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Antibacterial activity of *Bixa orellana* L. (achiote) against *Streptococcus mutans* and *Streptococcus sanguinis*

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ABSTRACT

Objective: To evaluate the cytotoxic and antibacterial effect of *Bixa orellana* L. (*B. orellana*) (achiote) methanol extract against *Streptococcus mutans* (ATCC 25175) (*S. mutans*) and *Streptococcus sanguinis* (ATCC 10556) (*S. sanguinis*).

Methods: Two methanol extracts of *B. orellana* were prepared *in vitro*, from the seeds and leaves. The antibacterial activity of extracts against *S. mutans* and *S. sanguinis* was evaluated using the cup-plate agar diffusion method. The minimum inhibitory concentration (MIC) was determined using the microdilution method and the cytotoxic activity was determinated by using the cell line MDCK.

Results: A stronger antibacterial effect was observed with the leaves methanolic extract with an inhibition zone of (19.97 ± 1.31) mm against *S. mutans* and (19.97 ± 1.26) mm against *S. sanguinis*. The methanolic extract of the seeds had an activity of (15.11 ± 1.03) mm and (16.15 ± 2.15) mm against *S. mutans* and *S. sanguinis*, respectively. The MIC of the leaf and the seed extracts against *S. sanguinis* was 62.5 and 125 μg/mL, respectively, and the MIC of the leaf extract against *S. mutans* was 62.5 μg/mL, and for the seed extract it was 31.25 μg/mL. The 50% cytotoxic concentration was 366.45 and 325.05 μg/mL for the leaves and seeds extracts, respectively.

Conclusions: The experimental findings demonstrated the antibacterial effect of the methanolic extract of *B. orellana* (achiote) on *S. mutans* and *S. sanguinis*. The extract of this plant is cytotoxic at high concentrations.

1. Introduction

The Peruvian flora has an immense variety of species, which is famous for its colorant properties as well as its medicinal values. However, there are a lot of plants that have not been studied, and their phytotherapeutic values are not fully understood.

Bixa orellana (*B. orellana*), also known as achiote or annatto, is an American plant widely used in Peru as nourishment, seasoning, as well as a colorant in the cosmetic and paint industry [1–3]. The achiote is frequently used in the Peruvian Amazonia as a preparation, extracted from the *B. orellana* leaves, for snake bites treatment, as a food digestive and for cough treatment [2]. *B. orellana* is recognized for its medicinal applications as an antioxidant, analgesic, wound healer, hemostatic and diuretic among others [3–5]. Apart from its antibacterial properties that have been postulated for treatment in certain gastrointestinal and pulmonary diseases [6,7], *B. orellana* is also commonly used by urologists for prostate cancer prevention [4,8].

The main objective of the study is to evaluate the cytotoxic and antibacterial effects of the *B. orellana* methanolic extract on the bacterial strains of *Streptococcus mutans* (ATCC 25175) (*S. mutans*) and *Streptococcus sanguinis* (ATCC 10556) (*S. sanguinis*) as potential applications in the odontology field.

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2. Materials and methods

2.1. Plant material and extracts

B. orellana was purchased from natural stores and the six of them had sanitary registration. Seeds and leaves were chopped and soaked in absolute methanol (1:2, w/v), and stored without sunlinght for 10 days at room temperature. The mixtures were filtered through a Whatman No. 4 filter paper, and the filtrates were evaporated at 50 °C [9]. All extracts were stored at 4 °C until used.

2.2. Bacteria strain

Strains of *S. mutans* and *S. sanguinis* were used (Genlab del Peru S.A.C., Peru). The cultivation medium was brain heart infusion (BHI) agar (Oxoid, Hampshire, UK). Cultures were grown anaerobically for 72 h at 37 °C. For antibacterial activity assay, three or four isolated colonies were inoculated in 3 mL of BHI broth and incubated under anaerobic condition for 72 h at 37 °C. The cultures were later diluted with fresh medium to approximate density of 0.5 McFarland standard, which represents an estimated concentration of 1.5×10^8 CFU/mL.

The McFarland standard was prepared by inoculating colonies of the bacterial test strain in sterile saline and adjusting the cell density to the concentration specified before [10].

2.3. Antibacterial screening of the methanolic extracts

2.3.1. Determination of antibacterial activity

To determinate the antibacterial activity of the studied extracts, the cup-plate agar diffusion method was used [11]. BHI agar was autoclaved for 15 min at 121 °C and cooled to about 55 °C. The medium was then inoculated with the prepared bacterial suspension, mixed gently and finally poured into sterile Petri dishes. Sugar tubes containing molten agar (10 mL) were sterilized and cooled to about 40–42 °C. The tubes were then inoculated with 0.1 mL of the appropriate culture suspension of bacteria. These agar plates were incubated under sterile condition for 8 h at room temperature. Three wells per plate of 6 mm in diameter and 4 mm in depth were made with a sterile cork borer, preserving a distance of 3 cm between them. The wells were filled with 100 µL of the corresponding methanolic extract. The chlorhexidine (0.12%) was used as positive control [12]. The Petri dishes were incubated under the same growth conditions mentioned above. At the end of the period, the inhibition zones formed were measured in millimeters using a vernier. The inhibition zones with less than 12 mm in diameter were not considered for the antibacterial activity analysis. For each extract, 12 replicates were assayed.

2.3.2. Determination of minimum inhibitory concentration (MIC)

The MIC was determined using the microdilution method as described by Jayaraman *et al*. [13] and Sader *et al*. [14]. Serial two-fold dilutions of all the extracts were prepared with sterile saline in a 96-well microtiter plate, obtaining a concentration range from 500 to 15.62 µg/mL. Then, 5 µL of *S. mutans* or *S. sanguinis* suspension (optical density at 550 nm = 0.6) were added to the wells containing the dilutions. Each dose was assayed in quadruplicate. Uninoculated wells containing sterile saline or saline and extract were used as controls. After incubation under anaerobic condition for 72 h at 37 °C, the samples were observed and MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity.

2.4. Cytotoxicity assay of B. orellana

2.4.1. Cell lines

MDCK cells were obtained from American Type Culture Collection, USA. The cells were grown in minimum essential medium with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 25 µg/L gentamicin and 200 mmol/L L-glutamine (growth medium). The cells were maintained in minimum essential medium with 1% fetal bovine serum, 25 µg/L gentamicin and 200 mmol/L L-glutamine (maintenance medium). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2-95% air.

2.4.2. Cytotoxicity assay

Cytotoxicity of *B. orellana* seeds and leaves extract was assessed using an assay based on the color change subsequent to the reduction of MTT by mitochondrial enzymes [15–17]. The assays were performed using MDCK cells at 1×10^4 cells/well in 200 µL of medium which were cultured in 96-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2-95% air. When cell cultures were confluent, the culture medium was removed from the wells, which were replenished with 0.2 mL of the maintenance medium containing *B. orellana* extract prepared by dilution. *B. orellana* concentrations had a range from 0 to 1 000 µg/mL. Each dose was assayed in quadruplicate. The wells with 0.2 mL maintenance medium but without *B. orellana* extract were used as cell controls. All cultures were incubated at 37 °C for 6 days. Cell morphology was inspected daily for alterations. The 50% cytotoxic concentration (CC_{50}) is defined as the concentration of compound that reduces the viability of mock-infected cells by 50%. This index is commonly estimated by MTT assay. In our study, 20 µL of MTT stock solution (3 mg/mL in phosphate-buffered saline) was added to each well. After 3 h of incubation under culture conditions, the medium was carefully removed and formazan crystals were solubilized by adding 200 µL dimethyl sulfoxide. Finally, cell viability was expressed as the percentage of the absorbance value determined for the control cultures. Absorbance (A_{570} nm) was measured in an ELISA reader. CC_{50} was then determined using Pharm/PCS software [18]. To confirm MTT results, the monolayers were also observed microscopically to estimate the cytopathic effect (*i.e*. rounding and other marked morphological changes with respect to control cells) [19].

3. Results

3.1. Antibacterial activity of the plant extracts

B. orellana methanolic extract *in vitro* antibacterial effect was measured on *S. mutans* and *S. sanguinis* strains and inhibition zones over 12 mm were considered positive. For *S. mutans* the seed extract produced an inhibition zone of 15.11 mm and the leaves extract an inhibition zone of 19.97 mm. Moreover, the Petri dishes with *S. sanguinis* showed an