IN VITRO ANTIMALARIAL ACTIVITY OF EXTRACTS OF THREE PLANTS USED IN THE TRADITIONAL MEDICINE OF INDIA

G. PRAVEEN BHAT AND NAMITA SUROLIA

Abstract. In an attempt to search for new antimalarial drugs, we studied plants used by traditional healers of southwest India to treat malaria. Aqueous and organic solvent extracts obtained from specific parts of the plants Swertia chirata, Carica papaya, and Citrus sinensis were tested on malaria strain Plasmodium falciparum FCK 2 in vitro. The temperatures of extraction were the same as that used by the traditional healers in their plant preparations. Visual evaluation of the antimalarial activity of the plant extracts on thin blood smears was followed by quantification of the activity by use of \[^{35}\text{S}\]-methionine incorporation into parasite proteins to determine the value that inhibits 50% (IC\(_{50}\)). Among the 3 plants tested, 2 had significant inhibitory effect on \(P. falciparum\) in vitro.

INTRODUCTION

Malaria continues to cause morbidity and mortality on a large scale in tropical countries. The alarming rate at which the parasite, particularly \(P. falciparum\), has developed resistance to currently used antimalarial drugs makes it imperative to search for newer, more effective therapeutic agents. The antimalarial potential of compounds derived from plants is proven by examples such as quinine, obtained from \(Cinchona\) species, and artemisinin, obtained from \(Artemisia annua\). The selection of plants to be screened for antimalarial activity is done on the basis of traditional reputation of particular plants for efficacy in the treatment of malaria.

Here, we studied 3 plants used by traditional healers of the Western Ghats (southwest India, where certain regions are endemic for malaria) to treat “recurrent fever” and evaluated them in vitro for antimalarial activity. For each plant, aqueous and organic solvent extracts were prepared either at room temperature or at higher temperatures depending on the plant’s mode of use by traditional healers. \(P. falciparum\) growth inhibition was studied first by microscopic examination of thin blood smears of synchronized culture to determine morphological changes of parasites in the presence of extracts. The value that inhibits 50% (IC\(_{50}\)) of each extract was determined by the incorporation of \[^{35}\text{S}\]-methionine in parasite proteins.\(^3\)

MATERIALS AND METHODS

Plant and extracts. Three plants were tested (Table 1). The parts of the plants tested were air-dried (\(S\. chirata\)) or sun-dried (\(C\. papaya\) and \(C\. sinensis\)) and pulverized into powder. The powdered rind and pulp of unripe fruits of \(C\. papaya\) and powdered rind of ripe fruits of \(C\. sinensis\) (5–7 g each) were separately stirred overnight at room temperature in petroleum ether, methanol, and water (50–75 mL each). The aqueous extracts were filtered sterilized by passing through cellulose acetate membranes (0.45 \(\mu\)m; Millipore Corp., Bedford, MA). The methanolic extract of \(S\. chirata\) leaves and stem (pulverized together) was obtained as above, and the water extract was prepared by boiling the powder (5–7 g) in water (100 mL) for 5 minutes. In addition, an ethanol extract also was made of \(S\. chirata\) by Soxhlet extraction with ethanol for 30 hr. The solvent extracts were evaporated to dryness, and the residue was stored in screw-capped vials at room temperature until tested.

Parasite strain and in vitro culture. FCK 2, a local strain of \(P. falciparum\) from Karnataka state, India (IC\(_{50}\) for chloroquine, 10.7 ng/mL), was cultured continuously according to the candle-jar method of Trager and Jensen\(^2\) in vitro in human red blood cells (blood type O\(^+\)) with 5% hematocrit in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 25 mM HEPES (Sigma), 0.2% sodium bicarbonate (Sigma), and 10% human O\(^+\) serum.

Estimation of \(P. falciparum\) growth inhibition. Stock solutions of extracts were prepared by dissolving known quantities of the dried ethanol and petroleum ether extracts in ethanol. The dried methanolic extracts were dissolved in methanol, dimethyl sulfoxide, and distilled water (1:1:3 by volume).\(^3\) The stock solutions were further diluted with RPMI 1640 to achieve the required concentrations before being tested in culture. Known quantities of the water extracts were diluted directly in RPMI 1640.

Serial double dilutions of the extracts were made in quadruplicate in 96-well microtiter plates (Corning Glassworks, Corning, NY). Each well contained 20 \(\mu\)L of the diluted extract and 200 \(\mu\)L of parasitized red blood cells (2% hematocrit) in RPMI 1640 (1–2% rings, growth rate > 4-fold in 48 hr). The extract concentrations tested ranged 0.5–1,000 \(\mu\)g/mL. The cultures, before testing, were synchronized by treatment with 5% \(d\)-sorbitol\(^4\) (Sigma). Solvent controls containing the same concentration of the solvent as that used in the test wells (\(\leq 0.05\%\)) were incorporated, and parasitic growth was not found to be affected at this solvent concentration. The titer plates were incubated at 37°C in the candle jar.

After 24, 48, and 72 hr, thin smears were made from duplicate wells, fixed in methanol, and stained with Giemsa stain, then observed through a microscope to study the morphology of parasites at various concentrations of extracts. Fresh medium with extracts were added at the end of 24 and 48 hr. The IC\(_{50}\) value of each of the extracts was estimated by measuring the parasite incorporation of \[^{35}\text{S}\]-methionine in the presence of the respective extract, as follows. A fresh set of wells was made in microtiter plates with the same volume of culture and concentration of the extracts as previously used. Ten microcuries of \[^{35}\text{S}\]-methionine (NEN Life Science Products; specific activity, 1,175.0 Ci/mmol) was added to each well at the end of 72 hr of incubation with
ANTIMALARIAL ACTIVITY OF INDIAN PLANTS

Table 1

<table>
<thead>
<tr>
<th>Plant Aspect</th>
<th>Part used</th>
<th>Temperature of extraction</th>
<th>Solvents used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carica papaya</td>
<td>Rind and pulp of unripe fruit</td>
<td>25–30°C</td>
<td>Methanol, petroleum ether, water</td>
</tr>
<tr>
<td>Citrus sinensis</td>
<td>Rind of ripe fruit</td>
<td>25–30°C</td>
<td>Methanol, petroleum ether, water</td>
</tr>
<tr>
<td>Swertia chirata</td>
<td>Stem, leaves</td>
<td>100°C (water), 70°C (ethanol), 25–30°C (methanol, petroleum ether)</td>
<td>Methanol, petroleum ether, water, ethanol</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Extract</th>
<th>Time 24 hr</th>
<th>Time 48 hr</th>
<th>Time 72 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (1–2% of rings at start)</td>
<td>Trophozoites (1–2%)</td>
<td>Rings (4–8%); schizonts (0.5–1%)</td>
<td>Young and mature trophozoites (8–10%); rings (2–4%)</td>
</tr>
<tr>
<td>Swertia chirata</td>
<td>Ethanol extract, IC_{50} = 21.69 μg/mL</td>
<td>Normal trophozoites (0.5–1%)</td>
<td>Rings (2–4%)</td>
</tr>
<tr>
<td></td>
<td>Other trophozoites (1.5–2%) are smaller in size than those in control</td>
<td>Abnormal trophozoites (1.5–2%) similar to those seen at 24 hr</td>
<td>Abnormal trophozoites (2–4%)</td>
</tr>
<tr>
<td>Carica papaya</td>
<td>Petroleum ether extract of pulp, IC_{50} = 18.09 μg/mL</td>
<td>Rings (0.5–1%)</td>
<td>Rings (1–2%)</td>
</tr>
<tr>
<td></td>
<td>Normal trophozoites (0.5)</td>
<td>Schizonts (0.5%)</td>
<td>Normal trophozoites (2–4%)</td>
</tr>
<tr>
<td></td>
<td>Other trophozoites (0.5–1%) are smaller than in control</td>
<td>Abnormal trophozoites (0.5–1%) similar to those seen at 24 hr</td>
<td>Abnormal trophozoites (1–2%)</td>
</tr>
<tr>
<td>Petroleum ether extract of rind, IC_{50} = 15.19 μg/mL</td>
<td>Normal trophozoites (0.5–1%)</td>
<td>Rings (2–4%)</td>
<td>Rings (1–2%)</td>
</tr>
<tr>
<td></td>
<td>Other trophozoites (1.5–2%) are smaller in size than in control and show vacuolation</td>
<td>Abnormal trophozoites (1.5–2%) similar to those seen at 24 hr</td>
<td>Abnormal trophozoites (1.5–2%)</td>
</tr>
</tbody>
</table>

*IC_{50} = value that inhibits 50%.

The extracts and further incubated for 2 hr. Controls, consisting of parasitized red blood cells and 10 μCi of [³⁵S]-methionine, were also incorporated with the above set of wells.

After the incubation period, the parasitized red blood cells were collected in 1.5-mL Eppendorf tubes, washed with 0.01 M phosphate-buffered saline (4 times), lysed with 4 volumes of ice-cold lysis solution (100 mM KCl, 7mM Mg(ΟAc)₂), 380 mM sucrose, 6.5 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.4, 0.14% (vol/vol) Triton X-100, sonicated, and spotted onto Whatman 3 discs pretreated with 10% trichloroacetic acid (TCA). The spotted discs were air dried, treated with hot TCA, TCA with hydrogen peroxide (3% v/v), cold TCA (10% throughout), absolute alcohol and diethyl ether (2:1 v/v), and finally with diethyl ether, each for 7 min, and then dried. Radioactivity was measured as counts per minute (cpm) in a liquid scintillation counter (Model 1409; Wallac, Gaithersburg, MD). The IC_{50} values of the extracts were calculated by use of a modification of the method of Watkins and others. The values from the controls provided a midpoint cpm value where the parasite growth would be at 50%. The regression function log cpm = [a + (b × log extract concentration)] was calculated by use of one set of data points above and one set of data points below that cpm midpoint. The IC_{50} values were estimated by interpolation. The IC_{50} values were retested twice by [³⁵S]-methionine incorporation to improve the accuracy of the estimates.

RESULTS

The study of parasite morphology in the thin blood smears pointed to the fact that different solvent extracts of any particular plant had different antimalarial activity. None of the water extracts produced change in morphology or number of parasites, even at high concentrations (>1,000 μg/mL). Table 2 shows the parasite morphology and parasitemia at the concentrations giving IC_{50} values (as determined later by [³⁵S]-methionine incorporation). The antimalarial activity of the extracts was discernible as a change in the morphology and number of the parasites.
Each extract produced discrete morphological changes (Figure 1). In the presence of the ethanolic extract of *S. chirata*, the trophozoites were smaller in size compared with controls. (Figure 1c, d). These abnormal trophozoites failed to develop into schizonts. The petroleum ether extract of *C. papaya* pulp caused a delay in the development of the ring stage as seen at the end of 24 hr of incubation with the extract (Table 2). In rings that had developed into the trophozoite stage, we saw abnormal trophozoites with smaller size. The petroleum ether extract of *C. papaya* rind produced vacuolation and a reduced size of the trophozoites (Figure 1e, f).

The specific changes in morphology produced by particular extracts may hint at the different modes of action of the putative active principles in the extracts. The parasitemia also decreased with increasing concentrations of the extracts, reflecting an inhibitory activity on the parasite replication.

The results obtained with the [35S]-methionine incorporation are summarized in Table 3. The petroleum ether extract of the rind of *C. papaya* had the highest antimalarial activity of all the extracts we tested (IC50 = 15.19 μg/mL). This may be indicative of a significant potential for isolating purer compounds with much higher antimalarial activity from this fraction; crude plant extracts with much lower activity have yielded purer compounds with potent antimalarial activity. None of the water extracts showed antimalarial activity, thus pointing to the hydrophobic nature of the putative active principles.

### DISCUSSION

In the traditional Indian pharmacopoeia, plants are often used by healers to treat recurrent fever. Three of these plants, used in the Western Ghats (southwest India), were tested for their antimalarial activity in vitro. Both aqueous and organic solvent extracts were made at temperatures that did not exceed the ones used by the native healers in their plant preparations to prevent denaturation of the active principles by heat. Although the parasite morphology was studied every 24 hr until 72 hr of incubation with the extracts, the IC50 values were determined at 72 hr of incubation, to account for potential cumulative effects on essential biochemical pathways of the parasite.

We used [35S]-methionine incorporation into parasites to determine the IC50 values, instead of the conventionally used [3H]-hypoxanthine, for the following reasons. Protein synthesis in the parasite begins soon after merozoite invasion, reaches its peak in ~24 hr, and persists at about this level for another 24 hr, thus spanning the entire parasite erythrocytic life cycle of 48 hr. However, DNA synthesis starts only ~28–31 hr after merozoite invasion, persists at its peak for 2–4 hr, declines sharply, and falls to levels undetectable by radiolabeled precursors. Thus, the antiparasitic activity of the extracts on any of the erythrocytic stages (ring, trophozoite, or schizont) would be reflected in the [35S]-methionine incorporation into parasite proteins, whereas [3H]-hypoxanthine incorporation would reflect such activity predominantly on late trophozoites only. Moreover, inhibitory activities of the extracts on nucleic acid synthesis, if present, would eventually be manifested as an inhibition of parasitic protein synthesis.

Extracts of *S. chirata* and a few other plants in combination are marketed commercially in the traditional Indian system of medicine (known as Ayurveda) as an antimalarial. *Swertia chirata* has also been shown to lower blood sugar and to possess antiulcerogenic and antihepatotoxic activities.

The rind of raw *C. papaya* fruit showed the highest antimalarial activity of the plant extracts tested. There may be significant commercial potential in extracting the active element from this plant, which grows abundantly throughout the tropics and the rind of which is discarded as waste. The *C. papaya* fruit has also been studied for its antifungal, antibacterial, and antihelmintic activity.

Currently, no data are available regarding the clinical use of these traditional drugs, nor to our knowledge are there any reports of double-blind clinical trials having been carried out to assess their antimalarial efficacy. Even though no toxicity test has yet been performed, the use of these plants by native healers suggests that no highly toxic compounds are present in the extracts. Efforts are under way to isolate the putative active principles and characterize their toxicities.
REFERENCES