IN VITRO ANTIPROTOZOAL ACTIVITY OF FIVE PLANT EXTRACTS FROM ALBAHA REGION

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ABSTRACT
Five methanol extracts obtained from five plant species selected according ethnobotanical data were screened for their antiprotozoal activity against Plasmodium falciparum, Leishmania infantum, Trypanosoma cruzi and Trypanosoma brucei. Cytotoxicity was evaluated on MRC-5 cells. Withania somnifera methanol extract showed low activity against P. falciparum (IC50 17.7 μg ml⁻¹), but with non-selective antimalarial activity. Most of the extracts tested against L. infantum exhibited low activity with IC50 ranging from 20 to 32 μg ml⁻¹, except W. somnifera which showed a remarkable but non-selective leishmanicidal activity (IC50 of 2.0 μg ml⁻¹). W. somnifera methanol extract also exhibited a marked activity against T. cruzi (IC50 0.6 μg ml⁻¹, SI=3.5).

KEYWORDS: In vitro, Plasmodium, Leishmania, Trypanosoma; cytotoxicity; plant extracts.

1. INTRODUCTION
Parasitic diseases produced by Trypanosoma, Leishmania and Plasmodium species are some of the major causes for high mortality and morbidity in developing countries. According to
the latest estimates, released in 2014 by WHO, there were about 198 million cases of malaria in 2013 and an estimated 584,000 deaths. Human African trypanosomiasis (HAT) is a major disease with half a million people in sub-Saharan Africa at risk of developing the disease. Leishmaniasis is distributed worldwide, especially in tropical and subtropical areas and more than 12 million people are currently infected. About 20,000 to 30,000 deaths occur annually. Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis and causes skin lesions, mainly ulcers. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East, and Central Asia. The chemotherapy of these tropical protozoal diseases has been undermined by the fact that the presently used drugs are relatively toxic or become ineffective by the spreading of drug resistance. Hence, there is a continuous need to explore new therapeutic agents, and ethnobotanical knowledge may be useful to open new ways in this field. Considerable attention has been given to search for new anti-infective and in particular antiprotozoal drugs. Several antiprotozoals in the market today were directly obtained from plants, such as quinine or structurally modified plant compound artemisinin. Some reports were already published about the antiprotozoal activity potential of Saudi medicinal plants. The Albahe region in Southern Saudi Arabia is very rich in flora with a great diversity of native and naturalized medicinal plants that have yet been very poorly explored. For this reason, this region was chosen for collecting the specimens. To the best of our knowledge, this study represents the first report on antiprotozoal activity for some of the tested methanol extracts, obtained from five plant species growing in Albahe region, KSA, and used in traditional medicine for the treatment of infectious diseases.

MATERIAL AND METHODS

Plant Material

The plant material was collected between March – April 2014 from different locations in Albahe town and outskirts of Albahe (Table 1). The plants were taxonomically identified at the Faculty of Science, Department of Botany, Aden University, Yemen. Voucher specimens of the plant material are deposited at the Pharmacognosy Department, Faculty of Clinical Pharmacy, Albahe University, KSA.

Preparation of Extracts

The harvested plant parts were air-dried at ambient temperature and powdered with a blender. The powdered plant material (40 g) was extracted in methanol (powdered material:
solvent = (1:10). The extraction was done at room temperature (29-32 °C) with constant shaking of the extraction set-up. Thereafter, the extracts were filtered, and evaporated to dryness in vacuo at 40 °C to obtain the dried extracts. The yields of each dried extract were calculated in %. The resulting dried crude extracts were stored at 4 °C.

Antiprotozoal activity

Standard Drugs

For the different tests, appropriate reference drugs were used as positive control: tamoxifen for MRC-5, chloroquine for P. falciparum, miltefosine for L. infantum, benznidazole for T. cruzi and suramin for T. brucei. All reference drugs were either obtained from the fine chemical supplier Sigma-Aldrich (tamoxifen, suramin) or from WHO-TDR (chloroquine, miltefosine, benznidazole).

Biological Assays

The integrated panel of microbial screens and standard screening methodologies were adopted as previously described. All assays were performed in triplicate at the Laboratory of Microbiology, Parasitology and Hygiene at the University of Antwerp (Belgium). Plant extracts were tested at 5 concentrations (64, 16, 4, 1 and 0.25 μg/mL) to establish a full dose-titration and determination of the IC50 (inhibitory concentration 50%). The final in-test concentration of DMSO did not exceed 0.5%. The selectivity antiprotozoal potential was assessed by simultaneous evaluation of cytotoxicity on a lung fibroblast normal cell line (MRC-5). The criterion for activity was an IC50<10 μg/mL and a selectivity index (SI) of >4.

Antiplasmodial Activity

Chloroquine-resistant P. falciparum K 1-strain was cultured in human erythrocytes O+ at 37 °C under a low oxygen atmosphere (3% O2, 4% CO2, and 93% N2) in RPMI-1640, supplemented with 10% human serum. Infected human red blood cells (200 μL, 1% parasitaemia, 2% hematocrit) were added to each well and incubated for 72 h. After incubation, test plates were frozen at −20 °C. Parasite multiplication was measured using the Malstat assay, a colorimetric method based on the reduction of 3-acetylpyridine adenine dinucleotide (APAD) by parasite-specific lactate-dehydrogenase (pLDH).
Antileishmanial Activity

L. infantum MHOM/MA(BE)/67 amastigotes were collected from the spleen of an infected donor hamster and used to infect primary peritoneal mouse macrophages. To determine in-vitro antileishmanial activity, $3 \times 10^4$ macrophages were seeded in each well of a 96-well plate. After 2 days outgrowth, $5 \times 10^5$ amastigotes/well, were added and incubated for 2 h at 37 °C.

Pre-diluted plant extracts were subsequently added and the plates were further incubated for 5 days at 37 °C and 5% CO$_2$. Parasite burdens (mean number of amastigotes/macrophage) were microscopically assessed after Giemsa staining on 500 cells, and expressed as a percentage of the blank controls without plant extract.

Antitrypanosomal Activity

Trypanosoma brucei Squib-427 strain (suramin-sensitive) was cultured at 37 °C and 5% CO$_2$ in Hirumi-9 medium$^{[16]}$, supplemented with 10% fetal calf serum (FCS). About $1.5 \times 10^4$ trypomastigotes/well were added to each well and parasite growth was assessed after 72 h at 37 °C by adding resazurin$^{[17]}$. For Chagas disease, T. cruzi Tulahuen CL2 (benznidazole-sensitive) strain was maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM-L-glutamine, 16.5 mM sodium hydrogen carbonate and 5% FCS. In the assay, $4 \times 10^3$ MRC-5 cells and $4 \times 10^4$ parasites were added to each well and after incubation at 37 °C for 7 days, parasite growth was assessed by adding the β-galactosidase substrate chlorophenol red β-D-galactopyranoside$^{[18]}$. The color reaction was read at 540 nm after 4 h and absorbance values were expressed as a percentage of the blank controls.

Cytotoxicity against MRC-5 Cells

MRC-5 SV2 cells were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% FCS. For the assay, $10^4$ MRC-5 cells/well were seeded onto the test plates containing the pre-diluted sample and incubated at 37 °C and 5% CO$_2$ for 72 h. Cell viability was assessed fluorimetrically after 4 h of addition of resazurin. Fluorescence was measured (excitation 550 nm, emission 590 nm) and the results were expressed as % reduction in cell viability compared to control and CC$_{50}$ was calculated using Origin program.
RESULTS AND DISCUSSION

Five methanol extracts of five plant species belonging to five families collected from Alba ha region (Table 1) were investigated for their antiprotozoal and cytotoxic activity against P. falciparum, L. infantum, T. cruzi, T. brucei and MRC-5 cells, respectively (Table 2). The listed IC50 values are the means of three determinations. Particularly for natural products, evaluation of selectivity is essential and represented as the selectivity index (SI): ratio (CC50 MRC-5 cells/IC50 protozoa). An acceptable criterion for activity was an IC50<10 μg ml\(^{-1}\) and a selectivity index (SI) of >4. Indeed, in evaluating the selectivity against protozoan versus mammalian cells, it is noted that the antiprotozoal activities of most active extracts may be due to their cytotoxicity to mammalian cells, as is demonstrated by the moderate, low or very low SI-values between <1 and 3.5.

Among the extracts tested, W. somnifera and L. dentata extracts displayed a low non-selective antiplasmodial activity with IC50 values of 17.7 and 23.4 μg ml\(^{-1}\) respectively. Most of the plant extracts showed non-selective low antileishmanial activity with IC50 values ranging from 20.3 to 32.0 μg ml\(^{-1}\), except W. somnifera which showed a non-selective antileishmanial activity with an IC50 of 2.0 μg ml\(^{-1}\). The latter also showed the highest activity against T. cruzi with an IC50 of 0.6 μg ml\(^{-1}\) and moderate SI of 3.5, followed by extract of L. dentata with an IC50 of 7.1 μg ml\(^{-1}\) and SI of 3.4. Similar inhibitory activity of L. dentata extract was also recorded against T. brucei (IC50: 8.1 μg ml\(^{-1}\), SI: 3).

In our study, L. dentata possessed moderate antitrypanosomal activity against both T. cruzi and T. brucei with moderate SI of 3.4 and 3.0. This result partially agrees with literature data on L. dentata published by^\(^{10,11,12}\) who reported antiprotozoal activity of the methanol extract of L. dentata with better selectivity (in comparison to our results. This activity is probably linked to the presence of linalool-rich essential oil that has been reported in L. dentata\(^{7,19}\), and the difference in the antiprotozoal activity may be due to variation in the time and area of plant collection and other ecological factors which exert great influence on the quality and quantity of the active constituents, in particular of essential oil components.\(^{17}\)

In the present study, W. somnifera (IC50 of 2.0 μg ml\(^{-1}\)) exhibited more potent antileishmanial activity than those reported previously and collected from Oman (IC50: 22.10 ± 1.62, SI: 0.89)\(^{20}\) and India (IC50: 63 ± 6).\(^{21}\) It was suggested that withaferin A, the inhibitor of protein kinase C, is responsible for the high anti-leishmanial activity.\(^{22,23}\) E. helioscopia
has been subjected to abundant phytochemical and biological investigations, but no antiprotozoal activity has been found in the literature. Several compounds have been isolated from E. helioscopia, such as diterpenoids, triterpenoids, that may be responsible for the antitrypanosomal activity.\textsuperscript{[24]} Several triterpenoids isolated from E. resinifera and E. officinarum were reported to have antitrypanosomal activity.\textsuperscript{[25]}

**Table: 1. Selected plants studied: ethnobotanical information and characteristics.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Plant Family (Voucher specimen No.)</th>
<th>Part tested\textsuperscript{a} (%) (Yield in %)</th>
<th>Local name</th>
<th>Traditional uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euphorbia helioscopia L.L.</td>
<td>Euphorbiaceae (CP-051)</td>
<td>AP (4.2)</td>
<td>Al-dehin</td>
<td>Antiseptic\textsuperscript{2}</td>
</tr>
<tr>
<td>Lavandula dentata L.</td>
<td>Lamiaceae (CP-041)</td>
<td>AP (2.9)</td>
<td>Al-shiah</td>
<td>As antispasmodic, antiseptic\textsuperscript{1} when the leaves chewed\textsuperscript{1}</td>
</tr>
<tr>
<td>Rumex nervosus Vahl</td>
<td>Polygonaceae (CP-001)</td>
<td>L (4.8)</td>
<td>Ah-athrub</td>
<td>Paste of leaves used for treating wounds\textsuperscript{2}</td>
</tr>
<tr>
<td>Ruta chalepensis L.</td>
<td>Rutaceae (CP-121)</td>
<td>L (5.2)</td>
<td>Al-shathab</td>
<td>Antimicrobial\textsuperscript{2}</td>
</tr>
<tr>
<td>Withania somnifera L.</td>
<td>Solanaceae (CP-011)</td>
<td>F (4.6)</td>
<td>Aloeb</td>
<td>Chronic dermatitis\textsuperscript{2}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} AP, aerial parts; F: fruits; L: leaves. 1 information obtained from reference [13]. 2Interviewing with local people.

**Table: 2 Antiprotozoal activity of the extracts of the investigated plants and their cytotoxicity against MRC-5 cell line.**

<table>
<thead>
<tr>
<th>Plant species (part tested)\textsuperscript{a}</th>
<th>P. falciparum</th>
<th>L. infantum</th>
<th>T. cruzi</th>
<th>T. brucei</th>
<th>CC\textsubscript{50} \textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\textsubscript{50} \textsuperscript{b}</td>
<td>SI\textsuperscript{c}</td>
<td>IC\textsubscript{50}</td>
<td>SI</td>
<td>IC\textsubscript{50}</td>
</tr>
<tr>
<td>E. helioscopia (AP)</td>
<td>&gt;64</td>
<td>-</td>
<td>27.3</td>
<td>-</td>
<td>7.2</td>
</tr>
<tr>
<td>R. nervosa (L)</td>
<td>&gt;64</td>
<td>-</td>
<td>&gt;64</td>
<td>-</td>
<td>32.6</td>
</tr>
<tr>
<td>L. dentata (AP)</td>
<td>23.4</td>
<td>&gt;1</td>
<td>20.3</td>
<td>&gt;1</td>
<td>7.1</td>
</tr>
<tr>
<td>R. chalepensis (L)</td>
<td>&gt;64</td>
<td>-</td>
<td>32.0</td>
<td>-</td>
<td>21.3</td>
</tr>
<tr>
<td>W. somnifera (F)</td>
<td>17.7</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.3 ± 0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>-</td>
<td>3.3 ± 0.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benznidazole</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Suramin</td>
<td>-</td>
<td>-</td>
<td>0.03 ± 0.02</td>
<td>-</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Tamofoxen</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.0 ± 2.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} AP, aerial parts; F: fruits; L: leaves
\textsuperscript{b} IC\textsubscript{50}: Concentration of extract causing 50\% growth inhibition.
SI: Selectivity index. —: Not done

CC: Cytotoxic concentration of the extracts causing death to 50% of viable cells.

CONCLUSION
The in vitro antiprotozoal activity against P. falciparum, L. infantum, T. brucei and T. cruzi is reported for the first time for E. helioscopia and R. chalensis, which all have been used traditionally for treating infectious diseases. The methanol extract of W. somnifera showed the best activity against T. cruzi and L. infantum. W. somnifera extract was selected for further bioactivity-guided fractionation in order to isolate the active pure compound(s).

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