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Evaluation of antiparasitic activity of hydroethanolic extracts from root, stem and leaf of *Bixa orellana L.* ON *Leishmania amazonensis* samples

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RESUMO:

Os extratos hidroetanólicos de *Bixa orellana* L. têm apresentado atividade antimicrobiana. Neste sentido e com a justificativa de obter informações sobre alternativas terapêuticas no tratamento das leishmanioses, o presente trabalho avaliou a atividade antiparasitária dos extratos hidroetanólicos de raiz, caule e folha de *B. orellana* L. em culturas de *Leishmania amazonensis* (M2269). Estes extratos hidroetanólicos foram adicionados às culturas *L. amazonensis* na fase exponencial de crescimento nas concentrações de 0,12 a 2,5 mg/mL. A avaliação foi feita após 72 h de incubação à 22º C, por exame a fresco (contagem, viabilidade e mobilidade); e por método de coloração Giemsa (presença de granulações citoplasmáticas, estrutura nuclear e do cinetoplasto), comparando com o controle. Pelo cálculo de IC50 verificou-se que o extrato da folha (0,14 mg/mL) foi o que apresentou melhor atividade, seguido pelo extrato de caule (0,21 mg/mL) e raiz (0,44 mg/mL). Na análise a fresco, observou-se mobilidade em concentrações abaixo de 1,0; de 0,75 e de 1,5 mg/mL dos extratos de raiz, de folha e de caule, respectivamente. Todos os extratos em concentrações acima de 0,25 mg/ml apresentaram uma ação antiparasitária evidenciada pela presença de granulações citoplasmáticas sobre as culturas de *L. amazonensis*.
Unitermos: *Bixa orellana L.*, extratos hidroetanólicos de raiz, caule e folha, *Leishmania amazonensis*

Avaliação da atividade antiparasitária de extratoshidroetanólicos de raiz, caule e folha de *Bixa orellana L* em amostras de promastigotas de *Leishmania amazonensis*

ABSTRACT:

Hydroethanolic extracts of *Bixa orellana L.* were shown to present antimicrobial activity. In this sense and for the purpose of obtaining data about the therapeutic alternatives in leishmaniases treatment, this study has evaluated the anti-parasitic activity of hydroethanolic extracts from root, stem and leaf of *B. orellana L.* on *Leishmania amazonensis* (M2269) samples. These hydroethanolic extracts were added to *L. amazonensis* cultures in exponential-phase of growth in concentrations ranging from 0.12 to 2.5 mg/mL. The evaluation, by smears analyses (counting, availability and mobility), and by Giemsa staining method (presence of cytoplasmic granules, nuclear structure and kinetoplast), was carried out 72 hours after the incubation at 24ºC by comparing the results obtained to the control. It was observed by the IC50 calculation that the leaf extract (0.14 mg/mL) presented the highest activity, followed by the stem extract (0.21 mg/mL) and the root extract (0.44 mg/mL). Mobility was observed in the smears analyses at concentrations lower than 1.0; 0.75 and 1.5 mg/mL for the root, leaf and stem, respectively. In concentrations higher than 0.25 mg/mL, all extracts presented antiparasitic activity, evidenced by the presence of cytoplasmic granules on *L. amazonensis* cultures.
Keywords: *Bixa orellana L.*, leaves, stem roots ethanolic extracts, *Leishmania amazonensis*

INTRODUCTION

Bixa orellana L. (*B. orellana*) is a small tree, native in tropical America. Besides its traditional culinary (Colorau®) and cosmetic use in Brazil it is also employed in popular medicine in the treatment of kidney, grastroenteric and lung disorders, as well as in burns and fevers (Teske & Trentini, 1994; Lorenzi & Matos, 2002).

Cogo et al. (2002) verified the anti-*Helicobacter pylori* activity in extracts used traditionally in the treatment of gastroenteric disorders. Huhtanen (1980) studied the fruit tincture for the treatment of *Clostridium botulinum*, determining the MIC value at 31ppm. Recently, Braga et al. (2007) reported the leishmanicidal activity of methanolic extracts of *B. orellana L* seeds and fruit on *L. amazonensis* and *L. chagasi*, however they did not research the effect of stem and leaves.

Leishmania sp. is a protozoon of the *Trypanosomatidae* family, which reproduces asexually by bipartition. It presents two fundamental forms: amastigote in the vertebrate host, and promastigote in the invertebrate host or in appropriate culture media. They are etiological agents of leishmaniasis which present chronicle evolution that split pathologically in two different types: the tegumental and the visceral forms whose manifestations depend on the parasite species as well as the immune response of the infected individual (Almeida et al., 2003; Murray et al., 2005). The leishmaniasis is prevalent on four continents and is considered to be endemic in 88 countries. In terms of distribution, 90% of all cases of visceral form occur in Bangladesh, Brazil, India, Nepal, and the Sudan; and 90% of the cutaneous form occur in Afeghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria (WHO, 2009).

The first choice treatment for all kinds of leishmaniasis prescribes pentavalent antimonial drugs. In the case of antimony therapeutic failure, the second choice drugs are Amphotericin-B and the pentamidine isothiocyanate. Despite their efficacy, these drugs present highly-toxic potential and are abortive. They may cause kidney and liver malfunctions as well as electrocardiographic alterations. Therefore, it is necessary that these drugs be administered to in-patients under close evaluation of heart, kidney and liver clinic and laboratory functions, under surveillance by a specialized team (Goto & Lindoso, 2010). Besides, in accordance with the FUNASA studies (2000), adding to the severe side-effects presented by these drugs, healing of the patients may not occur, mainly in the skin-diffused form, which usually does not respond to the traditional treatments, and present many recurrences.

Thus, new therapeutic alternatives for leishmaniasis have to be studied due to the little attention given to these diseases. In this

context, the present study has as goal to deepen the knowledge about *B. orellana L.* when evaluating antiparasitic activity of the hydroethanolic extracts from root, stem and leaf of this plant on promastigote cultures of *Leishmania amazonensis* (M2269).

MATERIAL AND METHODS

***B. orellana* preparations**: Root, stem and leaf samples of *B. orellana* L. were collected in Sitio da Lagoa, in Alfenas-MG (Latitude 21°6'36, 74" S and Longitude 45°38'3,67" W), in October, 2005 and sent to Universidade Federal de Lavras, where they were botanically identified and deposited in the herbarium under number 15.817. According to the technique described by Cáceres et al. (1990, 1995), the material was placed in a domestic blender with ethanol at 70°GL to be triturated (at the ratio of 800 mL of ethanol to 200 g of sample). Then, the mixture was allowed to macerate, protected from light and kept at room temperature, for seven days. Extracts were filtered and concentrated with a rotatory concentrator (Fisatom) . Extracts were lyophilized (Christ Beta 2-16) and their masses were quantified.

***L. amazonensis* preparations:** *L. amazonensis* (MHOM/BR /1972/ M2269) was used as reference strain. It was supplied by the Parasitology Laboratory cryobank of the Institute of Biological Sciences of Universidade Federal de Ouro Preto-MG. Promastigote forms were cultivated at 24ºC in Schneider medium supplemented with fetal bovine serum (FBS) at 10% and collected in an exponential-phase of growth. Cultures were rinsed once in PBS at 4 ° C and centrifuged (Eppendorf) at 1500 rpm for 10 minutes. After rinsing, the promastigotes were placed in Schneider medium and counted in a Neubaeur chamber and were employed at a concentration of 10^7 cells/mL.

Co- Cultivation : After cultivating the promastigotes for 6 hours at 24º C , root, stem and leaf extracts of *Bixa orellana L.* were added to the cultures at concentrations ranging from 0.12; 0.25; 0.50; 0.75; 1.00; 1.50; 2.00 and 2.50 mg/mL.

After incubation for 72 hours at 24ºC the promastigotes were evaluated in smears analyses as for mobility and viability. Additionally, the surviving parasites were counted in a Neubauer's chamber and compared with controls, with DMSO only in concentration of 0.6% v/v, for the determination of 50.0% inhibitory growth concentration (IC50). Promastigote cultures without addition of extract were used as control. Amphotericin-B ® (Eurofarma) was used as the reference drug at 5 µg/mL. All tests were performed in triplicate on three different occasions. Cells structure and the number of morphologically healthy promastigotes, i.e., promastigotes with nucleus and evident kinetoplast, besides the

presence or absence of citoplasmatic granules were analyzed by the staining method with Giemsa. These analyses were carried out by optical microscopy (Olympus) evaluating 100 fields of the created smears besides being compared to the control. The leishmanicidal activity was expressed as growth inhibition. Statistical analysis was performed by using nonlinear regression to obtain the values of IC50.

RESULTS AND DISCUSSION

In our study it was possible to observe cells with altered morphology in the analysis of *L. amazonensis* promastigotes in stained slides after contact with different concentrations of the tested extracts. Among the main alterations, irregular nucleus and kinetoplast were observed, besides granules in the cytoplasm (Figure 1).

Changes in the cell structures (nucleus and kinetoplast) were observed at concentrations of 0.25; 0.50 and 0.75 mg/mL of root, leaf and stem extracts, respectively. The presence of citoplasmatic granules in the *L. amazonensis* promastigotes occurred at concentrations higher than 0.25 mg/mL with every extract used.These data are compared in Table 01.

The treatment of illnesses caused by microorganisms such as protozoon is complex mainly due to the fact that they are eukaryotes, i.e., they share many characteristics with cells of mammals. Thus, the activity of the antiparasitaries occurs in vias common to both parasite and host (Murray et al., 2000). For Calixto (2000), a quite promising alternative which has been in use since the dawn of civilization is the use of plants. One reason for this is the smaller number of side-effects presented by phytotherapic agents when compared to those of synthetic drugs. Chan-Bacab & Peña-Rodriguez (2001) mention the traditional use of the *B. orellana L* leaf in the treatment of leishmaniases. These authors evaluated the action of the methanolic extract from the fruit and the seed of *B. orellana L* on *L. amazonensis* and *L. chagasi* cultures. From the 11 plants evaluated in that study, *B. orellana L* was the one that presented more intense activity.

The analysis of antiparasitc activity of *B. orellana L.* on *L. amazonensis* promastigotes is not only feasible but also opportune since the new therapeutic alternatives for leishmaniasis have to be investigated due to difficulties in the treatment of these diseases. The wide distribution of leishmaniases, the difficult access to healthcare by the mostly low-income affected populations, and the consequent impairment it represents to human beings, make the research on the possible antiparasitic activity of *B. orellana L.*

Phytochemical characterization of *B. orellana L.* hydroethanolic extracts was carried out by Pinto et al. (2003) who reported the presence of saponins, flavonoids, tannins, alkaloids and steroids with antimicrobial activity on several species of microorganisms (bacteria). Comparing hydroethanolic extracts from stem, flower, leaf, fruit and root, only the extract from the dried fruit did not present antimicrobial activity. Other authors showed that the ethanolic extracts of the leaves and seeds of this species presented activity against this yeast and also against some Gram-positive and Gram-negative bacteria (Fleisher et al., 2003).

Preliminary phytochemical analysis revealed that almost all the bioactive extracts showed flavonoids, alkaloids and tannins in their chemical composition. Flavonoids are a broad class of phenolic compounds of plant origin that are known to possess a well-established protective effect against membrane lipoperoxidative damages and have long been under investigation for antiparasitic activity (Sen et al., 2005). The mode of action of antiprotozoal flavonoids remains unclear (Ribeiro et al., 1997). A recent study reveals the efficacy of five naturally occurring flavonoids in arresting the development of anemia during the visceral leishmaniasis post-infection period. The smears analyses of the leishmanicidal activity of the hydroethanolic extracts, considering both qualitative and quantitative aspects, reveal results similar to those observed in the morphological analyses of stained slides with the promastigote forms of *L. amazonensis*. In some analysis, the leaf extracts presented more effective action than the others. In accordance with Cáceres et al. (1990, 1995) studies about the antimicrobial activity of *B. orellana L*, the extracts obtained from the fruit, the root and the leaf presented positive results, while the seeds extract did not present such activity. In this sense, it is recommended that chromatographic fractions of the leaf extract be obtained and tested separately. Finally, these first results of *B. orellana L* extracts showed a leishmanicidal potential that must be studied in detail, mainly from the mechanism of action point of view.

Table 01: Overall morphological and viability analysis of *L. amazonensis* promastigotes after exposure to *B. orellana* hydroethanolic extracts

Extract Source	Extract Concentration (mg/mL)	Viability/Mobility (smears analyses)	Nucleus and Kinetoplast Structure (Giemsa)	Cytoplasmic Granules (Giemsa)
Stem	2.50	-	-	+
	2.00	-	-	+
	1.50	-/+	-	+
	1.00	-/+	-	+
	0.75	+	-	+
	0.50	+	+	+
	0.25	+	+	-
	0.12	+	+	-
Root	2.50	-	-	+
	2.00	-	-	+
	1.50	-	-	+
	1.00	+	-	+
	0.75	+	+/-	+
	0.50	+	+/-	+
	0.25	+	+/-	-
	0.12	+	+	-
Leaf	2.50	-	-	+
	2.00	-	-	+
	1.50	-	-	+
	1.00	-	+/-	+
	0.75	+	+/-	+
	0.50	+	+/-	+
	0.25	+	+	-
	0.12	+	+	-

Source: The author - Note: Viability/Motility and Cytoplasmic Granules: Presence = (+) / Absence = (-)
Nucleus/Kinetoplast: Preserved = (+) / Non-preserved = (-)

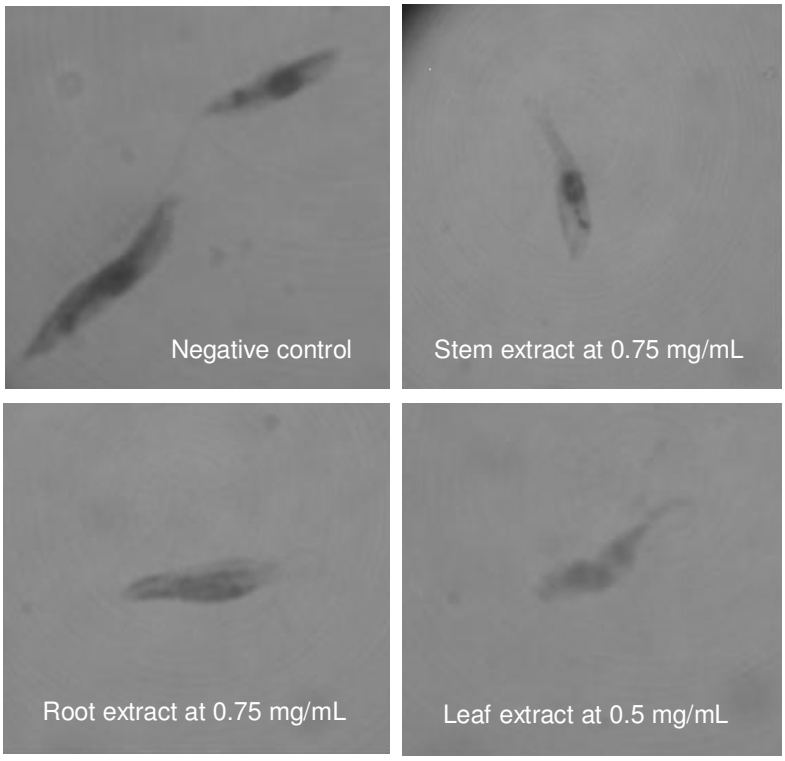

Figure 01 – Morphologic analysis of *Leishmania amazonensis* promastigotes in stained slides after contact with different concentrations of the tested extracts (Stem, Root and Leaf). Source: The author

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