* had a good antimalarial activity *in-vitro* [13]. While, nothing could be found for *M. platycalyx*. This provoked us to carry out extensive studies on this plant including *viz*., *in-vitro* antimalarial in addition to antileishmanial activities.

Materials and Methods

Materials

Amphotericin B, Chloroquine, Artemisin and Amikacin (Sigma-Aldrish, USA). Solvents used in this work, e.g. light petroleum ether, chloroform, ethyl acetate, ethanol, methanol and distilled water were purchased from El-Nasr Company for Pharmaceuticals and Chemicals, Egypt.

Apparatus

Rotary evaporator (Laborota Heidolph, Germany) was used for distillation the solvents. Circulating hot-air oven (Carbolite, Germany) was used for drying. Sonicator (Wise Clean WUC

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Table 1: *In-vitro* antimalarial activity (1ry screening) of *M. platycalyx* leaves against *P. falciparum* D_6 strain.

Extract/Fraction/Compound	% of Inhibition
TEE	8
Petroleum ether fraction	87
DCM fraction	47
EtOAc fraction	24
Aqueous fraction	14
CP	1
Chloroquine	100
All (15867 ng/mL) except Chloroquine (79 ng/mL)	

A-10H, Dahan Scientific Co. Ltd, Korea) was used for dissolving the materials used. Water distillater (Bhanu Basic/PH4, MK-I, India).

Plant material

The leaves of *M. platycalyx* Sprague were collected in May 2012 from El-Zohria Botanical Garden, Giza, Egypt. The plant was identified by Dr. Mamdouh Shokry, Director of El-Zohria Botanical Garden, Giza, Egypt. A voucher specimen (Mn-Ph-Cog-015) was kept in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Minia University, Minia, Egypt.

Preliminary Phytochemical Screening

Preliminary qualitative phytochemical screening of the total ethanol extract (TEE) of *M. platycalyx* leaves was carried out using standard reported phytochemical procedures [14,15].

Extraction and Fractionation of *M. platycalyx* leaves

The air dried powdered leaves (5 kg) of *M. platycalyx* were extracted by maceration with 95% ethanol (3x, 5 L each) and then concentrated under reduced pressure to give (500 g) of a viscous residue. It was suspended in the least amount of distilled water (600 mL), transferred to a separating funnel and partitioned successively with light petroleum ether, dichloromethane (DCM) and ethyl acetate (EtOAc) (each solvent; 3x, 350 mL each). The obtained fractions were concentrated under reduced pressure using rotary evaporator to afford three fractions: petroleum ether (95 g), DCM (19 g), EtOAc (30 g). The remaining aqueous layer was concentrated to afford (250 g).

Preparation of Crude Polysaccharide (CP)

A part of the concentrated aqueous fraction (25 g) was gradually added to a flask containing one liter methanol with vigorous shaking and kept in a refrigerator overnight, then filtrated using Büchner funnel under reduced pressure. The residue was dried in the oven 40°C to yield (3 g) of crude polysaccharide powder.

In-vitro antimalarial activity

Antimalarial activity was estimated in-vitro against chloroquine sensitive (D_6, Sierra Leone) and resistant (W_2, Indo China) strains of *Plasmodium falciparum* by determining Plasmodial Lactate Dehydrogenase (PLDH) activity [16]. A 200 µL suspension of *P. falciparum* culture (2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mLAmikacin) was added to the wells of a 96-well plate containing 10 µL of serially diluted samples. The plate was flushed with a gas mixture of 90% N_2, 5% O_2 and 5% CO_2 and incubated at 37°C for 72 h in a modular incubation chamber. PLDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR). Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat™ reagent and incubated for 30 min. Then, 20 µL of a 1:1 mixture of NBT/PES (Sigma, St. Louis, MO) was added and the plate is further incubated for an hour in dark. The reaction was stopped by adding 100 µL of a 5% acetic acid solution. The plate was measured at 650 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments, Vermont). IC_{50} values were obtained from the dose-response curves generated by plotting percent growth versus drug concentration. Chloroquine was included in each assay as positive control. DMSO (0.25%) was used as a vehicle control. The TEE and different fractions of *M. platycalyx* leaves were initially tested against *P. falciparum* D_6 strain in a 1ry phase screening at 15867 ng/mL and the percentage of inhibition was calculated relative to the negative and positive controls. The tested samples that resulted in % inhibition ≥ 50% proceeded to 2nd assay.

In the 2nd phase assay, the tested extract and fractions passing 1st phase screening dissolved to 20 mg/mL and tested at three concentrations 47600, 15867, 5289 ng/mL and IC_{50} were determined. The Selectivity Index (SI) was calculated. All ICs_{50} were calculated using the XLfit curve. Artemisin and Chloroquine (standard antimalarial drugs) were used as positive controls. All experiments were carried out in duplicate. The results of the antimalarial activity were listed in tables 1 and 2. The *in-vitro* cytotoxicity was also determined against mammalian kidney fibroblasts (VERO cells). The assay was performed in 96-well tissue culture-treated plates as described earlier [17]. Briefly, cells were seeded in the wells of a 96-well plate (25,000 cells/well) and incubated for 24 h. Samples were added and plates were again incubated for 48 h. The number of viable cells was determined by neutral red assay. IC50 values were determined from dose-response curves. Amphoterecin B was used as a positive control, while DMSO was used as vehicle control. The results were showed in table 2.

In-vitro antileishmanial activity

The antileishmanial activity of the TEE and different fractions of *M. platycalyx* leaves were tested *in-vitro* against a culture of *Leishmania donovani* promastigotes [18]. The promastigotes were grown in RPMI 1640 medium supplemented with 10% fetal calf

Table 2: *In-vitro* antimalarial activity (2nd assay) of the petroleum ether fraction of *M. platycalyx* leaves.

Fraction/Compound	*P. falciparum* D_6		*P. falciparum* W_2		VERO cells
	IC_{50}	SI	IC_{50}	SI	IC_{50}
Petroleum ether fraction (47600- 5289 ng/mL)	26760	> 1.8	22430	> 2.1	> 47600
Artemisin (238-26.4 ng/mL)	< 26.4	> 9	172.6	> 1.4	> 238
Chloroquine (238-26.4 ng/mL)	< 26.4	> 9	< 26.4	> 9	> 238

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Table 3: *In-vitro* antileishmaial activity (1ry phase screening) of *M. platycalyx* leaves.

Extract/Fraction/Compound	% of Inhibition		
	L. donovani_Pinh	*L. donovani AMAST_Pinh*	*L. donovani AMASTTHP_Pinh*
TEE	0	9	13
Petroleum ether fraction	6	8	31
DCM fraction	18	48	86
EtOAc fraction	1	12	0
Aqueous fraction	2	12	0
CP	2	13	5
Amphotericin B	99	96	81
All (20 µg/mL) except Amphotericin B (0.4 µg/mL)			

serum (Gibco Chem. Co.) at 26°C. A 3-day-old culture was diluted to 5×10^5 promastigotes/mL. Drug dilutions were prepared directly in cell suspension in 96-well plates. Plates were incubated at 26°C for 48 h and growth of *L. donovani* promastigotes was determined by the Alamar blue assay. Standard fluorescence was measured on a Fluostar Galaxy plate reader (BMG Lab Technologies) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Amphoterecin B was used as the standard antileishmanial agents. IC_{50} values were computed from dose-response curves.

The tested samples that showed % inhibition ≥ 50% proceeded to 2nd assay. In the 2nd assay, the tested extract and fractions were dissolved to 20 mg/mL and tested at (40.0, 8.0 & 1.6 µg/mL). All ICs_{50} and ICs_{90} were calculated using the XLfit curve. The Amphotericin B (standard drug) was used as positive control [19,20]. All experiments were carried out in duplicate. The results of the antileishmanial (1ry phase) were listed in Table 3.

Results and Discussion

Preliminary phytochemical screening

The TEE of *M. platycalyx* leaves showed the presence of carbohydrates and/or glycosides, flavonoids, unsaturated sterols and/or triterpenes and anthraquinones. On the other hand, it was free from crystalline sublimate substances, saponins, alkaloids, tannins, cardenolides and coumarins. This preliminary phytochemical screening showed many classes of secondary metabolites indicating various expected biological activities.

In-vitro antimalarial activity

The petroleum ether fraction showed the highest percentage of inhibition (87%) against *P. falciparum* D_6 strain relative to chloroquine. Any drug caused ≥ 50% inhibition; it proceeded to 2nd phase assay. On the other hand, the DCM fraction exhibited a good antimalarial activity 47% inhibition, followed by EtOAc fraction 24%, while the aqueous fraction showed weak activity 14%, but higher than that of the TEE (8%) as shown in Table 1.

The 2nd phase assay of the petroleum ether fraction (Table 2) revealed IC_{50} of 26760 ng/mL against *P. falciparum* D_6 and 22430 ng/mL against *P. falciparum* W_2. It did not show any cytotoxic activity against the VERO mammalian cells line up to the maximum dose tested; 47600 ng/mL, as recorded in Table 2, indicating the safety of the petroleum ether fraction.

The previous phytochemical review showed various classes of compounds isolated and identified from genus Markhamia *viz.*, phenylpropanoids, lignans, naphthoquinones, anthraquinones, sterols, cycloartane triterpenes and their glycoside derivatives, phenolic glycosides and triterpene acids. These compounds are isolated from different plant parts including roots, leaves, stem, root bark and heart wood [21].

Moreover, sterols and triterpenes were found in the preliminary phytochemical screening of TEE. Consequently, petroleum ether has an ability to extract sterols and triterpenes from TEE. Therefore, the activity may be due to presence of sterols and triterpenes in this fraction [22,23]. Our findings are in line with the previous studies [11,13].

In-vitro antileishmanial activity

The 1ry phase screening of antileishmanial activity in Table 3 revealed that the DCM fraction was the most active against *L. donovani* amastigotes in THP1 with 86% inhibition, which is even higher than Amphotericin B. While, the other fractions showed weak or no activity. The DCM fraction in 2nd phase assay showed IC_{50} and IC_{90} against *L. donovani* amastigote > 20 µg/mL.

Leishmaniasis is a vector-borne disease, affecting 72 developing countries and 13 of the least developed countries. Visceral leishmaniasis due to *L. donovani* is the most severe form of Leishmania infections. The annual incidence of visceral leishmaniasis is estimated to be 500,000 cases. The overall prevalence of visceral leishmaniasis is 12 million people and the population at risk is 350 million [24]. The drug of choice for the treatment is still a problem. Therefore, there is an urgent need to discover new drugs with high activity and low side effects. Natural products have become a key source of new drugs in the last years [19,25].

As mentioned before, the earlier phytochemical review exhibited the presence of different classes of compounds isolated and identified from Markhamia species as phenyl propanoids, lignans, naphthoquinones, anthraquinones, sterols, cycloartane triterpenes and their glycoside derivatives, phenolic glycosides and triterpene acids [21]. Moreover, sterols and triterpenes were found in the preliminary phytochemical screening of TEE. Consequently, DCM has an ability to extract sterols and triterpenes from TEE. Therefore, the activity may be due to presence of sterols and triterpenes [20,22]. Our results are in line with the previous studies on another two species [11,13].

Conclusion

In this study, the significant antimalarial and antileishmanial activities make *Markhamia platycalyx* leaves a potential source for the antiprotozoal drugs that are strongly recommended for further development.

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