Antimycobacterial activity evaluation and MIC determination of liophilized hydroalcoholic extracts of *Bixa orellana* L., Bixaceae

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**INTRODUCTION**

Brazilian flora is very rich and composed by approximately twenty thousand different species being *Bixa orellana* L. is one of them, which is part of the family of the Bixaceae and commonly known as annatto. It is a small tree growing 3 to 5 m in height, with a developed treetop. The leaf is simple, beardless, measuring 8 to 15 cm in length. It is a species from Tropical America including the Brazilian Amazon (Lorenzi & Matos, 2002). Besides being a food additive, annatto is used in the popular medicine against coronary illnesses, stomach and intestine affections, burns, respiratory affections and also as aphrodisiac. The leaves are used to combat fever and kidney affections (Lorenzi & Matos, 2002).

Brazil is one of the major world annatto grain producers. From this production 70% are used in cooking, 20% as natural dye or as food coloring, and 10% to

**RESUMO:** “Avaliação da atividade antimicobacteriana e a determinação da CIM de extratos hidroalcoólicos liofilizados de *Bixa orellana* L., Bixaceae.” *Bixa orellana* é usada na medicina popular em várias doenças. Extratos do fruto, raiz e folhas apresentaram atividade antimicrobiana, enquanto extratos do caule mostraram resultados negativos. Este estudo teve por objetivo verificar a atividade antimicobacteriana de extratos hidroalcoólicos liofilizados de *B. orellana* frente *Mycobacterium tuberculosis* e determinar a Concentração Inibitória Mínima para cinco bactérias. A atividade antimicobacteriana foi determinada por difusão e a CIM por difusão e análise colorimétrica. As CIM foram 0,3, 0,5 e 0,2 mg/mL para os extratos de folhas, raiz e caule frente *M. tuberculosis*. Os extratos de folhas mostraram CIM de 1,12 mg/mL (*B. cereus*), 1,53 mg/mL (*S. aureus* e *S. typhimurium*), 4,50 mg/mL (*P. aeruginosa*), 8,01 mg/mL (*P. mirabilis*); extratos de folhas mostraram 0,66 mg/mL (*P. aeruginosa*), 0,94 mg/mL (*P. mirabilis*), 1,88 mg/mL (*S. aureus*), 3,95 mg/mL (*B. cereus* e *P. mirabilis*). Extratos de folhas e caule mostraram atividade antimicobacteriana. As CIM por colorimetria foram menores que na difusão em agar. Os extratos revelaram atividade bacteriostática sobre as cinco espécies bacterianas.

**Unitermos:** *Mycobacterium tuberculosis, Bixa orellana*, Bixaceae.

**ABSTRACT:** *Bixa orellana* is used in popular medicine against several diseases. Extracts of its fruit, root and leaf presented antimicrobial activity, while seed extract showed negative results. This study aimed at verifying the antimycobacterial activity of *B. orellana* lyophilized hydroalcoholic extracts over *Mycobacterium tuberculosis*, and determine the Minimum Inhibitory Concentration against five bacteria. Antimycobacterial activity was determined by diffusion technique, while MIC was assessed by diffusion and colorimetric analysis. MICs were 0.3, 0.5 and 0.2 mg/mL respectively, for leaf, root and stem extracts, against *M. tuberculosis*. Stem’s extract showed 1.2 mg/mL for *B. cereus*, 1.53 mg/mL for *S. aureus* and *S. typhimurium*, 4.50 mg/mL for *P. aeruginosa* and 8.01 mg/mL for *P. mirabilis*. Leaf extracts showed 0.66 mg/mL for *P. aeruginosa*, 0.94 mg/mL for *P. mirabilis*, 1.88 mg/mL for *S. aureus*, 3.95 mg/mL for *B. cereus* and 8.37 mg/mL for *S. typhimurium*. Root’s extracts showed 0.25 mg/mL for *P. aeruginosa*, 0.31 mg/mL for *S. aureus*, 0.62 mg/mL for *S. typhimurium* and 3.00 mg/mL for *B. cereus* and *P. mirabilis*. Extratos de folhas e caule mostraram atividade antimicobacteriana. As CIM por colorimetria foram menores que na difusão em agar. Os extratos revelaram atividade bacteriostática sobre as cinco espécies bacterianas.

**Keywords:** *Mycobacterium tuberculosis, Bixa orellana*, Bixaceae.
exportation. Agner et al. (2005) studied effect of annatto on preneoplastic lesions induced by DMH and on DNA damage on rat's colons. Under experimental conditions, annatto did not develop any adverse effect and results suggested possible chemopreventive effects by its cellular proliferation modulation, but not in the carcinogenesis initial stage (Agner et al., 2005). In studies with rabbits, bixin and norbixin, carotenoids found in annatto, yielded promising results for a possible future use to either prevent or treat cardiac illness (Lima et al., 2001). Intrapерitoneal doses of 500 mg/kg to 1000 mg/kg in rats caused an increase of diuresis and reduction on muscular activity, without toxicity apparent signal. In this situation LD was of 700 mg/kg. Since 1970 FAO/WHO permit a temporary daily ingestion of 1.25 mg/kg body weight of annatto extracts.

Antimicrobial activity has been demonstrated B. orellana in extracts of fruits, root and leaf but not in seed extract (Caceres et al., 1990; Caceres et al., 1995). Huhtanen (1980) studied annatto fruit dyeing with Clostridium botulinum and determined the MIC value in 31 ppm. Pinto Coelho (2003) reported antimicrobial activity of annatto against several microbial species using hydroalcoholic extracts of stem, flower, fruit, and root only dry fruit extract did not present antimicrobial activity (Pinto Coelho et al., 2003). The importance of studies to verify the antimicrobial activity of plants is increasing due to the increasing resistance of microorganisms to the antibiotics currently commercialized. As reported in the literature, Mycobacterium tuberculosis is also becoming resistant to the antibiotics proposed by World Health Organization to combat tuberculosis, probably because of the long time of treatment and the adverse effects of these drugs, factors that hinder the patients adapt to therapy (Trabulsi, 2005).

With the purpose of amplifying the knowledge of medical plants with antimicrobial potential, this study evaluated the antimicrobial activity of Bixa orellana and determined the Minimum Inhibitory Concentration (MIC) of lyophilized hydroalcoholic extracts of B. orellana L. leaf, root and stem against Salmonella typhimurium (ATCC 14028), Proteus mirabilis (ATCC 25933), Bacillus cereus (ATCC 11778), Staphylococcus aureus (ATCC 12228) and Pseudomonas aeruginosa (ATCC 27853).

MATERIALS AND METHODS

Samples of root, stem and leaf of Bixa orellana L. were collected in Sitio da Lagoa, in Alfenas-MG, in October 2005 and sent Universidade Federal de Lavras, where they were botanically indentified and deposited in the herbarium under number 15.817. According to the technique described by Cáceres et al. (1990, 1995), material was placed on domestic blender with ethanol 70 'GL to be triturated (in the ratio of 800 mL of ethanol to 200 g of sample). Then, the mixture was allowed to macerate, protected against light and ambient temperature during seven days. Extracts were filtered and concentrated with rotatory concentrator. Aliquots of each extract were used for microbiological trial and showed inhibition halos with all tested bacteria. Extracts were lyophilized and their masses were quantified.

With the adapted agar diffusion technique described by Bauer et al. (1966), the MIC of root, stem and leaf extracts were tested against Salmonella typhimurium, Proteus mirabilis, Bacillus cereus, Staphylococcus aureus e Pseudomonas aeruginosa and then submitted to colorimetric. In these tests different dilutions of root, stem and leaf extracts of B. orellana, prepared with bases on the MIC found to each microorganism were incorporated into Muller Hinton broth with 2% of Isopropil miristate and 100 µL of bacterial suspension, with a tube turbidity of approximately 0.5 on the McFarland Scale. Positive and negative controls were used for the tests. The tubes were incubated at 37 ºC during 18 h. At this stage, results were obtained by colorimetric reading of the tubes, after shaking them with a vortex. Each tube was read before and after incubation and the difference between the first and second readings values was obtained. Bacteriostatic or bactericidal activity was evaluated in each tube.

According to Dutta et al. (2004) antimycobacterial activity was determined by diffusion technique in Lowenstein Jensen medium (LJM). Distilled water (1.5 mL) was added to lyophilized extracts and filtered with a 0.22 µm membrane. After this, 7 µL of each extract were added to filter paper discs. Positive controls of LJM and negative controls with 7 uL of Rifamicine® (30 µg/mL) were used. Inoculation in LJM with strain ATCC 25177 (H37Ra) was done with turbidity approximated to 0.5 on the McFarland Scale tube. Than MICs were determined by adding decreasing extracts concentrations of 30.0 to 0.05 mg/mL in Lowenstein Jensen medium and observing in witch of the tubes the microorganism did not showed growth after twenty eight days at 37 ºC. Positive control was done using inoculated LJM, negative control of LJM and Rifamicine® (30 µg/mL) and sterility control with LJM only. These experiments run on triplicate.

RESULTS

Stem’s extract showed MIC of 1.12 mg/mL for B. cereus, 1.53 mg/mL for S. aureus and S. typhimurium, 4.50 mg/mL for P. aeruginosa and 8.01 mg/mL for P. mirabilis; leaf’s extract showed MIC of 0.66 mg/mL for P. aeruginosa, 0.94 mg/mL for P. mirabilis, 1.88 mg/mL for S. aureus, 3.95 mg/mL for B. cereus and 8.37 mg/mL for S. typhimurium; root’s extract showed MICs of 0.25 mg/mL for P. aeruginosa, 0.31 mg/mL for S. aureus, 0.62 mg/mL for S. typhimurium and 3.00 mg/mL for B. cereus and P. mirabilis (Table 1). MICs were 0.3 mg/mL, 0.5 mg/mL and 0.2 mg/mL for leafs, root and stem extracts, respectively against M. tuberculosis (Table 2).
DISCUSSION

Fleischer et al. (2003) evaluated the antimicrobial activity of B. orellana lyophilized hydroalcoholic extracts with 10 mg/mL against Candida albicans and Gram-positive and Gram-negative bacteria, three of them being Staphylococcus aureus, Salmonella typhimurium and Pseudomonas aeruginosa. Our extracts showed antimicrobial activity against five microorganisms. Leaves extract showed higher inhibition against S. typhimurium and P. aeruginosa when compared with stem and root extract. For P. aeruginosa halos were similar for roots and leaf extracts.

Rojas (2006) evaluated antimicrobial activity of ten plants extracts and determined the MIC against five bacteria, three of them being Staphylococcus aureus, Salmonella typhimurium and Pseudomonas aeruginosa. Our extracts showed antimicrobial activity against five microorganisms. Leaves extract showed higher inhibition against S. typhimurium and P. aeruginosa when compared with stem and root extract. For P. aeruginosam halos were similar for roots and leaf extracts.

As reported in the literature, variations can occur due to concentrations differences of antimicrobial substances on the botanical material and respective extracts, in environmental factors where plants are located, in the period when the material was collected or even because of genetic differences in plant species. In addition variations can also occur due to the use of different microorganism strains.

Inhibition halos with stem and leaf extracts against Mycobacterium tuberculosis confirm popular medicine reports of Bixa orellana utilization against tuberculosis (Table 2). In the diffusion test the leaf extract showed an inhibition halo of 30 mm, larger than the Rifamicine control. It was also generally show that the MICS of roots, leaf and stem extracts were smaller for M. tuberculosis when compared to those of other bacteria. Thus, B. orellana extract could have this activity due to phenolic or saponin components. Alkaloids are known to act on extra-cellular proteins and maybe this fact can explain antimicrobial activity.

<table>
<thead>
<tr>
<th>Tested Discs</th>
<th>Diameter of inhibition halos (mm)</th>
<th>Tested tubes</th>
<th>MIC of Bixa orellana against Mycobacterium tuberculosis (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Extract</td>
<td>30.0</td>
<td>Leaf extract</td>
<td>0.3</td>
</tr>
<tr>
<td>Root Extract</td>
<td>11.0</td>
<td>Root extract</td>
<td>0.5</td>
</tr>
<tr>
<td>Stem Extract</td>
<td>16.0</td>
<td>Stem extract</td>
<td>0.2</td>
</tr>
<tr>
<td>Rifamicine® (30 μg/mL)</td>
<td>26.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Determination of antimycobacterial activity of leaf, root, and stem extracts of Bixa orellana against Mycobacterium tuberculosis.

MICs determined by agar diffusion were higher than the ones obtained by colorimetric methods because extracts can have more contact with microorganisms when suspension is used and colorimetric tests allows more sensitive results. Using the agar diffusion technique, MIC varied from 14.17 mg/mL to 75.54 mg/mL for leaf extracts. The present results demonstrated lower MIC for Bacillus cereus with leaf extract, for Staphylococcus aureus and Salmonella typhimurium with root extract and for Pseudomonas aeruginosa with stem extract. Using colorimetric technique, MIC varied from 0.66 mg/mL to 8.37 mg/mL for leaf extracts. Our results demonstrated lower MIC for Pseudomonas aeruginosa with leaf extract, for Pseudomonas aeruginosa with root extract and for Bacillus cereus with stem extract (Table 1).

<table>
<thead>
<tr>
<th>Microrganism</th>
<th>Minimum Inhibitory Concentration (mg/mL)</th>
<th>Agar diffusion tests</th>
<th>colorimetric tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem extract</td>
<td>Root extract</td>
<td>Leaf's extract</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>34.71</td>
<td>14.09</td>
<td>18.88</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>34.71</td>
<td>14.09</td>
<td>75.54</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>105.93</td>
<td>28.18</td>
<td>18.88</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>17.77</td>
<td>75.06</td>
<td>75.54</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>34.71</td>
<td>28.18</td>
<td>14.17</td>
</tr>
</tbody>
</table>

Table 1. Results of tests to determine MIC of extracts of Bixa orellana.

Antimycobacterial activity evaluation and MIC determination of lyophilized hydroalcoholic extracts of Bixa orellana L., Bixaceae
CONCLUSIONS

The results obtained in these studies showed lower MIC of extracts evaluated by colorimetric technique when compared to agar diffusion technique used as trial tests. Stem, root and leaf hydroalcoholic extracts of *Bixa orellana* L. showed bacteriostatic activity against *Salmonella typhimurium* (ATCC 14028), *Proteus mirabilis* (ATCC 25933), *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 12228) and *Pseudomonas aeruginosa* (ATCC 27853) antimycobacterial activity was determined in leaf, root and stem hydroalcoholic extracts of *Bixa orellana* L. against *Mycobacterium tuberculosis* ATCC 25177 (H37Ra).

ACKNOWLEDGMENTS

The authors thank UFLA for botanical material identification, CNPq for financial support, UNIFAL-MG for collaboration and IAL-SP for Mycobacterium tuberculosis strains and Professor Vinicius Vieira Vignoli (UNIFENAS) for language review.

REFERENCES


