

Full Length Research Paper

***In vitro* preliminary study of antiprotozoal effect of four medicinal plants from Benin**

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Accepted 22 March, 2012

A total of fourteen extracts with different polarity obtained from four species *Crataeva religiosa*, *Baillonella toxisperma*, *Boswellia dalzielii* and *Khaya senegalensis*, traditionally used in Benin to treat malaria were tested for their *in vitro* antiprotozoal activity towards *Plasmodium falciparum*, *Trypanosoma rhodesiense*, *Trypanosoma cruzi* and *Leishmania donovani*. Selected plants were extracting with cyclohexane, methylene chloride, ethyl acetate, ether diethyl, methanol and ethanol. Tested extracts showed moderate to good antiparasitic effects at two different concentrations (9.6 and 1.6 µg/ml). Many extracts showed appreciable growth inhibition at 9.5 µg/ml. Ethanol extract of *B. dalzielii* was the most active against *T. rhodesiense* and *L. donovani* with growth inhibition percentage of 100 and 95.6%, respectively. Seven out of fourteen extracts also showed interesting growth inhibition percentage from 99 to 87%, on *P. falciparum* at 9.6 µg/ml. The most interesting activity against *L. donovani* was observed with the cyclohexane extract of *C. religiosa* with growth inhibition value of 55% at 1.6 µg/ml.

Key words: Traditional medicine, antimalarial, leishmanicidal, trypanocidal, Benin.

INTRODUCTION

Malaria is the most crucial problem of public health in African sub-Saharan countries. It is prevalent in about 100 countries and around 2,400 million people are at risk (Kager, 2002). 74% of the population lives in area of strong endemic disease and 18% in epidemic area. About 600 million persons are exposed to malaria (Weniger et al., 2008). Every year, about 300 million cases and 1 million deaths are recorded.

There is an urgent need to develop new drugs or vaccines for the treatment, prevention and management

of malaria, because of the devastating nature of malaria and the failure of the most affordable drugs (Asase et al., 2010; Waako et al., 2005). Leishmaniasis is an infection caused by protozoa of the genus *Leishmania* presenting several forms of the disease such as cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL), which can be fatal when untreated. The disease is endemic in some tropical areas of the world and in under developed countries (Alves et al., 2003). As malaria, leishmaniasis is also a major public health problem that affects around 12 million people in 80 countries and causes morbidity and mortality mainly in Africa, Asia, and Latin America (Pereira et al., 2010).

Human African trypanosomiasis, sleeping sickness, African lethargy, or Congo trypanosomiasis is a parasitic

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disease of people and animals, caused by protozoa of the species *Trypanosoma brucei* and transmitted by tsetse fly. The disease is endemic in some regions of sub-Saharan Africa, covering about 36 countries and 60 million people. It is estimated that 50,000 to 70,000 people are currently infected; the number having reduced somewhat in recent years (WHO, 2006). It is believed that many cases go unreported. About 48,000 people died of it in 2008 (Sarah, 2009). The treatment and control of the disease was based on the prophylactic and therapeutic use of trypanocides. Regrettably, their intensive use over decades leads to drug resistance which grows to a major problem (Peregrine, 1994).

The increasing global spread of drug resistance to most of the available and affordable antiprotozoal drugs is a major concern and requires innovative strategies to combat the disease. There is an urgent need for new therapeutic compounds, which are easy to administer and store, and which are of low cost (Asokan et al., 2011).

In Africa, up to 80% of the population still relies on traditional medicine as primary health care. Within the traditional medicine (WHO, 2008), the use of medicinal plants plays an important role and has a tradition lasting for millennia. Twenty-five percent of modern medicines are made from plants that were first used traditionally (Holtz, 2008).

Historically, the major conventional antimalarial drugs have been derived from plants or from structures modeled on plant-derived compounds (Klayman, 1985). Regrettably, available and effective medicines are expensive to the population. They combine the conventional and traditional medicine to look after themselves.

In this work, antiprotozoal activity of four plants species widely used by the traditional healers to treat parasitic diseases in Benin were investigated, by evaluated their inhibitory effect towards *Plasmodium falciparum* resistant K1 multidrug-resistant strain, *Trypanosoma brucei rhodesiense* STIB 900 strain, *Trypanosoma cruzi* Tulahuen C4 strain, and *Leishmania donovani* MHOM/ET/67/L82 strain.

MATERIALS AND METHODS

Collection of plant

The parts of each plant, traditionally use were collected between March and July, 2008 in different area in South Benin. All species were selected on the basis of their traditional use.

The leaves of *Crataeva religiosa* (Capparidaceae; AA 6366/HNB) were collected at Medicinal Plants Garden at Porto-Novo in the Ouémé Department (Southern Benin). *Boswellia dalzielii* (Burceraceae; AA 6365/HNB) and *Baillonella toxisperma* (Sapotaceae) barks were acquired by market plants sellers in Adjarra market in South Benin (Department of Ouémé). Entire plant of *Khaya senegalensis* (Meliaceae, AA 6367/HNB) was collected in Atlantic region. Botanical determination was performed by botanists from Herbar National d'Abomey-Calavi.

Antiplasmodial activity assay

The extracts of experimental plants were evaluated for their antimalarial activity against the multidrug-resistant *P. falciparum* K1 strain. *In vitro* antiplasmodial activity was determined by means of microculture radioisotope technique based on the method previously described by Desjardins et al. (1979) and modified by Ridley et al. (1996). The assay uses the uptake of [³H]hypoxanthine by parasites as an indicator of viability. Continuous *in vitro* cultures of asexual erythrocytic stages of *P. falciparum* were maintained following the methods of Trager and Jensen (1976). For this preliminary work, two concentrations (1.6 and 9.6 µg/ml) of each extract were tested. The aim of this preliminary work was to eliminate the extracts, which at these two concentrations do not inhibit the growth of parasites. After 48 h incubation of the parasites with the extracts at 37°C, [³H]hypoxanthine (Amersham 115 Int., Buckinghamshire, UK) was added to each well and the incubation was continued for another 24 h at the same temperature. The percentage of inhibition of growth was determined in comparison with negative wells. Artemisinin (0.018 and 0.003 µg/ml) was used as positive references. Each assay was run in duplicate.

Anti-*T. b. rhodesiense* activity assay

The assays were performed according to a slight modify procedures described by Freiburghaus et al. (1996). The extracts were dissolved in 10% dimethyl sulfoxide (DMSO), and tested solutions were prepared in serum containing culture medium. Each extract were tested in triplicate. After the addition of *T. b. rhodesiense* bloodstream-form trypanosomes from axenic culture, the concentrations of the extracts were 1.6 and 9.6 µg/ml. The total number of trypanosomes in each well was $2 \times 10^2/100 \mu\text{l}$. The plate was then incubated for 72 h at 37°C in 5% CO₂. Two hours before the end of the incubation, 10 µl of Alamar blue solution was added. Fluorescence was measured after 2 h of incubation with Alamar blue dye in a fluorescence plate reader at 530 nm excitation and 590 nm emission wavelength (Cytofluor 2300, Millipore, Bedford, MA, USA) (Răz et al., 1997). The percentage of inhibition of each extract was then determined in comparison with negative wells without extract. Melarsoprol (0.015 and 0.003 µg/ml) was used as positive references. Each assay was run in duplicate.

T. cruzi assay

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtiter plates at 2000 cells per well per 100 µl in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS) and 2 ml glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* were added in each well (100 µl) with or without extracts. The plates were incubated at 37°C in 5% CO₂ for 4 days. After 96 h, the inhibition of growth was determined microscopically. The values are means of two independent assays. Each assay was run in duplicate. Benznidazole (2.4 and 0.5 µg/ml) was used as positive control.

Leishmanicidal activity assay

Fifty microliters of culture medium, a 1:1 mixture of SM medium (Cunningham, 1977) and SDM-79 medium (Brun and Schönenberger, 1979) at pH 5.4 supplemented with 10% heat-inactivated FBS, was added to each well of a 96-well microtiter plate (Costar). Then, 10⁵ axenically grown *L. donovani* amastigotes (strain MHOM/ET/67/L82) in 50 µl medium were added to each well and the plate was incubated at 37°C under a 5% CO₂ atmosphere for 72 h. Ten microliters of resazurin solution (12.5 mg resazurin

Table 1. Antimalarial and antileishmanial activity of extracts of selected species.

Species	Extract	<i>L. donovani</i>		<i>P. falciparum</i>	
		C ₁	C ₂	C ₁	C ₂
<i>C. religiosa</i>	C	84.4±7.61	55±0.24	87.7±3.55	3.9±0.28
	D	71.3±1.84	42.4±0.74	88.1±2.38	13.1±0.10
	O	38.1±0.85	5.6±0.96	-	-
	A	14.8±1.41	-	53.6±1.03	8.7±0.34
	M	31.1±0.28	-	1.5±0.33	-
<i>K. senegalensis</i>	C	0.00	-	86.3±9.08	18.2±1.65
	D	42.7±0.42	-	92.7±0.83	-
	F	67.4±1.85	27.3±1.61	91.7±1.02	-
	O	20.8±2.39	7.5±0.00	98.3±1.23	23.4±2.11
	T	44.2±2.97	-	21.8±0.25	0.00
	A	-	-	50.8±5.18	5.8±0.25
	M	-	-	-	4.6±0.08
<i>B. toxisperma</i>	E	13.3±0.58	-	99.2±1.13	31±4.61
<i>B. dalzielii</i>	E	95.1±4.53	36.9±5.39	62.2±3.63	17.8±1.26
Control	-	-	-	-	-
Miltefosin	-	85.2	62.8	-	-
Artemisinin	-	-	-	100.0	79.6

C₁=9.6 µg/ml; C₂=1.6 µg/ml; C: cyclohexane; D: methylene chloride; F: chloroform; O: diethyl ether; A: ethyl acetate; M: methanol; E: ethanol.

dissolved in 100 ml distilled water) were then added to each well and incubation continued for a further 2 to 4 h. The plate was read in a Spectramax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and emission wavelength of 588 nm (Räz et al., 1997). Fluorescence development was measured and expressed as percentage of the control. Miltefosin (1.2 and 0.2 µg/ml) was used as positive control. Each assay was run in duplicate.

RESULTS AND DISCUSSION

In this study, four species from four different families were screened for their antiprotozoal potencies. The fourteen extracts obtained from these species were evaluated for their *in vitro* antiplasmodial, antitrypanosomal and leishmanicidal activities at two concentrations (1.6 and 9.6 µg/ml). Results are summarized as shown in Tables 1 and 2.

Antileishmanial activity

Regarding the *in vitro* antileishmanial assay, cyclohexane and methylene chloride extracts of *C. religiosa*, were the most active with growth inhibitory percentage of 55 and 42.4% at 1.6 µg/ml, respectively. This concentration of 1.6 µg/ml could correspond with the inhibitory concentration of 50 (IC₅₀) values of both extracts. At 9.6 µg/ml, ethanol extract of *B. dalzielii* was the most active

with an inhibitory percentage of 95.1%. Good potencies, over 50% inhibitory percentage, were also displayed by methylene chloride extract of *C. religiosa* and chloroform extract of *K. senegalensis* (71.3 and 67.4%, respectively). Similar study was conducted by Ahua et al. (2007) who reported methylene chloride and methanol extracts showed week leishmanicidal activity with survival percentage of 97.9 and 93.4, respectively. The activity of antileishmanial plant extracts has been attributed thus far to compounds belonging to diverse chemical groups, such as isoquinolines, indole alkaloids, quinones and terpenes (Berhaut, 1974; Arbonnier, 2002). These chemical groups are for most part stemming from non polar or averagely polar extracts. The results obtained by Araujo et al. (1998) on chloroform extract and isolated compounds from *Centrolobium sclerophyllum* confirmed the results obtained in our study.

Antiplasmodial activity

Ten extracts out of fourteen showed interesting activity up to 50% at 9.6 µg/ml. Ethanol extract of *B. toxisperma* and the diethyl ether extract of *K. senegalensis* were found to be more potent with inhibitory percentage of 99.2 and 98.2%, respectively. The strongest inhibitions obtained in our study should be attributed to limonoids isolated from *K. senegalensis* (Maneerat et al., 2008;

Table 2. Trypanocidal activity of extracts of selected species.

Species	Extract	Growth inhibition (%)			
		C ₁	C ₂	C ₁	C ₂
		<i>T. b. rhodesiense</i>		<i>T. cruzi</i>	
<i>C. religiosa</i>	C	49.9±0.57	11.5±0.19	-	-
	D	42.5±0.15	7.7±0.14	-	-
	O	16.2±0.86	-	-	-
	A	40.6±0	2.2±0.42	-	-
	M	15.5±0.45	-	-	-
<i>K. senegalensis</i>	C	30.4±0.01	0.1±0.14	58.6±10.30	4±1.13
	D	46.6±0.64	13.4±0.28	-	-
	F	68.8±2.40	6.9±0.57	21.1±3.07	8.3±0.66
	O	66.2±5.36	2.8±0.71	69.4±1.33	18.5±1.41
	T	35.6±2.14	0.7±0.06	-	2.3±0.25
	A	24.6±4.84	7.1±0.96	-	18.9±1.06
	M	27.2±1.98	-	-	-
<i>B. toxisperma</i>	E	73.7±2.21	6.3±5.94	11.5±0.98	-
<i>B. dalzielii</i>	E	100±0	10.8±1.50	-	-
Control	-				
Melarsoprol	-	99.1	90.1		
Benznidazole	-			95.1	58.2

C₁=9.6 µg/ml; C₂=1.6 µg/ml; C: cyclohexane; D: methylene chloride; F: chloroform; O: diethyl ether; A: ethyl acetate; M: methanol; E: ethanol.

Khalid et al., 1998). Five other extracts inhibited over 50% of the growth of *P. falciparum* (86.3 to 92.7%). At 1.6 µg/ml, the tested extracts showed a weak percentage of inhibition (0 to 31%). The highest inhibition at this concentration was obtained with the ethanol extract of *B. toxisperma* (31.0%). The methanol extract of *K. senegalensis* and diethyl ether of *C. religiosa* showed no inhibition towards *P. falciparum*.

Antitrypanosomal activity

Eight extracts out of fourteen showed a good inhibitory activity against *T. b. rhodesiense* at 9.6 µg/ml (40 to 100%). The EtOH extract of *B. dalzielii* at 9.6 µg/ml was the most potent with a percentage of inhibition of 100%. The chloroform and diethyl ether extracts of *K. senegalensis* showed also an interesting activity with a percentage of inhibition value of 68.8 and 66.2%, respectively.

Our results are more consistent than those obtained by Aderbauer et al. (2008), in which the IC₅₀ value of methylene chloride extract of *K. senegalensis* was 100 µg/ml. Four other extracts also showed interesting activity against *T. b. rhodesiense* (42.5 to 73.7%). At 1.6 µg/ml, all tested extracts showed weak inhibition (0 to 13.4%). Regarding the inhibitory percentage of extracts against *T.*

cruzi, only cyclohexane and diethylether extracts of *K. senegalensis* were potent with percentage inhibition of 58.6 and 69.4, respectively. A very weak inhibition was observed for the other extracts (0 to 21%).

Conclusion

Our results indicate that the studied plants possess therapeutic effects especially on *L. donovani* with inhibitory percentage values of 55 and 42.4%, respectively for cyclohexane and methylene chloride extracts. The results give a scientific basis to the traditional uses of these plants. Further investigations are in progress in our laboratory to determine the inhibitory concentration 50 (IC₅₀) of active extracts.

ACKNOWLEDGEMENTS

The authors wish to thank the traditional healers from Benin for their willingness to share their knowledge about plants and Agence Universitaire de la Francophonie (AUF) for the travel support to University of Strasbourg and International Foundation for Sciences and OPCW for financial support.

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