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In vitro ANTIMALARIAL ACTIVITY AND CYTOTOXICITY OF SOME SELECTED CUBAN MEDICINAL PLANTS

Aymé Fernández-Calienes VALDÉS(1), Judith Mendiola MARTÍNEZ(1), Ramón Scull LIZAMA(2), Yamilet Gutiérrez GAITÉN(2), Deyanira Acuña RODRÍGUEZ(1) & Juan Abreu PAYROL(2)

SUMMARY

Terrestrial plants have been demonstrated to be sources of antimalarial compounds. In Cuba, little is known about antimalarial potentials of plant species used as medicinals. For that reason, we evaluated the antimalarial activity of 14 plant species used in Cuba as antimalarial, antipyretic and/or antiparasitic. Hydroalcoholic extracts were prepared and tested *in vitro* for the antimalarial activity against *Plasmodium falciparum* Ghana strain and over human cell line MRC-5 to determine cytotoxicity. Parasite multiplication was determined microscopically by the direct count of Giemsa stained parasites. A colorimetric assay was used to quantify cytotoxicity. Nine extracts showed IC_{50} values lower than 100 µg/mL against *P. falciparum*, four extracts were classified as marginally active (SI < 4), one as partially active (*Parthenium hysterophorus*) exhibiting SI equal to 6.2 and two extracts as active (*Bambusa vulgaris* and *Punica granatum)*, showing SI > 10. *B. vulgaris* showed the most potent and specific antiplasmodial action (IC_{50} = 4.7 µg/mL, SI = 28.9). Phytochemical characterization of active extracts confirmed the presence of triterpenoids in *B. vulgaris* and polar compounds with phenol free groups and fluorescent metabolites in both extracts as major phytocompounds, by thin layer chromatography. In conclusion, antimalarial use of *B. vulgaris* and *P. hysterophorus* was validated. *B. vulgaris* and *P. granatum* extracts were selected for follow-up because of their strong antimalarial activity.

KEYWORDS: Malaria; Ethnopharmacology; Ethnomedicine; Antiparasitic; Antiprotozoal; Toxicity.

INTRODUCTION

Malaria is one of the major threats concerning world public health. There are around 250 million clinical cases every year and almost one million deaths, mostly children, which are attributable to this disease[35]. The main reasons that explain this worsening situation are therapy problems[30]: resistance to the current antimalarial drugs[33], unavailability and unaffordability of antimalarial drugs[4] and lack of new therapeutic targets[3]. These facts have led to searches for new antimalarial compounds.

Plant species have demonstrated their potential to provide effective drugs for the treatment of malaria. Two of the most effective antimalarial drugs available, quinine and artemisinin, are derived from terrestrial plants. For that reason, many research groups screen plant extracts searching for new therapeutic alternatives.

A search of Cuban medicinal species with antimalarial activity is being developed in our laboratory. Products with antimalarial activity have been isolated from plants used against other parasites[5,10] and some antimalarial natural products have shown good activity against a broad spectrum of parasites[1,13]. So, we included in our investigation, in addition to antimalarials and antipyretics, plant species used in our country as antiparasitic.

Pharmacological evaluation of seven species used in Cuba as antimalarial or antipyretic by the Cuban population was previously developed by our group[11,29] obtaining low activity or non-specific action against *P. falciparum*. In this study, the activity of 14 medicinal plant species against *P. falciparum* was assayed. Cytotoxicity on human fibroblasts was also evaluated in order to determine the selectivity of antimalarial action.

MATERIALS AND METHODS

Plant material: Plant species were selected after a revision of the classic Cuban book "Plantas medicinales, venenosas y aromáticas de Cuba", written by Juan Tomás Roig[28]. Identification and collection of the fourteen plant species were made by a botanical specialist (Dr. Ramón Scull) in the Cuban National Botanical Garden, Havana City, in August 2006. Plant specie, vernacular name, part of the plant studied and registration number in the garden are shown in Table 1. Medicinal use of interest (concerning to antimalarial, antipyretic and/or antiparasitic uses) is shown in Table 2.

Plant parts were carefully collected to avoid contamination with other parts of the plant and strange materials. Vegetal material was dried in a

(1) Departamento de Parasitología. Instituto de Medicina Tropical Pedro Kourí. Autopista Novia del Mediodía Km 6½. Marianao 13. La Habana. Cuba.
(2) Departamento de Farmacia. Instituto de Farmacia y Alimentos. Universidad de La Habana. Calle 222. La Coronela. La Habana. Cuba.
Correspondence to: Aymé Fernández-Calienes Valdés, Department of Parasitology, Institute of Tropical Medicine "Pedro Kourí", Apdo Postal No.601, Marianao 13, Havana City, Cuba. Tel.: 53-7-202 06 50. E-mail: ayme@ipk.sld.cu

Table 1
List of medicinal plant species tested for antimalarial activity

Specie (Family)	Vernacular name	Part tested	Voucher number
Bambusa vulgaris Schrad. Ex J.C. Wendl. (Poaceae)	Caña Brava	Aerial parts	9500087
Bursera simaruba (L.) Sarg. (Burseraceae)	Almácigo	Aerial parts	8603305
Cymbopogon citratus (D C.) Stapf. (Poaceae)	Caña santa	Aerial parts	8700008
Lepidium virginicum L. (Brassicaceae)	Mastuerzo	Aerial parts	8700259
Luffa aegyptiaca Mill. (Cucurbitaceae)	Estropajo	Aerial parts	0600366
Manguifera indica L. (Anacardiaceae)	Mango	Aerial parts	9700175
Melia azedarach L. (Meliaceae)	Paraíso	Bark	8603268
Ocimun sanctum L. (Lamiaceae)	Albahaca morada	Aerial parts	9200485
Parthenium hysterophorus L. (Asteraceae)	Escoba amarga	Root	9700175
Picramnia pentandra Sw. (Simaroubaceae)	Aguedita	Aerial parts	8603268
Punica granatum L. (Punicaceae)	Granado	Bark	8300050
Stachytarpheta jamaicensis (L.) Vahl. (Verbenaceae)	Verbena Cimarrona	Aerial parts	9200475
Tamarindus indica L. (Caesalpiniaceae)	Tamarindo	Bark	8300068
Turnera ulmifolia L. (Turneraceae)	Marilope	Aerial parts	8602024

ventilated incubator at 30 °C and subsequently milled in coarse particles of about 5 mm.

Preparation of extract: One extract for each collected species was prepared. Liquid extraction of the dried processed plant material was achieved by maceration during seven days[19] in ethanol 80%, using a relation of 1:10 vegetal material mass/solvent volume. The solvent was removed by evaporation at reduced pressure during 24 hours and the remaining residue was frozen at -70 °C for 48 hours before lyophilization. Stock solutions of each lyophilized sample were reconstituted in 100% dimethyl sulphoxide (DMSO) at 40 mg/mL and stored at 4 °C until use.

Antiplasmodial activity test: The chloroquine-susceptible *P. falciparum* Ghana strain (GHA) was kindly donated by the Laboratory for Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Belgium. Parasites were cultured in human A^+ erythrocytes at 37 °C under a low oxygen atmosphere (3% O_2, 4% CO_2 and 93% N_2) in a modular incubator chamber (ICN Biomedicals, USA). The culture medium was RPMI-1640, supplemented with 20% human serum.

The experiments were performed in 96-well culture plates (Nunc); extracts were tested at two-fold dilutions in a dose-titration range of 100 µg/mL to 1.56 µg/mL. One hundred microliters of infected human red blood cells suspension (1% parasitemia, 4% hematocrit), with more than 90% of ring forms, were added to each well containing 100 µL of extracts pre-diluted in RPMI-1640. Test plates were incubated for 48 hours. Parasite multiplication was determined microscopically after Giemsa staining and expressed as a percentage of the controls without extract. More than 2000 red blood cells were examined for parasite presence at each concentration per one experiment. The extract concentration showing inhibition of parasite multiplication was tested three times. Concentration of extracts that inhibits 50% of parasite growth (IC_{50}) was calculated by linear interpolation. Chloroquine sulphate (Sigma Chemical Co.) was used as reference drug.

Cytotoxicity assay: A human diploid embryonic lung cell line MRC-5, was used to assess the cytotoxic effects of plant extracts. Cells were cultivated in Minimum Essential Medium (MEM), supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% Fetal Calf Serum (FCS) at 37 °C and 5% CO_2. For the assay, 100 µL of cell suspension containing 15,000 cells were seeded onto each well of 96-well plate. After formation of the confluent monolayer, 2 µL of stocks pre-diluted in culture medium were added to each well for a final dose range of 500 µg/mL to 1.56 µg/mL. Fibroblasts were maintained for 72 hours under 5% CO_2 atmosphere. DMSO (0.1%) and untreated cultures were included as controls. The cytotoxicity was determined using the MTT assay[20]. Briefly, after incubation period, 10 µL of stock MTT solution (5 mg/mL) was added to each well and plates were incubated at 37 °C for four hours. Then, 100 µL of sodium dodecyl sulfate solution (SDS 10% in 0.01 M HCl) were added to each well and the amount of formazan formed was measured by scanning it with an ELISA reader at 560 nm and a background of 630 nm. The percentage of inhibition was calculated. The 50% cellular cytotoxic concentration (CC_{50}) of test extracts was calculated by linear interpolation. The selectivity index (SI) was calculated as the ratio of the CC_{50} to the IC_{50}.

Criteria of antiplasmodial activity: Extract exhibiting $IC_{50\ P.falciparum} >$ 100 µg/mL was considered inactive. Extract showing $IC_{50\ P.falciparum} <$ 100 µg/mL was classified as follows: Marginally active at SI < 4, partially active at SI 4-10 and active at SI > 10. Active extract showing $IC_{50\ P.falciparum}$ < 10 µg/mL should be selected for further bioassay-guided fractionation.

Phytochemical analysis: Samples of the most active extracts were characterized using the main chemical reactions for most significant metabolites[17]: Dragendorff reaction for alkaloids detection, Fehling for reductor substances, Baljet to detect lactonic compounds, resins test,

foam test to detect saponins, Lieberman-Burchard for triterpenoids and/or steroids, $FeCl_3$ for phenols and tanins, ninhidrin to detect aminoacids, Börntrager for quinones, Kedde for cardiotonic glycosides, anthocyanidin test and Shinoda to detect flavonoids.

Thin layer chromatography (TLC) coupled with chemical or physical methods was used for identification of different secondary metabolites. Glass sheets coated with silica gel 60 F_{254} (4 x 5 cm), were used. The chromatography was run in a chamber with ethyl acetate: methanol: water (10:1.3:1, v/v) as the mobile phase. Exposure to ultraviolet (UV) light at 254 and 366 nm, ammonia vapour and vainillin/sulphuric spray followed by heating at 105 °C, were used for the visualization of chromatograms.

RESULTS

All ethanolic extracts of the 14 species were assayed for antimalarial activity against *P. falciparum* chloroquine-susceptible GHA-strain. The IC_{50} values, CC_{50} values and selectivity indices (SI = ratio of cytotoxicity to antimalarial activity) of extracts are shown in Table 2. Extracts from nine species (64.28%) exhibited $IC_{50} < 100$ µg/mL. Four extracts were classified as marginally active and one as partially active (*P. hysterophorus*) exhibiting SI of 6.2. Only two extracts were found active, *B. vulgaris* and *P. granatum*, they showed SI > 10 and $IC_{50} < 10$ µg/mL. Chloroquine, used as a reference antimalarial drug, tested in parallel had an IC_{50} of 0.02 µM.

Phytochemical screening was consistent with the detection of alkaloids, lactones, triterpenoids and/or steroids, phenols, quinones, flavonoids and anthocyanidines in the two extracts, whereas, the presence of saponins and aminoacids were detected only in *B. vulgaris.* Figure 1 shows that the analyzed extracts were mixtures of compounds: strong spots at the baseline of chromatography (compounds with polar characteristics) were observed for the two extracts, these spots exhibited a yellow color after ammonia exposure confirming the presence of free phenols groups; spots in the solvent front were observed in *B. vulgaris*, treatment with sulphuric acid and vainillin was consistent with

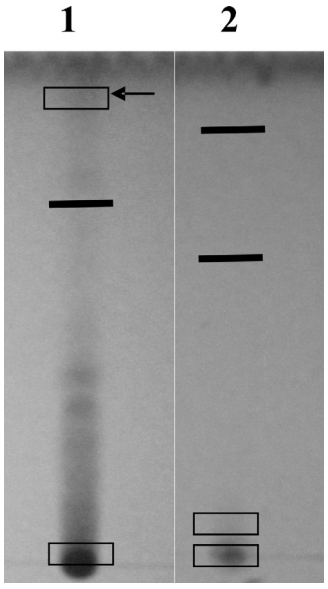

Fig. 1 - Combined profile of spots observed after thin layer chromatography of the *B. vulgaris* (1) and *P. granatum* (2) extracts, exposed to UV 254 nm, with spots observed at visible light (open rectangles) and UV 366 nm (black lines, blue fluorescence). Profile after sulphuric/vainillin treatment was very similar to the obtained after UV 254 nm exposure; arrow indicates the red spot position.

Table 2
In vitro antiplasmodial activity and cytotoxicity of plant extracts

Extract	Medicinal use	*P. falciparum* IC_{50} (µg/mL)	MRC-5 CC_{50} (µg/mL)	SI	Classification
B. vulgaris	Against paludic fevers	4.7	136.7	28.9	A
B. simaruba	Against intermitent fevers	>100	-	-	I
C. citratus	Antipyretic	>100	-	-	I
L. virginicum	Antihelminthic	>100	-	-	I
L. aegyptiaca	Antihelminthic	46.5	138.1	2.9	MA
M. indica	Antipyretic	64.0	20.0	<1	MA
M. azedarach	Antihelminthic	66.2	250.0	3.7	MA
O. sanctum	Antipyretic	40.1	63.1	1.5	MA
P. hysterophorus	Against paludic fevers	45.2	282.8	6.2	PA
P. pentandra	Quina substitute, against intermitent fevers	66.5	125.2	1.8	MA
P. granatum	Antihelminthic	9.1	121.5	13.3	A
S. jamaicensis	Antihelminthic, against tertian fevers	76.0	62.0	<1	MA
T. indica	Antipyretic	>100	-	-	I
T. ulmifolia	Antipyretic	>100	-	-	I

(I) Inactive; (MA) Marginally active; (PA) Partially active; (A) Active

triterpenoids (red color); at UV exposure fluorescent spots appear, blue spots were particularly significant at 366 nm.

DISCUSSION

The remarkable activity of quinine and related drugs and the success of artemisinin have stimulated the search for new plant-derived antimalarials. A large number of plants have been screened for antiplasmodial activity[15,26].

Selection of the plant species to be studied is obviously a crucial step for the ultimate success of the investigation. Three strategies are currently pursued: random collection of plant material, targeted collection based on consideration of chemotaxonomic relationships and the exploitation of ethnomedical information[26]. Identification of new plant-derived antimalarial using an ethnopharmacological approach appears to be more predictive compared with random screening[29].

In Cuba, malaria was eradicated in 1971 and no recent ethnobotanical data is available. However, Cuban ethnobotanical information has been compiled by several researchers. All species included in this study were selected after revision of the most important Cuban text about medicinal plants[28]. In another way, the selected plants are abundant in the wild and their cultivation is easy with a low cost, which guarantees enough quantities to perform the pharmacological evaluations.

Usually, plant species used as antimalarials and antipyretics, are the targets of antimalarial screening. We included in our study five species used as antipyretic and five which uses are more closely related with an antimalarial action (against paludic fevers, intermitent fevers, tertian and quartan fevers and/or to substitute quina) but we also evaluate four species used as antihelminthic. Last criteria was based on reports about activity of some products derived from plants, including current antimalarial drug artemisinin, against a wide number of parasites[1,16] and plants used against other parasitic infections which are sources of antimalarial compounds[5,10].

Multiple efficacy parameters for *in vitro* antimalarial activity have been proposed[4,7,8, 21,36,]. For crude extracts, IC_{50} values should certainly be below 100 µg/mL[4,7], although most promising antimalarial extracts exhibit IC_{50} values under 10 µg/mL[4,15,31]. To estimate the potential of molecules or extracts to inhibit parasite growth without toxicity, the selectivity index (SI) was introduced. Low SI indicates that the antiplasmodial activity is probably due to cytotoxicity rather than activity against the parasite themselves. In contrast, high SI should offer the potential of safer therapy[31]. We decided to define 4 as minimal SI value to validate a safe antimalarial use, whereas, SI greater than 10 and IC_{50} values under 10 µg/mL should be promising sources of antimalarial molecules.

We observed some activity against *P. falciparum* in a high percentage of tested species; the antipyretic group shows the highest percentage of inactive extracts. In the case of malaria, plants may function either by acting directly on the parasite and relieving the symptoms, i.e., fever, or by acting via a specific mechanism[4]. Obviously, *in vitro* study presumes a direct action on the parasite, and the antipyretic use could be more properly evaluated *in vivo*.

Although antimalarial activity has been detected in some Poaceae species[23,31] this is the first report for *B. vulgaris*. Phytochemical analysis of the extract suggests the presence of alkaloids, triterpenoids, flavonoids and lactonic compounds, chemical classes with widely demonstrated effective antimalarial activity[4].

In Cuba, as in other Latin American countries, *P. granatum* is used as antihelminthic and against intestinal disorders[6,27]. For this specie, amoebicidal[6], antibacterial[17], antifungal[21] and antileishmanial[12] activities have been reported. Antimalarial action has been studied using fruit rind extracts[9,26]. The activity was associated to the fraction enriched with tannins[9]. Ellagic acid (EA)[9], gallagic acid[26], EA glycoside[9] and ellagitannins[9,26] were considered to be responsible for activity. The presence of phenolic compounds in our extract was also demonstrated.

Phytochemical screening of our most potent antimalarial extracts revealed a positive reaction for lactonic compounds, whereas, TLC shows blue fluorescence after UV light at 366 nm exposure, it could suggest the presence of coumarins in both extracts[16]. Coumarins, have been isolated as active principle of plants used as antimalarials in Latin America[2,14], Africa[22] and Asian folk medicine[34].

In conclusion, the antimalarial use of *P. hysterophorus* and *B. vulgaris* was validated and extracts of *P. granatum* and *B. vulgaris* were selected for further follow-up because of their strong antimalarial activity and the presence of chemical classes with proved efficacy against *P. falciparum*.

RESUMEN

Actividad antimalárica *in vitro* y citotoxicidad de algunas plantas medicinales Cubanas seleccionadas

Las plantas terrestres han demostrado ser fuentes de compuestos antimaláricos. En Cuba, el conocimiento sobre el potencial antimalárico de las plantas medicinales es escaso. Por esta razón, evaluamos la actividad antimalárica de 14 especies de plantas usadas en Cuba como antimaláricas, antipiréticas y/o antiparasitarias. Se prepararon extractos hidroalcohólicos y se probaron *in vitro* frente a la cepa Ghana de *Plasmodium falciparum* para la actividad antimalárica y frente a la línea celular humana MRC-5 para determinar citotoxicidad. La multiplicación de los parásitos se determinó microscópicamente mediante el conteo directo de los parásitos teñidos con Giemsa. Un ensayo colorimétrico se utilizó para cuantificar la citotoxicidad. Nueve extractos mostraron valores de CI50 frente a *Plasmodium falciparum* por debajo de 100 µg/mL; cuatro extractos se clasificaron como marginalmente activos (IS < 4), uno parcialmente activo (*Parthenium hysterophorus*) exhibiendo IS de 6.2 y dos activos (*Bambusa vulgaris* y *Punica granatum*) mostrando IS>10. *B. vulgaris,* mostró la acción más potente y específica (CI_{50} = 4,7 µg/mL, IS = 28,9). La caracterización fitoquímica de los extractos más activos; confirmó la presencia de triterpenoides en *B. vulgaris* y de compuestos polares con grupos fenólicos libres y metabolitos fluorescentes en ambos extractos como fitocompuestos principales mediante cromatografía en capa delgada. En conclusión, se validó el uso antimalárico de *B. vulgaris* y *P. hysterophorus.* Los extractos de *B. vulgaris* y *P. granatum* se seleccionaron para seguimiento por su potente actividad antimalárica.

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