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New evidences of antimalarial activity of *Bidens pilosa* roots extract correlated with polyacetylene and flavonoids

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Abstract

Bidens pilosa is among the several plants used in Brazil to treat malaria. It was demonstrated that crude extracts from roots prepared with 80% ethanol by percolation are active in vitro against *Plasmodium falciparum* and the activity is correlated with the presence of polyacetylene and flavonoids. This extract was submitted to column chromatography with ether and ether methanol (1:1) and two fractions, enriched in polyacetylene and flavonoids, respectively, were obtained. The extract and the fractions were assessed by HPLC/DAD analysis and antimalarial tests in vivo. Ethanol extract showed by HPLC the presence of several peaks for polyacetylene and flavonoids, compounds corresponding to quercetin-3,3′-dimethoxy-7-0-rhamnoglucopyranose and the acetylene 1-phenyl-1,3-diyn-5-en-7-ol-acetate, previously identified in this extract. The peaks for flavonoids were absent in ether fraction and those ones for polyacetylene in ether:methanol. In in vivo tests, ethanol extract caused 36% of reduction of parasitaemia at fifth day, and 29% at seventh day. Ether:methanol fraction caused 38% of reduction at fifth day but was inactive at day 7. The survival of the animals treated with 80% ethanol extract was higher than in the fractions. The results showed that the in vivo activity of ethanol extract depends on the presence of polyacetylene and flavonoids.

Keywords: Antimalarial; *Bidens pilosa*; Polyacetylene; Flavonoids

1. Introduction

Since the 70s, the World Health Organization has recognised the importance of traditional medicine as an affordable source of health care, including treatment of tropical diseases. Sometimes traditional formulas represent the only alternative of treatment, especially for the world's poorest patients. Recently, the Research Initiative on Traditional Antimalarial Methods (RITAM) was created to encourage the use of validated, low-cost traditional antimalarial preparations to treat fever and malaria. Research priorities have included the assessment of efficacy and safety of new herbal antimalarials and standardisation of extracts (Willcox and Bodeker, 2000).

Bidens pilosa L. (Asteraceae) is among the several plants used in Brazil to treat fevers and malaria (Brandão et al., 1992; Oliveira et al., 2003). Experimental evidences have shown that crude extracts from roots, prepared with 80% ethanol by percolation, show in vitro activity against *Plasmodium falciparum*. Chemical study of the ethanol crude extract led to identification of two major groups of chemical constituents, the polyacetylenes and the flavonoids, the most likely active compounds (Brandão et al., 1997). In the present study, a correlation of in vivo antimalarial activity with the presence of polyacetylene and flavonoids was established using High Performance Liquid Chromatography coupled with Diode Array Detector (HPLC/DAD).

2. Materials and methods

2.1. Preparation of BR80 and its fractions

Samples of *Bidens pilosa* were collected in Betim (MG) in June 2001, and identified by J.R. Stehmann (BHCB 64919). The dried and powdered roots (100 g) were extracted by percolation with 80% ethanol, at room temperature, and the solvent was evaporated to dryness at maximum 45 °C. The ethanol crude extract was further submitted to

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flash chromatography on silica gel and eluted with ether and then with a mixture of 1:1 ether/methanol. Two fractions enriched in polyacetylene and flavonoids were obtained.

2.2. HPLC/DAD analysis

The assays were performed using a HPLC Hewlett Packard model 1100, with a DAD detector. Column: RP-18 Chromolith 100 mm × 4.6 mm (Cat. 1.02129.0001/Merck). The eluent was acetonitrile:water (EM Science, AX0142-1), with gradient of 10–90% of acetonitrile in 30 min. Detection: UV spectra at 254 nm. Injection volume: 20 μl. One millilitre of each sample was evaporated to dryness at less than 50 °C, dissolved in 20% acetonitrile, filtered (Minisart RC-15, Sartorius, 0.45 μm) and injected directly in HPLC. The identification of the compounds was carried out by analysis of retention time (Rt) versus the characteristic bands for polyacetylene and flavonoids in UV spectra (Brandão et al., 1997). The flavonoid quercetin-3,3′-dimethoxy-7-0-rhamnoglucopyranose, previously isolated by Brandão et al. (1998) was used as a standard and showed a Rt of 7.58 min.

2.3. Antimalarial tests

The antimalarial suppressive test described by Peters (1985) and modified by Carvalho et al. (1991) was used. Briefly, adult Swiss albino mice weighing 18–20 g were inoculated by the i.p. route with 1×10^5 *Plasmodium berghei*-infected red blood cells (strain NK-65, originally received from the New York University Medical School). The mice were randomly distributed in groups of five per cage, and treated during four consecutive days, with daily single doses of the drugs, per os. Two control groups were used in each experiment, one treated with chloroquine at a low non-curative dose (25 mg/kg), the other group was kept untreated. On the fifth and seventh days parasitaemia were determined in coded blood smears. Overall mortality was monitored daily in all groups during a period of 4 weeks following inoculation. The inhibition of parasite growth in the drug-treated groups was calculated and expressed as percentages. The extracts were considered partially active when parasitaemia was reduced by ≥30%. The studies were approved by the Ethical Committee for Using Animals at Fundação Instituto Oswaldo Cruz, FIOCRUZ (CEUA P0094-01).

2.4. Statistical analysis

The results of in vivo antimalarial tests were double-entered using the EPI Info 2000 (CDC, Atlanta GA, USA) and analysed using the Stata Software package (Stata Corporation, College Station, TX, USA). The Student's *t* test was used to compare the inhibition of parasite growth. The mice survival mortality was analysed by the Kruskal–Wallis test. The parameters that showed significant differences were subsequently incorporated in a multiple comparison analysis using the ANOVA/post hoc test statistical.

3. Results and discussion

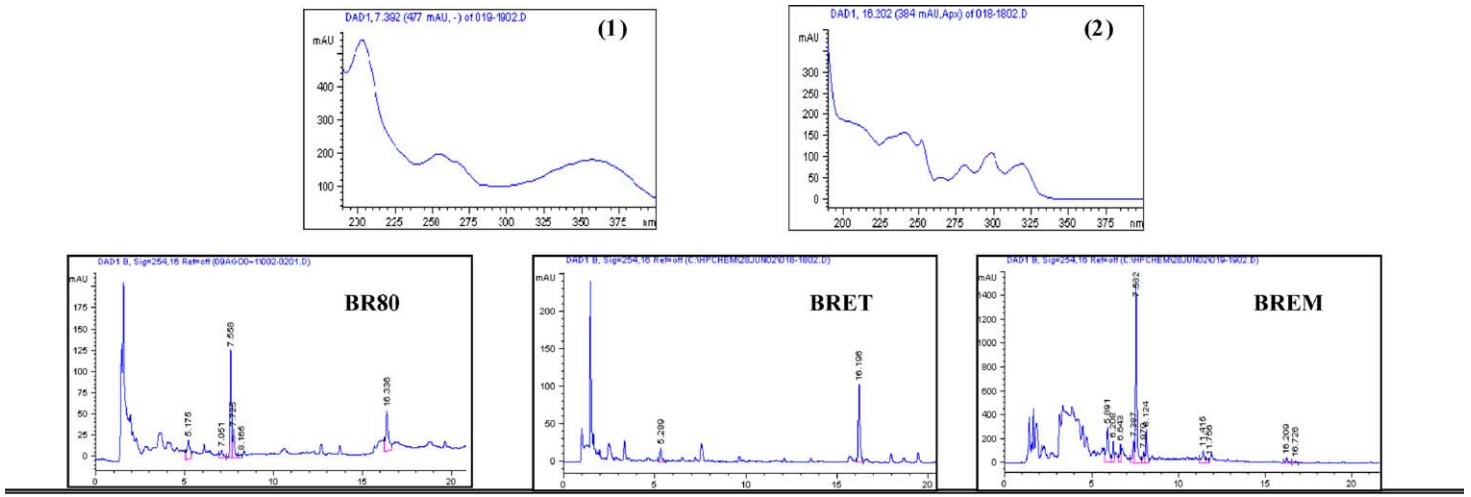
Fig. 1 shows the results obtained by HPLC analysis and in vivo antimalarial tests. The chromatograms for the ethanol crude extract (BR80) show peaks for several compounds between Rt 5.17 and 8.13 min, which were identified by their characteristic bands on UV spectra as flavonoids. A higher peak at Rt 7.58 min corresponded to the methoxylated flavonoid quercetin-3,3′-dimethoxy-7-0-rhamnoglucopyranose (**1**), previously characterised in *Bidens pilosa* roots (Brandão et al., 1998). From Rt 16.21 to 16.36 min polyacetylenes were identified by UV spectra. The peak at Rt 16.19 min was attributed to 1-phenyl-1,3-diyn-5-en-7-ol-acetate (**2**) also previously identified (Brandão et al., 1997). The chromatograms and the UV spectra of (**1**) and (**2**) are shown in Fig. 1.

The chromatograms for the ether and ether:methanol fractions are also shown in Fig. 1. They showed different profiles since the peaks for flavonoids were absent in ether fraction and those for polyacetylenes were absent in ether:methanol. The concentration of flavonoids was increased in ether:methanol and the peak for (**1**) is higher when compared to that as observed in BR80. The peak for (**2**) has also increased in ether fraction. These results demonstrate that the treatment with ether and ether:methanol was efficient to remove the flavonoids and polyacetylenes of the ethanol crude extract.

The in vivo tests showed that the ethanol extract (250 mg/kg) reduced parasitaemia at the fifth day (36% reduction, $\rho \leq 0.05$) in relation to the non-treated control mice; by seventh day reduction was 29%. Ether fraction reduced only 10% of the parasitaemia and was considered inactive. The ether:methanol fraction showed an antimalarial activity at fifth day similar to ethanol crude extract (38%, $\rho \leq 0.05$), but was inactive at the day 7 (10% reduction). These results show that the absence of polyacetylenes, and the increased concentration of flavonoids, have not contributed for increasing of the activity of ether:methanol fraction.

The mice survival was also increased by ethanol crude extract treatment. At 19th day, 20% of animals had survived in comparison with 60% of mice treated with chloroquine; all controls had died. For ether and ether:methanol fractions, the mortality was total at 13th and 15th days, respectively, similar for not treated mice (Fig. 1). These results demonstrated that whole extract of *Bidens pilosa* roots, containing both polyacetylene and flavonoids, is more effective to treat malaria (Fig. 1).

Polyacetylenes are hydrocarbons that strongly absorbs long-wave UV light and their biological activity is increased upon exposure to light (photoactivation). Extracts of *Bidens* and other plants containing polyacetylene inhibit



	Chloroquine	Ethanol crude extract/ BR80	Ether fraction/ BRET	Ether: methanol fraction/ BREM	Control non-treated
% Inhibiton of parasitaemia at days					
5 th	100**	36*	10	38*	--
7 th	98**	29	0	10	--
% Mice Survival at days					
7th	100	100	100	100	80
11 th	100	60	20	60	40
15th	80	40	0	0	0
19 th	60	20	0	0	0

Fig. 1. Inhibition of parasitaemia and mice survival per day after treatment with BR80, BRET, BREM (250 mg/kg) and standard antimalarial drug chloroquine (25 mg/kg), and respectives HPLC/DAD chromatograms. Significant difference in relation to control ($^{*}P \leq 0.05$; $^{**}P = 0.0001$). The peaks for quercetin-3,3′-dimethoxy-7-0-rhamnoglucopyranose (**1**) and 1-phenyl-1,3-diyn-5-en-7-ol-acetate (**2**) were observed at Rt 7.55 and 16.33 min, respectively.

various pathogenic organisms in laboratory conditions (Wat et al., 1980). The in vitro antiplasmodial activity of fractions containing polyacetylenes from *Bidens pilosa* roots has been previously demonstrated (Brandão et al., 1997). In the present study, however, a similar fraction was inactive in vivo which demonstrated the limitations of these isolated compounds as an internal remedy to treat malaria. In the ethanol crude extract, however, the antioxidative activity of flavonoids may avoid the degradation of such compounds (Lam and Thomasen, 1988; Brandão et al., 1997).

It has also been demonstrated that methoxylated aromatic compounds, like lignanes, showed moderate activity against the parasite in vitro (Kraft et al., 2002) as well as the flavonoid (**1**), previously isolated (Fig. 1; Krettli et al., 2001). Phytochemical studies of plants used as antimalarial in traditional medicine of different countries revealed the presence of methoxylated aromatic compounds (Nkunya et al., 1993; Reddy et al., 2003; Rao et al., 2002; Cimanga et al., 1995; Kittakoop et al., 2000). The methoxylated flavonoids also enhance the in vitro activity of artemisinin (Elford et al., 1987; Liu et al., 1989; Bilia et al., 2002). In the present study, the 80% ethanol extract and the fraction enriched in flavonoids (ether:methanol fraction) from *Bidens pilosa* roots proved to be active against malaria also in vivo. The whole extract, however, containing both polyacetylenes and flavonoids, was more effective.

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