academicJournals

Vol. 9(14), pp. 1020-1025, 8 April, 2015
DOI: 10.5897/AJMR2015.7390
Article Number: 9EAEA0252309
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

In vitro antibacterial activity of two plant extracts against *Enterococcus faecalis*

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Received 19 January, 2015; Accepted 13 March, 2015

The antimicrobial activity of two crude plant extracts– *Solanum paniculatum* L. (jurubeba) and *Bixa orellana* L. (annatto) against *Enterococcus faecalis* was tested *in vitro*, and their efficacy was compared with that of a chemical agent with known antibacterial activity – chlorhexidine digluconate at 0.12% concentration. Extracts were prepared from jurubeba roots and annatto seeds after selection by phytochemical screening. Microorganisms were divided into two groups: *E. faecalis* (ATCC 292012) and *E. faecalis* (44 - AB). The minimum inhibitory concentration (MIC) and the minimum inhibitory concentration of adherence (MICA) of the extract and the control were determined, and the cytotoxic potential and lethal dose (LD) of jurubeba extract were assessed because it was the only extract to exhibit inhibitory activity against bacteria. Only the *S. paniculatum* Linn (jurubeba) extract exhibited activity against the two bacterial strains– MIC: up to 1:64 against *E. faecalis* ATCC 292012, up to 1:32 against *E. faecalis* of the oral environment (44-AB); MICA: up to 1:512 against the two strains, exhibiting cytotoxicity at the 1:2 dilution (250 mg/mL) and LD = 0. The *B. orellana* Linn extract exhibited no inhibitory activity in any of the experiments; thus, it was not assayed in the cytotoxicity experiments, nor was its LD determined. The low toxicity of *S. paniculatum* L. extract (cytotoxicity only at the 1:2 dilution, or 250 mg/mL) in addition to its LD = 0 and good antibacterial performance in the tests suggest the potential use of this product for the treatment of endodontic and periodontal oral infections; however, further experiments strengthening these results should be performed. The *B. orellana* L. extract exhibited no antimicrobial activity.

Key words: Antimicrobial activity, plant extracts, phytotherapeutics, *Enterococcus faecalis,* endodontic infections, *Bixa orellana* L., *Solanum paniculatum* L.

INTRODUCTION

Brazilian studies on phytotherapeutics, especially with regard to the application of these compounds in clinical dentistry, are currently limited in number and results (Barbosa et al., 2012). Some of these studies have aimed to determine the antimicrobial activity of ethanolic and hydroethanolic plant extracts on oral microorganisms,

including *Enterococcus faecalis* strains, which are considered invasive bacteria of root canals and are frequently associated with persistent infections and lesions linked to unsuccessful endodontic treatments (Peciuliene et al., 2008; Rôças and Siqueira, 2008; Skucaite et al., 2010; Santos et al., 2011; Rôças and Siqueira, 2012).

According to Fisher and Phillips (2009), *Enterococcus* species were initially not considered important in clinical pathology until their association with nosocomial infections was demonstrated, which turned them into a major bacterial pathogen. As stated by those authors, these bacteria are resistant to a wide range of temperatures (from 5 to 50°C) and show great ability to survive in adverse environments. In root canal microbiota, they are present at low amounts in untreated infected canals (Peciuliene et al., 2008). However, the possibility of root canal system (RCS) invasion by *E. faecalis* is high considering their ability to survive in harsh environments as compared to other bacteria, making their presence common in persistent infections (Peciuliene et al., 2008; Skucaite et al., 2010; Rôças and Siqueira, 2012).

To treat these infections, irrigation solutions are used in combination with mechanical cleaning methods, and intracanal medication is used in the intervals between treatment sessions (Siqueira, 2011). Thus, phytotherapy may represent an interesting alternative as an antimicrobial treatment against this microorganism (Pereira et al., 2010).

Bixa orellana L., a shrub of the family Bixaceae, also known as annatto, occurs from northern to southern Brazil. The most commonly used parts are the seeds, although a few phytochemical and antimicrobial activity studies have also used the leaves, stems and roots (Tamil et al., 2011; Almeida et al., 2012). The roots and stems of *Solanum paniculatum* Linn. (jurubeba), a member of the family *Solanaceae*, are also commonly used by humans to disinfect wounds (Garcia et al., 2008). However, the healing and antibacterial activities of the plant have not yet been confirmed (Lobo et al., 2010).

This study was aimed at a preliminary research to investigate the potential antimicrobial effects of two Brazilian plant extracts against *E. faecalis* strains (standard and from the oral environment) and, according to the results, broaden the number of microorganisms to be tested.

MATERIALS AND METHODS

Extract preparation

After phytochemical screening by thin-layer chromatography to evaluate the phytocompounds of several Brazilian plants, the seeds of *B. orellana* L. (annatto) and the peels of *S. paniculatum* L. (jurubeba) were selected for this study due to the following components present in good concentrations: terpenes, tannins, saponins and essencial oils (found in both extracts – annatto and jurubeba) and flavonoids (found in jurubeba extract).

An ethanolic extract or vegetable tincture of *B. orellana* L. seeds harvested from shrubs registered in the Federal University of Maranhão (Universidade Federal do Maranhão - UFMA) Ático Seabra Herbarium (São Luís, Maranhão, Brazil) under voucher specimen no. 00815 was prepared in the laboratory of the UFMA School of Pharmacy. Seeds from mature fruits were collected in April 2014 by a collector wearing rubber gloves. The seeds were placed in paper bags and kept dry and ventilated until being sent to the laboratory two days later. In the laboratory, the annatto seeds were spread, dried at room temperature and pulverised in an electric mill to obtain powder. Next, the powder underwent extraction (maceration) for 48 h with 70% ethanol at a 1:3 ratio v/v and was filtered. This procedure was repeated three times, and the filtrate of the three extractions was pooled, concentrated in a rotary evaporator and named hydroethanolic extract (HE). To determine the dry weight and yield, three 1 mL aliquots of the HE were used, separated into three flasks. The aliquots were evaporated under a hot air stream, and the flasks were then cooled and weighed in a digital analytical balance. This procedure was also repeated three times until constant weight was obtained. The extraction yield was based on the powder weight, the final volume of the concentrated HE and the residue weight.

S. paniculatum L. (jurubeba) peels were collected in September 2010 in the city of Natal, Rio Grande do Norte, Brazil. Botanical identification was performed by Prof. Dr. Maria Iracema Bezerra Loyola (Department of Biology/UFRN), where one voucher specimen was deposited (no. 5468). After collection, stem samples were desiccated in a forced air oven at a mean temperature of 45°C for three to four days and then pulverised in a mechanical mill, turning them into powder. The dried plant powder underwent thorough maceration with two litres of 95% ethanol for 72 h, and this process was repeated three times to obtain the maximum extraction of the chemical compounds. The resulting extraction solution was filtered and concentrated in a rotary evaporator under reduced pressure at a temperature not higher than 40°C.

Microbial strains

Samples of *E. faecalis* (ATCC 292012) and *E. faecalis* (44 – AB, isolated from endodontic infections) were obtained upon request from the School of Dentistry of the Estácio de Sá University (Universidade Estácio de Sá) (Rio de Janeiro/RJ) and the School of Dentistry of the Federal University of Rio Grande do Norte (Universidade Federal do Rio Grande do Norte – UFRN) (Natal/RN). The samples were subsequently reactivated in the Laboratory of Microbiology of UFRN, Natal/RN.

Determination of the minimum inhibitory concentration of *S. paniculatum* linn and *B. orellana* linn extracts

The minimum inhibitory concentration (MIC) of the *S. paniculatum* L. and *B. orellana* L. extracts was determined using the method described by Bauer et al. (1966), with modifications, based on the lowest concentration of the extract able to inhibit bacterial growth as compared to the control (0.12% chlorhexidine digluconate), which was based on the presence of a zone of inhibition measured in

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millimetres with a calliper.

Determination of the minimum inhibitory concentration of adherence of *S. paniculatum* l. and *B. orellana* l. extracts

The minimum inhibitory concentration of adherence (MICA) of the extracts was determined from the crude extract (CE) up to the maximum dilution obtained– 1:512. Bacterial inocula were obtained and distributed as subcultures into haemolysis tubes with 0.2 mL of the solution corresponding to the respective extract dilution. The tubes were then incubated at 37°C under anaerobic conditions at a 30° inclination. Microorganism adherence to the tube wall after shaking was visually determined and compared with the controls (0.12% chlorhexidine). The MICA was determined based on the lowest concentration yielding adhered bacteria.

Toxicity tests

After determining the MIC and MICA, the cytotoxic potential of the *S. paniculatum* Linn (jurubeba) extract was assayed because it was the only one to exhibit an inhibitory effect against the *E. faecalis* strains. After approval from the Human Research Ethics Committee, protocol registration no. 743/10, cover sheet no. 390665, Certificate of Presentation for Ethical Appreciation (Certificado de Apresentação para Apreciação Ética - CAAE) no. 6396.0.000.126-10 of March 3, 2011, the extract was evaluated at different concentrations using the method of human erythrocytes - A, B, O and AB - (Rangel et al., 1997; Prokofeva et al., 2004), derived from blood that could not be used for transfusion (to be discarded). The material was obtained from the Transfusion Unit of the Lauro Wanderley University Hospital/Federal University of Paraíba (Hospital Universitário Lauro Wanderley/Universidade Federal da Paraíba - UFPB) and handled and disposed of in accordance with the Safety Guidelines followed by this unit.

Determination of the lethal dose

The LD of the *S. paniculatum* L. extract was also determined. The animal observation method was based on the experimental protocol developed by the Psychopharmacology unit of UFRN and performed using Swiss albino mice. The mice were provided by the UFRN vivarium, grouped in cages and kept under a mean temperature of 27°C without any medication and had free access to food (Purina® feed pellets) and potable water in graduated polyethylene bottles. The animals were maintained under a 12-h light/dark cycle prior to the experiment and transferred to the experimental room 30 min before the experiment. They were then divided into groups and treated with *S paniculatum* L. extract (at different concentrations), intraperitoneally (IP) with a single dose - 0.1 mL/animal. Distilled water was given to the control group.

The animals were observed for 24 h to map possible behavioural changes suggestive of effects on the central nervous system (CNS) and/or the autonomic nervous system (ANS). At the end of the experiment, the number of dead animals was recorded to determine the dose causing death of 50% of the experimental animals (lethal dose = LD_{50}).

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening tests showed the strong presence of toluene and formic acid in the seeds of *B. orellana* L., as well as low but evident concentrations of alkaloids and flavonoids. In the roots of *S. paniculatum* L., several chemical compounds were found, such as tannic acid, flavonoids and tannins. These findings were considered to be an important indication of antimicrobial activity due to the biological functions and biotechnological applications of these phytocompounds (Alves et al., 2009; Hammer et al., 1999; Chatuverdi et al., 2010).

Determination of the minimum inhibitory concentration and minimum inhibitory concentration of adherence of the extracts

Our results corroborate those from other studies reporting relative and limited microbicidal and antiseptic activity for annatto extract against several types of bacteria (Table 2 and 4). This limitation is associated with the different parts of the plant used and seasonal and geographical characteristics (Bertini et al., 2005; Agra et al., 2008).

Majolo et al. (2013) reported that different plant accessions harbour distinct levels of antimicrobial activity, perhaps due to different characteristics of the soil, climate, genetics and availability of phytonutrients, which affect the chemical content associated with this activity. Additionally, differences in soil site, climatic region, humidity level and sampling period may promote different concentrations of saponins, flavonoids, alkaloids and steroids. Assuming that these variables, combined or individually, may have influenced the results presented here, we suggest that further experiments be conducted in the near future using other seed samples as well as leaves collected from the same voucher specimen, though in periods different from when the seeds used in the present study were extracted (April 2014 – no rain, medium humidity level, very hot sun).

Giridhar and Venugopalan (2012) have observed antibacterial activity of annatto leaves only, corroborating the data reported by Coelho et al. (2003) and Fleischer et al. (2003). Silva et al. (2010), after testing the inhibitory activity against bacteria of *B. orellana* L. hydroethanolic extracts obtained from the fruit, stem, root and leaves of the plant, found that the products from the leaves and stems exhibited bacteriostatic activity against several bacteria. However, Majolo et al. (2013) and Mital et al. (2013) used seeds from multiple accessions and concluded that the hydroethanolic preparation exhibited the best inhibitory activity, with Gram-positive bacteria being the most sensitive, resulting in a moderate zone of inhibition. Chaturvedi et al. (2010) and Almeida et al. (2012), after testing extracts of *B. orellana* L. seeds on bacteria and fungi with no success, suggested further testing with the product.

The results obtained here with the jurubeba extract confirm the inhibitory potential of the plant on microorganisms (Tables 1 and 3), a result also found by Lobo et al. (2010), Rodrigues et al. (2013) and Costa (2011).

Table 1. Minimum inhibitory concentration (mean zone of inhibition in mm) in solid medium of *S. paniculatum* Linn extract and chlorhexidine digluconate.

Microbial Strains	***Solanum paniculatum* Linn extract**									
	CE	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
	500 mg/mL	**250 mg/mL**	**125 mg/mL**	**62.5 mg/mL**	**31.25 mg/mL**	**15.65 mg/mL**	**7.81 mg/mL**	**3.90 mg/mL**	**1.95 mg/mL**	**0.97 mg/mL**
E. faecalis 29212 ATCC	16	14	16	14	14	12	12	0	0	0
E. faecalis 44 AB	15	15	15	13	13	12	0	0	0	0
Microbial	0.12% Chlorhexidine digluconate									
Strains	PS	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
E. faecalis 29212 ATCC	20	17	17	16	15	15	15	15	13	10
E. faecalis 44 AB	21	18	18	17	16	14	0	0	0	0

*Crude extract; *PS– pure substance.

Table 2. Minimum inhibitory concentration (mean zone of inhibition in mm) in solid medium of the annatto extract and chlorhexidine digluconate.

Microbial	***Bixa orellana* L. extract**									
Strains	**CE**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
E. faecalis ATCC	0	0	0	0	0	0	0	0	0	0
E. faecalis AB	0	0	0	0	0	0	0	0	0	0
Microbial	**0.12 and 2% Chlorhexidine digluconate**									
Strains	**PS**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**	**1:512**
E. faecalis ATCC										
E. faecalis AB	21	18	18	17	16	14	0	0	0	0

*CE– Crude extract; *PS– pure substance.

Table 3. Minimum inhibitory concentration of adherence (MICA) of *S. paniculatum* Linn extract for *E. faecalis* when compared with MICA of the control.

Microbial Strains	***Solanum paniculatum* Linn extract (MICA)**	**0.12% CLX**
E. faecalis ATCC	1:512	1:512
E. faecalis AB	1:512	1:512

*CLX– chlorhexidine

However, few studies have demonstrated the antimicrobial effects of the plant *S. paniculatum* Linn.

The MIC of 250 mg/mL (1:2) was a good result as compared to the zone of inhibition of the controls with chlorhexidine (Table 1). The inhibition of microbial growth was homogeneous, according to the degree of concentration of the extract, showing a progressive decrease in the diameter of the halos with increasing dilutions. The *S. paniculatum* Linn extract exhibited inhibitory effects on *E. faecalis* up to a concentration of 7.81 mg/mL (1:64). Moreover, it also affected microbial adhesion up to the maximum dilution (1:512 and 0.97 mg/mL) (Table 3). Out of the two groups of microorganisms, *E. faecalis* 29212 ATCC was the strain most sensitive to jurubeba. It could not establish a statistical analysis due to the small number of strains tested (two only).

Table 4. Minimum inhibitory concentration of adherence of *B. orellana* L. extract (annatto) for *E. faecalis* when compared with MICA of the control.

Microbial strains	*Bixa orellana* Linn extract (MICA)	0.12% CLX
E. faecalis ATCC	0	1:512
E. faecalis AB	0	1:512

*CLX – chlorhexidine.

Jurubeba cytotoxicity

The *S. paniculatum* (jurubeba) extract haemolysed 41.2% of human erythrocytes type A, 45.1% of type B, 16.4% of type O and 24.8% of type AB. However, cytotoxicity was observed at the 1:2 dilution (250 mg/mL). Since this is a preliminary study, these results demonstrate a promising non-toxic behavior of jurubeba extract.

Determination of jurubeba lethal dose

No mortality was observed in the animals treated intraperitoneally with the *S. paniculatum* Linn extract at any dilution tested (from non-diluted up to 1:512) at 24, 72 h or 15 days after the experiment. Thus, LD = 0. Moreover, no significant behavioural changes were observed.

Conclusions

The low toxicity of *S. paniculatum* L. extract (cytotoxicity only at the 1:2 dilution or 250 mg/mL), its LD = 0, and its good antifungal and antibacterial performance revealed in the *in vitro* tests suggest the potential use of this plant in the treatments of endodontic and periodontal oral infections; however, further experiments providing more evidence should be performed. The *B. orellana* L. extract exhibited no antimicrobial activity, and further studies with the product are also recommended.

Conflict of interests

The authors did not declare any conflict of interest.

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