



HOSTED BY



ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtbOriginal article <http://dx.doi.org/10.1016/j.apjtb.2016.03.005>Antibacterial activity of *Bixa orellana* L. (achiote) against *Streptococcus mutans* and *Streptococcus sanguinis*Dyanne Medina-Flores¹, Gabriela Ulloa-Urizar², Rosella Camere-Colarossi¹, Stefany Caballero-García¹, Frank Mayta-Tovalino¹, Juana del Valle-Mendoza^{1,3*}¹School of Dentistry, Faculty of Health Sciences, Peruvian University of Applied Sciences-UPC, Lima, Peru²Research and Innovation Center of the Faculty of Health Sciences, Peruvian University of Applied Sciences-UPC, Lima, Peru³Molecular Biology Laboratory, Nutrition Research Institute, Lima, Peru

ARTICLE INFO

ABSTRACT

Article history:

Received 13 Nov 2015

Received in revised form 26 Nov,

2nd revised form 30 Nov 2015

Accepted 2 Jan 2016

Available online 18 Mar 2016

Keywords:

Antibacterial effect

Medicinal plants

Bixa orellana L.

Cytotoxicity

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 determined 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.

*Corresponding author: Juana del Valle-Mendoza, Peruvian University of Applied Sciences (UPC), Av. San Marcos cdra. 2, Cedros de Villa, Lima, Peru.

Tel: +51 13133333, ext. 2704

Fax: +51 13496025

E-mail: jdelvall@upc.edu.pe

Foundation Project: Supported by Research Center of the Peruvian University of Applied Sciences (Grant-UPC-401-2014).

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

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 sunlight 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% CO₂-95% 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% CO₂-95% 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 (CC₅₀) 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. CC₅₀ 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

inhibition zone of 16.15 mm and 19.97 mm for seeds and leaves extract, respectively. In both bacterial cultures a larger inhibition zone was observed in the leaves methanolic extracts (Table 1).

Table 1

B. orellana methanolic extract *in vitro* antibacterial effect on *S. mutans* and *S. sanguinis* strains. mm.

Micro organism	Treatments	Inhibition zone			
		Mean \pm SD	Median	Minimum	Maximum
<i>S. mutans</i>	Seed extract	15.11 \pm 1.03	14.78	14.02	17.11
	Leaf extract	19.97 \pm 1.31	19.82	17.92	22.08
	Chlorhexidine	23.97 \pm 1.75	23.41	21.12	27.82
<i>S. sanguinis</i>	Seed extract	16.15 \pm 2.15	15.78	13.76	19.18
	Leaf extract	19.97 \pm 1.26	19.71	18.84	23.16
	Chlorhexidine	19.80 \pm 1.18	19.70	18.00	21.80

3.2. MIC of *B. orellana* methanolic extract

The MIC of the *B. orellana* methanolic extract from seeds and leaves were calculated for both *S. mutans* and *S. sanguinis* strains. For *S. mutans*, we observed a MIC between 25 and 50 $\mu\text{g/mL}$ for the seeds extract and a MIC between 50 and 75 $\mu\text{g/mL}$ for the leaves extract. On the other hand, for the *S. sanguinis* strains a MIC of 125 $\mu\text{g/mL}$ was observed for the seeds extract and a lower MIC between 50 and 75 $\mu\text{g/mL}$ was observed for the leaves extract (Table 2).

Table 2

Determination of the MIC of the *B. orellana* methanolic extract in *S. mutans* and *S. sanguinis* strains.

Extract concentration ($\mu\text{g/mL}$)	<i>S. mutans</i>		<i>S. sanguinis</i>	
	Leaves extract	Seeds extract	Leaves extract	Seeds extract
500.000	–	–	–	–
250.000	–	–	–	–
125.000	–	–	–	MIC
62.500	MIC	–	MIC	–
31.250	–	MIC	–	–
15.625	–	–	–	–

3.3. Cytotoxicity of *B. orellana* extract

The cytotoxicity of the methanolic extracts of *B. orellana* was determined using MDCK cells for virus propagation. MDCK cells were incubated with increasing concentrations (from 0 to 1 000 $\mu\text{g/mL}$) of *B. orellana* seeds and leaves extracts; cell viability was determined by MTT method. Relative viability of MDCK cells was calculated by comparison with untreated cultures (control, 0 $\mu\text{g/mL}$). Results indicated that the *B. orellana* extract did not produce adverse effects on MDCK cells cultures at low concentrations. However, the methanolic extract from *B. orellana* leaves produced 50% cellular viability inhibition with a concentration of 366.45 $\mu\text{g/mL}$, and the CC_{50} of seeds extract was 325.05 $\mu\text{g/mL}$. These values were confirmed by microscopic observation of the cytopathic effect.

4. Discussion

Phytotherapy has been used for the treatment of many conditions, and its applications expand from the most simple

extract preparations introduced by natives, to the more complex pharmacological processes to identify and obtain bioactive substances that are commonly used in modern medicine [18].

B. orellana antibacterial effect has been demonstrated in many investigations [1]. Some of these studies have been done in bacteria that can potentially be pathogenic in humans [20–22]. However, the information about the antibacterial effect of *B. orellana* on oral bacteria is limited.

The human oral mucosa is colonized by a very wide bacterial microbiota. *S. mutans* and *S. sanguinis* are important members of the oral flora. Although they live in homeostasis with other oral bacteria, both of them have been also identified as potential pathogens of dental caries and periodontal disease [23]. *S. mutans* is the most important cause of dental caries and the viridans group of streptococci (e.g. *S. sanguinis*) can cause bacteremia and endocarditis [24].

The agar diffusion method showed that the *B. orellana* methanolic extract presents antibacterial activity against *S. mutans* and *S. sanguinis* strains. This method corroborates that methanol is an effective organic solvent to extract phenols and flavonoids from plants just as Leyva *et al.* have previously demonstrated [25]. Similar antibacterial properties have been previously described in other bacterial strains [22,26]. The inhibitory activity of seed extract of *B. orellana* could be attributed to the presence of flavonoids. Flavonoids have the ability to assemble extracellular complexes with soluble proteins and the bacterial cell wall. Alternatively, lipophilic flavonoids may also disrupt bacterial membranes [27].

Furthermore, the methanolic extract showed different MIC for leaves and seeds. This could be related to the fact that the active antibacterial principles obtained in the plant leaves and seeds might be isolated in different concentrations, and greater efficiency in leaf extract compared to seed extract may be due to the absence of alkaloids in seeds.

The methanolic extract was proved to be cytotoxic for the MDCK cells at high concentrations. The information from previous studies is not enough to compare the CC_{50} from their extracts with our results. Nonetheless, a study in 2011 reported a CC_{50} of 60.2 $\mu\text{g/mL}$ for a seed hydro-alcoholic extract of *B. orellana* [28]. This might indicate that the hydro-alcoholic extracts can reach very low cytotoxic concentrations, whereas methanolic extracts appear to be safe.

The achiote appears to be a non-toxic natural product with a lot of potential uses in the odontology field due to their antibacterial properties against *S. mutans* and *S. sanguinis*. However, in Peru investigations regarding *B. orellana* and other plants with potential medical uses are poor. Further studies are needed to understand more about its antimicrobial applications, as well as potential cytotoxic effects, adverse events, other drugs interactions and contraindications.

The study results partially validate the *B. orellana* applications in the odontology field, with antimicrobial properties and a non-toxic profile. This natural product may bring new alternatives for the antibiotic treatments used in oral infections. Our results also highlight the importance of the ethnobotany properties evaluation in the selection of future possible bioactive components. In this regard, our study wants to demonstrate the great value of plants metabolites used in traditional medicine and their possible applications in the development of new medicines.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The present research was supported by Research Center of the Peruvian University of Applied Sciences, Lima-Peru (Grant-UPC-401-2014).

References

- [1] García M, Monzote L, Montalvo AM, Scull R. Effect of *Bixa orellana* against *Leishmania amazonensis*. *Forsch Kompleimentmed* 2011; **18**(6): 351-3.
- [2] Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect* 2001; **109**(Suppl 1): 69-75.
- [3] del Prado GLG, Hernández MEG, Castillo MQ, Hernández NG, Puig JF. [*Bixa orellana* L: a potential substance for detection of dentobacterial plaque]. *Rev Cuba Estomatol* 2009; **46**(2): 1-11. Spanish.
- [4] Yong YK, Chiong HS, Somchit MN, Ahmad Z. *Bixa orellana* leaf extract suppresses histamine-induced endothelial hyperpermeability via the PLC-NO-cGMP signaling cascade. *BMC Complement Altern Med* 2015; **15**: 356.
- [5] Lourido Pérez HC, Sánchez GM. [The *Bixa orellana* L. in treatment of stomatology affections: a subject that hasn't studied yet]. *Rev Cubana Farm* 2010; **44**(2): 231-44. Spanish.
- [6] Vilar Dde A, Vilar MS, de Lima e Moura TF, Raffin FN, de Oliveira MR, Franco CF, et al. Traditional uses, chemical constituents, and biological activities of *Bixa orellana* L.: a review. *ScientificWorldJournal* 2014; **2014**: 857292.
- [7] Cogo LL, Monteiro CLB, Miguel MD, Miguel OG, Cunico MM, Ribeiro ML, et al. Anti-*Helicobacter pylori* activity of plant extracts traditionally used for the treatment of gastrointestinal disorders. *Braz J Microbiol* 2010; **41**(2): 304-9.
- [8] Agner AR, Bazo AP, Ribeiro LR, Salvadori DM. DNA damage and aberrant crypt foci as putative biomarkers to evaluate the chemopreventive effect of annatto (*Bixa orellana* L.) in rat colon carcinogenesis. *Mutat Res* 2005; **582**(1–2): 146-54.
- [9] Del Valle Mendoza J, Pumarola T, Gonzales LA, Del Valle LJ. Antiviral activity of maca (*Lepidium meyenii*) against human influenza virus. *Asian Pac J Trop Med* 2014; **7**(Suppl 1): S415-20.
- [10] Andrews JM, Howe RA, BSAC Working Party on Susceptibility Testing. BSAC standardized disc susceptibility testing method. *J Antimicrob Chemother* 2011; **66**(12): 2726-57.
- [11] Witkowska-Banaszczak E, Michalak A, Kędzia A. *In-vitro* antimicrobial activity of bronchosol. *Acta Pol Pharm* 2015; **72**(2): 367-75.
- [12] Karupiah P, Rajaram S. Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. *Asian Pac J Trop Biomed* 2012; **2**(8): 597-601.
- [13] Jayaraman P, Sakharkar MK, Lim CS, Tang TH, Sakharkar KR. Activity and interactions of antibiotic and phytochemical combinations against *Pseudomonas aeruginosa* *in vitro*. *Int J Biol Sci* 2010; **6**(6): 556-68.
- [14] Sader HS, Flamm RK, Jones RN. Antimicrobial activity of daptomycin tested against gram-positive pathogens collected in Europe, Latin America, and selected countries in the Asia-Pacific Region (2011). *Diagn Microbiol Infect Dis* 2013; **75**(4): 417-22.
- [15] Sylvester PW. Optimization of the tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. *Methods Mol Biol* 2011; **716**: 157-68.
- [16] Kwon HJ, Kim HH, Ryu YB, Kim JH, Jeong HJ, Lee SW, et al. *In vitro* anti-rotavirus activity of polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*. *Bioorg Med Chem* 2010; **18**(21): 7668-74.
- [17] Mehrbod P, Ideris A, Omar AR, Hair-Bejo M. Prophylactic effect of herbal-marine compound (HESA-A) on influenza A virus infectivity. *BMC Complement Altern Med* 2014; **14**: 131.
- [18] Taherkhani R, Farshadpour F, Makvandi M. *In vitro* antiretroviral activity of *Achillea kellalensis*. *Jundishapur J Nat Pharm Prod* 2013; **8**(3): 138-43.
- [19] Fattahi S, Zabihi E, Abedian Z, Pourbagher R, Motevalzadeh Ardekani A, Mostafazadeh A, et al. Total phenolic and flavonoid contents of aqueous extract of stinging nettle and *in vitro* anti-proliferative effect on HeLa and BT-474 cell lines. *Int J Mol Cell Med* 2014; **3**(2): 102-7.
- [20] Rojas JJ, Ochoa VJ, Ocampo SA, Muñoz JF. Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. *BMC Complement Altern Med* 2006; **6**: 2.
- [21] Stohs SJ. Safety and efficacy of *Bixa orellana* (achiote, annatto) leaf extracts. *Phytother Res* 2014; **28**(7): 956-60.
- [22] Fleischer TC, Ameade EP, Mensah ML, Sawyer IK. Antimicrobial activity of the leaves and seeds of *Bixa orellana*. *Fitoterapia* 2003; **74**: 136-8.
- [23] Kouidhi B, Al Qurashi YM, Chaieb K. Drug resistance of bacterial dental biofilm and the potential use of natural compounds as alternative for prevention and treatment. *Microb Pathog* 2015; **80**: 39-49.
- [24] Babii C, Bahrin LG, Neagu AN, Gostin I, Mihasan M, Birsa LM, et al. Antibacterial activity and proposed action mechanism of a new class of synthetic tricyclic flavonoids. *J Appl Microbiol* 2016; <http://dx.doi.org/10.1111/jam.13048>.
- [25] Leyva JM, Pérez-Carlón JJ, González-Aguilar GA, Esqueda M, Ayala-Zavala JF. [Antibacterial and antioxidant functionality hydroalcoholic extracts of *Phellinus merrillii*]. *Revi Mex Micol* 2013; **37**: 11-7. Spanish.
- [26] Ongsakul M, Jindarat A, Rojanaworarit C. Antibacterial effect of crude alcoholic and aqueous extract of six medicinal plants against *Staphylococcus aureus* and *Escherichia coli*. *J Health Res* 2009; **23**(3): 153-6.
- [27] Clements JM, Coignard F, Johnson I, Chandler S, Palan S, Waller A, et al. Antibacterial activities and characterization of novel inhibitors of LpxC. *J Antimicrob Agents Chemother* 2002; **46**: 1793-9.
- [28] Fernández-Calienes Valdés A, Mendiola Martínez J, Acuña Rodríguez D, Scull Lizama R, Gutiérrez Gaitén Y. [Antimalarial activity of a hydroalcoholic extract of *Bixa orellana* L.]. *Rev Cuba Med Trop* 2011; **63**(2): 181-5.