Research Paper

In vitro activity of Amazon plant extracts against *Enterococcus faecalis*

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Abstract

Previous studies analyzing 2,200 plant extracts indicated anti-enterococcal activity in 25 extracts obtained from Brazilian forests' plants. In the present study, these extracts were subjected to microdilution broth assay (MDBA) and disk diffusion assay (DDA) using planktonic *Enterococcus faecalis* ATCC® 29212™ and were submitted to phytochemical analysis in TLC and HPLC. Three extracts obtained from *Ipomoea alba* (MIC < 40 μg/mL), *Diclinanona calycina* (MIC ≤ 40 μg/mL) and *Moronobea coccinea* (40 < MIC < 80 μg/mL; MBC = 80 μg/mL) showed significant bactericidal activity in the MDBA and four extracts obtained from *I. alba* (14.04 ± 0.55 mm diameter) *S. globulifera* (14.43 ± 0.33 mm and 12.18 ± 0.28 mm diameter) and *Connarus ruber* var. *ruber* (13.13 ± 0.18 mm diameter) were active in DDA. Residues H_2O obtained from *Psidium densicomum* (mean of 16.78 mm diameter) and from *Stryphnodendron pulcherrimum* (mean of 15.97 mm diameter) have shown an improved antibacterial activity after fractionation if compared to that obtained from the respective crude extracts. Antioxidant activity was observed in some residues of the active extracts. TLC analysis showed that phenolic compounds are likely to be found in active extracts. Three molecules were isolated from *S. globulifera* and were identified by ^{13}C NMR lupeol, α-amyrin and 3β-hydroxyglutin-5-ene. The present chemical and biological findings suggest that these extracts are a potential source of new anti-*Enterococcus* compounds to be introduced in endodontic therapy.

Key words: *Enterococcus faecalis*, endodontitis, antibacterial activity, Amazon rain forest, bioprospection.

Introduction

Enterococcus faecalis is one of the main nosocomial pathogens (Horner *et al.*, 2005) which may be resistant to a wide range of antimicrobial agents, causing several diseases. These bacteria inhabit the gastrointestinal and genitourinary tracts and have been related to infectious endocarditis and surgical wound infection (Pasticci *et al.*, 2008). In the oral cavity, *E. faecalis* is likely to be found in caries lesions, in periodontal diseases (Souto *et al.*, 2006), and mainly in endodontic infections (Hancock *et al.*, 2001, Duggan and Sedgley, 2007), in which pulp may become infected via dentinal tubules, through carious lesions, and via periodontal disease. Studies have shown that remaining *E. faecalis* contributes to the failure of endodontic therapy. The suppression and control of *E. faecalis* in dental procedures is paramount to diminish invasion of bacteria in dentinal tubules (Love, 2001), as well as to avoid strains to get resistance to several antibiotics (Aslangul *et al.*, 2005) and irrigating solutions, to avoid bacteria adaptation to environments where nutrients are scarce, and to abolish the establishment of any relationship with other bacteria, as in biofilms, environment and virulence factors (Kayaoglu and Orstavik, 2004).

Chemical and mechanical site preparation in the treatment of caries lesions and periodontal and endodontic infections aim to reduce infection and provide a favorable

environment for an effective treatment. Chemical preparation in endodontics is synonym of sodium hypochloride (SH). Currently, there is a limited number of irrigating substances with antimicrobial properties that can be used in periodontal and endodontic treatment, as well as in the treatment of carious lesions. Most of the chemical substances show adverse factors, such as toxicity, non-physiological pH, unpleasant odor and taste, and inability to degrade organic matter and smear layer. One of the most widely indicated substance is SH diluted to 1% (Estrela *et al.*, 2008).

The use of SH as irrigation solution is part of the protocols established to prepare root canals to endodontic treatments, considering that removal of any microorganism is fundamental to avoid any further relapse. One of the main microorganisms found in recurrent failure in endodontic treatment is *E. faecalis*, which is a bacterium that exhibits increased virulence in pathological conditions of endodontic disease. For that reason, this bacterium is one of the main target for drug action in the treatment of this oral pathological condition (Pinheiro *et al.*, 2004; Pinheiro *et al.*, 2003). Considering the undesirable effects related to SH, it has become increasingly necessary to find new tools to assist in the fight against *E. faecalis*.

Systematic studies with plant and animal extracts (Alviano *et al.*, 2008) have shown that it is possible to find compounds with pharmaceutical potential, such as some antibacterial chemotherapeutic agents (penicillin, erythromycin, tetracyclin). Universidade Paulista (UNIP), Brazil, introduced in 1996 a large-scale bioprospecting program targeting the identification of antibacterial compounds in plant extracts native to Brazilian forests. Throughout the program, over 2,000 plant extracts were tested against several microorganisms, such as *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Streptococcus mutans*, *S. sanguinis*, and *Enterococcus faecalis* (Suffredini *et al.*, 2006a; Suffredini *et al.*, 2006b; Suffredini *et al.*, 2004; Suffredini *et al.*, 2002). Based on these results, the present study aimed the study of the antibacterial activity of 25 plant extracts and their residues against traditional antibacterial assays and to search for chemical groups that may occur in the active extracts and residues, emphasizing an organic extract obtained from *Symphonia globulifera*, which was available in higher amounts to develop initial chemical studies.

Material and Methods

Plant collection and extraction preparation

For plant collection and extraction, our team obtained a collecting permit issued by the Brazilian Institute of Environment and Renewable Natural Resources - IBAMA and a permit to access genetic resources issued by the Genetic Heritage Management Council - CGen of the Brazilian Ministry of the Environment. Collected plants were taxonomically identified and voucher specimens were deposited at UNIP Herbarium, São Paulo, Brazil. Organic and aqueous extracts were obtained by a 24-h maceration with dichloromethane: methanol (1:1), followed by a 24-h maceration with distilled water (Millipore®, Bedford, MA, USA) (Younes *et al.*, 2007; Younes *et al.*, 2000).

Test compounds and controls

Twenty-five previously selected extracts, here designed as EB 55, EB 321, EB 352, EB 429, EB 841, EB 1247, EB 1257, EB 1259, EB 1298, EB 1373, EB 1389, EB 1395, EB 1461, EB 1493, EB 1497, EB 1525, EB 1543, EB 1549, EB 1637, EB 1743, EB 1765, EB 1905, EB 1923, EB 1991 and EB 1999, which taxonomical and collection data are listed in table 1, were diluted to a series of dilutions that led to final test concentrations in the microdilution broth assay of 12,500, 10,000, 7,500, 5,000, 2,500, 1,300, 600, 310, 160, 80, and 40 µg/mL,, being 20 times more diluted than the initial extracts dilutions, according to the established protocol). Dimethylsulfoxide and water were used as solvent in the dilution procedure. Sodium hypochlorite 1% (SH) was used as positive control, and was obtained from a formulated SH 10% solution (Fórmula & Ação - Compounding Pharmacy). A 1% SH commercial solution (Asfer®, São Paulo, SP, Brazil) was also used as positive control. The same extracts were tested in disk diffusion assay at a concentration of 200 mg/mL, diluted in dimethylsulfoxide 50% in water. All organic extracts were diluted in dimethylsulfoxide 50% (DMSO50).

Enteroroccus faecalis

The biosafety level 2 bacteria used in all assays were obtained from ATCC. Bacteria was acquired lyophilized, in Loops® (Oxoid Ltd, London, England), and was seeded in Müeller-Hinton agar (Oxoid Ltd, London, England), put in an incubator for 24 h, at 36 °C. this plate was called "mother-plate", and was left to be used within 30 days, if kept under refrigeration. 24-h fresh colonies were acquired before each assay, so, bacteria were at the same 4th passage during every experiment performed in the present work.

Disk diffusion assay and the determination of the growth inhibition zone diameter

Disk diffusion assay was done as usually described for plant extracts analysis (Souto *et al.*, 2006), in strict sterile conditions. A 0.5 McFarland saline suspension was prepared from a fresh colony of *Enterococcus faecalis* ATCC® 29212™. Assay was performed in sterile Müeller-Hinton agar prepared in Petri dishes. Sterile swabs were used to seed bacteria on the medium surface. Six paper disks measuring 6 mm diameter were distributed over inoculated medium surface. Then, 10 µL of extracts and residues were added to each disk, in triplicate. Dishes were incubated at 36 °C for 24 h. After that, the diameters of growth inhibi-

Table 1 - List of extracts showing activity against *Enterococcus faecalis* ATCC® 29212™ and the corresponding minimal inhibitory concentrations obtained from microdilution broth assay.

Extract number*	Herbarium reference	Collect date	Organ	Family	Gender	Species	1×10^5 cfu MIC (μg/mL)
55	PSC 396	05/12/1997	Leaves	Fabaceae Caesalpinioideae	*Macrolobium*	*multijugum*	5,000 < MIC < 12,500
321	PSC 414	06/12/1997	Leaves and flowers	Myrtaceae	*Psidium*	*densicomum*	5,000 < MIC < 12,500
352	IBS 9	25/06/1998	Stem	Fabaceae Mimosoideae	*Stryphnodendron*	*pulcherrimum*	2,500 < MIC < 5,000
429	PSC 144	30/05/1997	Stem	Proteaceae	*Roupala*	*sp.*	7,500 < MIC < 12,500
841	AAO 3458	26/08/1999	Aerial organs	Myrsinaceae	*Rapanea*	*parvifolia*	200 < MIC < 300
1247	IBS 53	30/09/2001	Aerial organs	Myrtaceae	*Psidium*	*densicomum*	5,000 < MIC < 12,500
1257	AAO 3717	10/08/2001	Aerial organs	Clusiaceae	*Symphonia*	*globulifera*	200 < MIC < 300
1259	IBS 56	30/09/2001	Aerial organs	Piperaceae	*Piper*	*sp.*	7,500 < MIC < 12,500
1298	AAO 3721	11/08/2001	Stem	Lauraceae	*Ocotea*	*myriantha*	300 < MIC < 600
1373	IBS 110	06/12/2001	Leaves	Clusiaceae	*Garcinia*	cf. *macrophylla*	> 12,500
1389	IBS 142	08/12/2001	Stem	Clusiaceae	*Moronobea*	*coccinea*	1,300 < MIC < 2,500
1395	IBS 143	08/12/2001	Leaves	Fabaceae Caesalpinioideae	*Hymenaea*	*parvifolia*	80 < MIC < 200
1461	AAO 4015	12/05/2002	Aerial organs	Sapindaceae	*Paullinia*	cf. *alata*	7,500 < MIC < 12,500
1493	AAO 4031	13/05/2002	Aerial organs	Convolvulaceae	*Ipomoea*	*alba*	< 40
1497	IBS 164	13/06/2002	Aerial organs	Moraceae	*Sorocea*	*duckei*	200 < MIC < 300
1525	IBS 63	01/11/2001	Stem	Connaraceae	*Connarus*	*ruber var. ruber*	600 < MIC < 1,300
1543	AAO 3711	10/08/2001	Aerial organs	Clusiaceae	*Tovomita*	*longifolia*	> 12,500
1549	AAO 4027	12/05/2002	Aerial organs	Combretaceae	*Combretum*	*rotundifolium*	200
1637	AAO 4120	20/07/2002	Aerial organs	Annonaceae	*Diclinanona*	*calycina*	≤ 40
1743	IBS 204	12/07/2003	Aerial organs	Connaraceae	*Connarus*	cf. *ruber var. sprucei*	1,300 < MIC < 2,500
1765	IBS 142	08/12/2001	Flores	Clusiaceae	*Moronobea*	*coccinea*	40 < MIC < 80
1905	AAO 4148	21/07/2002	Stem	Clusiaceae	*Symphonia*	*globulifera*	2,500 < MIC < 5,000
1923	AAO 4029	12/05/2002	Aerial organs	Fabaceae Faboideae	*Vatairea*	*guianensis*	2,500 < MIC < 5,000
1991	AAO 4148	21/07/2002	Aerial organs	Clusiaceae	*Symphonia*	*globulifera*	80 < MIC < 200
1999	AAO 4199	22/08/2002	Aerial organs	Myristicaceae	*Iryanthera*	*laevis*	> 12,500

* Odd numbers refer to organic extracts and even numbers refer to aqueous extracts. MIC = minimal inhibitory concentration obtained from microdilution broth assay.

tion zones were measured horizontally and vertically with a caliper rule.

Statistical analysis for disk diffusion assays

One-way ANOVA and Tukey's post-test analysis was applied in the evaluation of growth inhibition zone diameters resulted from the antibacterial activity of plant extracts and their residues, SH1%F = formulated sodium hypochlorite 1%; SH1%C = commercial sodium hypochlorite 1% (standard drugs used as positive control), against *Enterococcus faecalis* ATCC® 29212™. Results were significant if $p < 0.05$.

Microdilution broth assay and determination of minimal inhibitory concentration and minimal bactericidal concentration

The extracts were tested by the microdilution broth assay (MDBA), in completely sterile conditions, adapted to high-throughput conditions (Suffredini *et al.*, 2006b), in a biosafety level 2 Laboratory Unit, using sterilized Müeller-Hinton broth medium (Oxoid Ltd, London, England), in 96-well microplates, with inocula adjusted to a 0.5 McFarland standard, or 1×10^8 colony-forming unit per mL (cfu/mL), obtained from fresh colonies of *Enterococcus faecalis* ATCC® 29212™ (Suffredini *et al.*, 2006a; Suffredini *et al.*, 2006b; Suffredini *et al.*, 2004) grown on sterilized Müeller-Hinton agar medium (Oxoid Ltd, London, England). Concentrations of bacterial suspension, such as 1×10^2, 1×10^3, 1×10^4, 1×10^5, 1×10^6, and 1×10^7 cfu/mL, were prepared in sterilized Müeller -Hinton broth medium (Oxoid Ltd, London, England) starting from the 1×10^8 colony-forming unit per mL (cfu/mL) suspension. Thus, a 190-μL aliquot of the inoculum was dispensed into the wells, and 10 μL aliquots of extract, residue or standard compound solutions were then added to the inoculum. Microplates were incubated at 37 °C for 24 h. Inhibition of bacterial growth was assessed visually, and bacterial suspensions from all test wells were subcultured in sterile agar medium in order to evaluate bactericidal activity.

Following a procedure similar to that described above in sterile MDBA, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were obtained for the 25 plant extracts (PE), against *E. faecalis*. Solvent dimethylsulfoxide were tested as negative control.

Liquid-liquid partition and biological assay of residues

Each one of the 25 extracts was partitioned with three solvents of different polarities resulting in non polar residues (chloroform residue, or Residue CHCl₃, or EB-CHCl₃ where EB refers to the extract number), intermediate-polarity residues (butanol residue, or Residue BuOH, or EB-BuOH where EB refers to the extract number), and polar residues (aqueous residue, or Residue H₂O, or EB-H₂O where EB refers to the extract number). The solvents were

completely evaporated, followed by lyophilization, resulting in 75 residues. Organic solvents were completely removed under vacuum. All residues were re-suspended in 50% dimethylsulfoxide (DMSO50) or in distilled water (both tested as negative controls), at the concentration of 200 mg/mL, to be tested, as follows.

For preparation of 200 mg/mL solutions, aqueous residues were diluted with Milli-Q distilled water (Millipore®, Bedford, MA, USA) and chloroform and butanol residues were diluted in DMSO50, in order to be assayed by the MDBA, according to the technique previously described, in bacterial suspensions containing 1×10^6 cfu/mL.

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed with extracts and residues. Silica gel GF chromatography sheets (Merck®, Whitehouse Station, New Jersey, USA) were used and the following mobile phase were prepared: System 1 - hexane and ethyl acetate (4:1) applied on chromatographic sheet 1; System 2 - chloroform and ethyl acetate (1:1) applied on chromatographic sheet 2; and System 3 - chloroform, ethyl acetate and methanol (2:2:1); System 4 - ethyl acetate, methanol and water (100:13.5:10); System 5 - ethyl acetate, glacial acetic acid, formic acid and water (100:11:11:26). Revelation of spots was done with potassium hydroxide (A); Kedde Reagent (B), Dragendorff Reagent (C), NP Reagent (D), 25% sulfuric acid solution (E) and β-caroten (F). Ultraviolet light at 254 and/or 366 nm (uv254 or uv356) was used to develop some of the chromatograms (Suffredini *et al.*, 2004).

Evaluation of antioxidant potential

Evaluation of the antioxidant potential of extracts and residues was performed using the β-carotene technique (Pratt and Miller, 1984), in System 3 described above. A mixture of two solutions composed of (a) 9-mg β-carotene dissolved in 30 mL of chloroform and (b) two drops of linoleic acid dissolved in 60 mL of ethanol was used to reveal antioxidant substances in chromatogram. The chromatograms were exposed to natural light for at least 2 h in order to catalyze the antioxidant reaction between the natural product and the development system.

Fractionation of EB 1257

Extract EB1257, obtained from the aerial organs of *S. globulifera*, was used in the chemical assays.

Column chromatography fractionation

In order to isolate major compounds, 16.7668 g of extract 1257 were submitted to liquid-liquid partition using the technique first described, which resulted in Residue CHCl₃ (7212.6 mg, or 43.02% yield) Residue BuOH (3815.3 mg, or 22.76% yield) and Residue H₂O (5738.9 mg, or 34.23% yield). Residue CHCl₃ was submitted to open CC fractionation with Sephadex LH-20 as sta-

tionary phase, and 100% hexane, 100% dichloromethane and 100% methanol, as mobile phase, and resulted in hexane fraction (3031.2 mg, or 42.03% yield), dichloromethane fraction (1230.9, or 17.07% yield mg) and methanol fraction (2950.5 mg, or 40.91% yield).

Chromatographic techniques used in the isolation of major compounds - thin layer chromatography and normal phase open column chromatography

The hexane fraction obtained from Residue CHCl$_3$, which was obtained from EB1257, was submitted to fractionation in column chromatography using silica gel 60-200 μm as stationary phase and a gradient with increasing polarity composed by mixtures of hexane, ethyl acetate and methanol as mobile phase, which resulted in 28 fractions. The fractions were compared by TLC and were grouped according to their similarity, after analysis with sulfuric acid 25% and heating, resulting in fractions 10-11, 12, 13, 14-15, 16, 17,.., 28. Fraction 13, named UNIP147 was sent to ^{1}H NMR and e ^{13}C NMR, in CDCl$_3$.

Fraction 13 (or UNIP147) was obtained as a crystal cream sample (30 mg). ^{1}H NMR was obtained but it showed to be complex. So, a ^{13}C NMR was obtained in order to support molecules' identification. Three molecules were identified and their spectra were compared to that in the literature.

Results

Taxonomic information of the 25 plants included in the present study is provided in Table 1 and extracts are referred to in the text by their extract number.

Figure 1 represents the comparison among means obtained from inhibition growth zone diameters of the extracts and positive / negative controls, in the disk diffusion assay against 0.5 McFarland inoculum of *E. faecalis*. One-way ANOVA followed by Tukey's post-test indicated that the means were significant different ($F_{(26, 135)}$ = 32.06; $p < 0.0001$; R^2 = 0.8606, 27 groups). Results generated from both DMSO solutions (media = 0.00 mm growth inhibition zone diameters) were not included in statistical analysis due to the lack of homocedasticity if those groups were included. The following treatments showed to be as efficient as SH1%F ($p > 0.05$): 55.PE, 352.PE, 841.PE, 1247.PE, 1257.PE, 1298.PE, 1373.PE, 1389.PE, 1497.PE, 1549.PE, 1743.PE, 1765.PE, 1905.PE, 1923.PE, 1999.PE. The following treatments showed to be significantly more efficient than SH1%F ($p < 0.05$): 1493.PE, 1525.PE and 1991.PE. Considering SH1%C, the following treatments showed equivalent antibacterial activity ($p > 0.05$): 1257.PE, 1298.PE, 1389.PE, 1497.PE, 1525.PE and 1999.PE.Lastly, the following treatments were significantly more efficient than SH1%C ($p < 0.05$): 1493.PE 1991.PE.

Figure 2 represents the comparison among means obtained from inhibition growth zone diameters of the extracts, residues and standard drugs, in the disk diffusion assay against 0.5 McFarland inoculum of *E. faecalis* using one-way ANOVA followed by Tukey's post-test. Results generated from both DMSO solutions (mean = 0.00 mm growth inhibition zone diameters) were not included in statistical analysis due to the lack of homocedasticity if those groups were included. The following treatments showed to be as efficient as SH1%F ($p > 0.05$): 55.BuOH, 321.BuOH, 352.BuOH, 1257.CHCl$_3$, 1257.Aq, 1389.Aq, 1525.Aq and 1991.CHCl$_3$. The following treatments were more effective than SH1%F ($p < 0.05$): 321.Aq, 352.Aq, 841.BuOH, 1257.BuOH, 1259.BuOH, 1298.BuOH, 1389.BuOH, 1493.CHCl$_3$, 1493.BuOH, 1525.BuOH and 1991.BuOH. In relation to SH1% C, the following treatments were statistically equivalent antibacterial activity ($p > 0.05$):
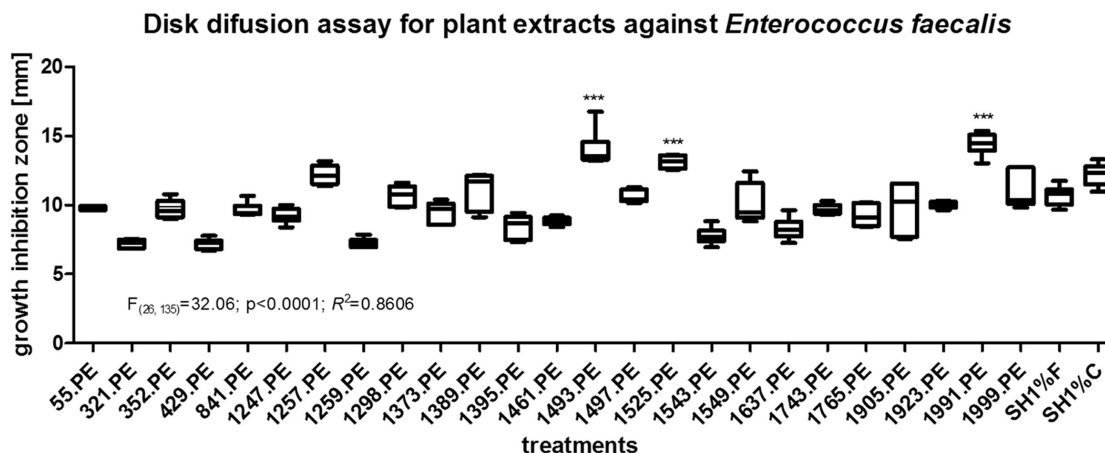

Figure 1 - Two-way ANOVA and Bonferroni's post-test analysis related to growth inhibition zone diameters obtained from the antibacterial activity in disk diffusion assay of plant extracts and their chloroform, butanol and aqueous residues (concentration of 200 mg/mL), formulated and commercial sodium hypochloride 1% (used as positive controls) and dimethylsulfoxide 50% and pure (used as negative control), against *Enterococcus faecalis* ATCC® 29212^{TM}. SH1%F = formulated sodium hypochlorite 1%; SH1%C = commercial sodium hypochlorite 1%. Residues are nominated as XX.CHCL3, XX.BuOH, XX.H2O and XX.PE, where XX corresponds to the extract number. Significance were considered if $p < 0.05$.
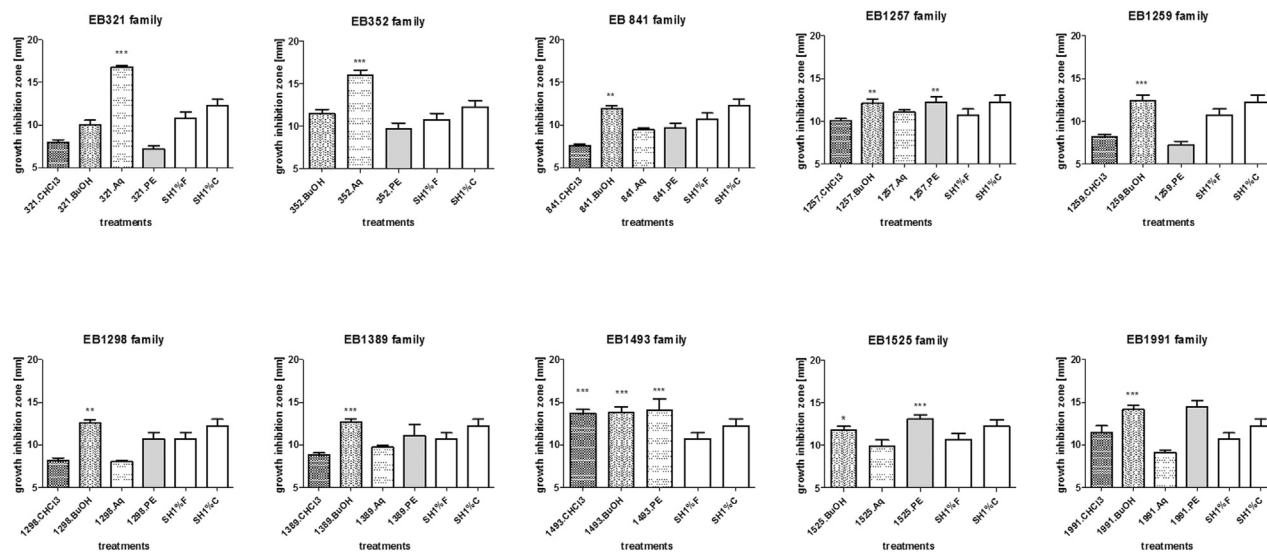
Disk diffusion assay with extracts and their residues against *Enterococcus faecalis*

Figure 2 - One-way ANOVA and Tukey's post-test analysis related to growth inhibition zone diameters obtained from the antibacterial activity in disk diffusion assay of plant extracts, residues (concentration of 200 mg/mL), formulated and commercial sodium hypochloride 1% (used as positive controls), against *Enterococcus faecalis* ATCC® 29212™. SH1%F = formulated sodium hypochlorite 1%; SH1%C = commercial sodium hypochlorite 1%. Significance were considered if p < 0.05.

55.BuOH, 352.BuOH, 841.BuOH, 1257.BuOH, 1259.BuOH, 1298.BuOH, 1389.BuOH, 1525.BuOH and 1991.CHCl₃. Finally, the following treatments were more effective than SH1%C (p < 0.05): 321.Aq, 352.Aq, 1493.CHCl₃, 1493.BuOH and 1991.BuOH.

Table 2 shows the antioxidant activity of residues. Antioxidant compounds are prone to be found in residues BuOH obtained from extracts EB 55, EB 841, EB 1257, EB 1373, EB 1389, EB 1525, EB 1905, EB 1991 and EB 1999. Also, antioxidant compounds are prone to be found in residues CHCl₃ obtained from extract EB 1637 and in residues H₂O obtained from extracts EB 1525 and EB 1905.

TLC analysis of EB 321, EB 352, EB 1257, EB 1493, EB 1525, EB 1765, EB 1991 and EB 321 indicated that phenolic compounds might be present, once a weak blue fluorescence could be seen after NP and uv356 development. Triterpenes or essential oils (due to sulfuric acid development) may be present in EB 321. EB 352 may also have phenolic compounds (due to the yellow spots that could be seen after NP and uv356 development) and triterpenes or essential oils. EB 1257 may have coumarins or anthrone-like compounds, once the development with KOH showed a brown spot under visible light, and may have phenolic compounds related to orange spots observed after NP and uv 356 development. Triterpenes may also be present in this extract. EB1493 may have triterpenes as major compounds, once only the sulfuric acid solution revealed the the chromatogram. EB 1525 may have coumarins and triterpenes. EB 1765 may have antrone-like compounds (development under KOH and visible light), a

brown spot was observed after development with Dragendorff's Reagent and NP followed by uv356 observation showed two orange and one yellow-green spots, suggesting the presence of phenolic compounds. Lastly, EB 1991 showed a yellow spot after development with KOH, suggesting anthrone-like compounds, 3 orange and 1 yellow-green spot, after development with NP and uv356.

EB 1257 was fractionated and compounds lupeol, β-amyrin and 3β-hydroxyglutin-5-ene were isolated (Figure 3) and their structure was identified by ¹³C NMR (125 MHz, CDCl₃) analysis and comparison with literature (Table 3).

Discussion

E. faecalis is an important pathogen related to diseases that affect the oral cavity. In endodontic infections, especially in recurrent infection, its presence is commonly associated with treatment failure (Kayaoglu *et al.*, 2011; Cowan, 1999). SH is a compound of choice in Dentistry, and is widely used in different concentrations, in Endodontics. Nonetheless, the compound has undesirable effects, which are often disregarded when these substances are elected because of their antimicrobial therapeutic benefit. Therefore, substances that have similar therapeutic efficacy and fewer side effects should be tracked and used in dental therapy. Natural products, such as propolis (Kayaoglu *et al.*, 2011), are being investigated for their antibacterial activity.

Two experimental models were selected to evaluate the potential antimicrobial activity of plant extracts against

Table 2 - Results corresponding to the antioxidant activity of residues, which represent reaction to β-caroten.

Extract number	CHCl₃	BuOH	H₂O	Extract number	CHCl₃	BuOH	H₂O
55	-	X	-	1493	-	-	-
321	-	-	-	1497	-	-	-
352	-	-	-	1525	-	X	X
429	-	-	-	1543	-	-	-
841	-	X	-	1549	-	-	-
1247	-	-	-	1637	X	-	-
1257	-	X	-	1743	-	-	-
1259	-	-	-	1765	-	-	-
1298	-	-	-	1905	-	X	X
1373	-	X	-	1923	-	-	-
1389	-	X	-	1991	-	X	-
1395	-	-	-	1999	-	X	-
1461	-	-	-				

CHCl₃ = chloroformic residue; BuOH = butanolic residue; H₂O = aqueous residue; H = horizontal; V = vertical. (-) = not antioxidant; X = antioxidant.
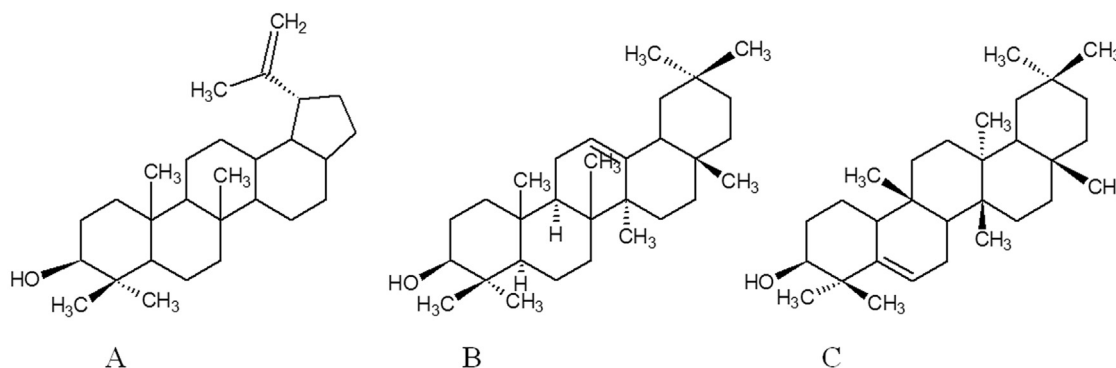
Figure 3 - Molecular structure of UNIP-147 isolated compounds (A) lupeol, (B) β-amyrin and (C) 3β-hydroxyglutin-5-ene.

E. faecalis: DDA and MDBA, understanding that both together might contribute to the prospection of new lead compounds in a complementary way. It is not the scope of the present manuscript to determine which assay is more suitable to be used - and discussions on the subject have widely been made before (Cowan, 1999). Briefly, natural products are complex mixture of substances that have different physical-chemical characteristics. Such differences may interfere with the characteristics of each DDA or MDBA assay due to the way chemicals can diffuse (in DDA) or dilute (in MDBA) in each type of medium. The use of both experiments seems to be complementary so far.

Based on analyses done with extracts in MDBA (Table 1) - which compared data from various concentrations of treatments tested against bacterial concentration of 1.0 x 10e5 CFU/mL, it was possible to identify three promising extracts: EB 1493, EB 1637 and EB 1765. EB 1493 was obtained from aerial organs of *Ipomoea alba* L. (Convolvulaceae). *I. alba* was barely studied in terms of its chemical constituents or pharmacological activity. The use of its latex was reported (Hosler *et al.*, 1999). Sweet potato is one of the species belonging to *Ipomoea* genus. Clavine alkaloids occur in *I. muricata* (Maruia and Srivastava, 2009), as well as ipobscurines that are indol alkaloids from the macrolactam-type (Jenett-Siems *et al.*, 2003) and alkaloids occur in some toxic species, as in *I. carnea* (Haraguchi *et al.*, 2003). Other compounds as anthocyanins (Steed and Truong, 2008) and polyphenolic compounds (Kurata *et al.*, 2007) also occur in *Ipomoea* species.

Extract 1637, obtained from *Diclinanona calycina* (Annonaceae), has shown a significant activity in the assay. Previous study made with *D. calycina*, tested against diverse microorganisms in an agar-diffusion model, has shown its activity against *Streptococcus oralis* and *Myco-*

Table 3 - ^{13}C NMR (125 MHz, CDCl$_3$) data for sample UNIP-147.

Carbon	δ (ppm)					
	lupeol (Mahato *et al*, 1994)	UNIP-147	β-amyrin (Mahato *et al*, 1994)	UNIP-147	3β-hidroxiglutin-5-ene (Gonzalez *et al.*, 1987)	UNIP-147
1	38.7	38.72	38.7	38.6	18.3	18.22
2	27.4	27.46	27.3	27.38	27.93	27.98
3	78.9	79.04	79	79.02	76.44	76.37
4	38.8	38.93	38.8	38.86	40.90	40.84
5	55.3	55.30	55.3	55.19	141.76	141.61
6	18.3	18.32	18.5	18.42	122.10	122.05
7	34.2	34.29	32.8	32.78	23.74	23.54
8	40.8	40.80	38.8	38.96	47.55	47.24
9	50.4	50.45	47.7	47.64	34.94	34.84
10	37.1	37.17	37.6	37.84	49.83	49.69
11	20.9	20.94	23.6	23.64	33.24	33.12
12	25.1	25.15	121.8	121.74	30.45	30.36
13	38	38.07	145.1	145.19	37.95	37.84
14	42.8	42.84	41.8	41.72	39.40	39.30
15	27.4	26.95	26.2	26.16	34.72	34.61
16	35.5	35.59	27	27.19	35.19	35.25
17	43	43.01	32.5	32.49	30.18	30.09
18	48.2	48.32	47.4	47.43	43.21	43.07
19	47.9	47.99	46.9	46.84	35.19	35.08
20	150.9	150.96	31.1	31.09	28.32	28.14
21	29.8	29.69	34.8	34.75	32.2	32.08
22	40	40.01	37.2	37.29	30.05	29.85
23	28.0	28.06	28.2	28.25	29.04	28.97
24	15.4	15.39	15.5	15.55	25.51	25.45
25	16.1	16.12	15.6	15.61	16.26	16.21
26	15.9	15.94	16.9	16.82	18.47	18.39
27	14.5	14.57	26	26.00	19.69	19.62
28	18	18.01	28.4	28.41	32.46	32.41
29	109.3	109.34	33.3	33.35	32.12	32.05
30	19.3	19.31	23.7	23.71	34.6	34.53

bacterium smegmatis (Carneiro *et al.*, 2008). Annonaceae species were chemically studied before. Alkaloids (Pérez and Cassels, 2010) and acetogenins (McLaughlin, 2008) are the main classes of active compounds occurring in the family.

DDA showed a different group of active extracts: EB 1257, EB 1493, EB 1525 and EB 1991. Extracts 1257 and 1991 were obtained from aerial organs of *S. globulifera*, Clusiaceae in different periods of time. Chemistry of *Ipomoea* (EB 1493) species was previously discussed. Extract 1525 was obtained from the stem of *Connarus ruber* var. *ruber* (Connaraceae). It was observed that there is a lack of chemical data related to the genus *Connarus*. Extracts EB 1257 and EB 1991 were obtained from the same species, *S. globulifera*, Clusiaceae, but plants were collected in different periods of time. Reports relate biological activity maybe related to the presence of polycyclic polyprenylated acylphoroglucinols and oxidized derivatives (Marti *et al.*, 2010). Xanthones are also present in the species (Pana *et al.*, 2010). Chemical studies were performed with EB 1257 due to extract availability. So, isolation and identification of major compounds were performed and re-

Extract EB 1765 was obtained from the flowers of *Moronobea coccinea*, a species belonging to Clusiaceae. Polycyclic polyprenylated acylphoroglucinols and oxidized derivatives (Marti *et al.*, 2010) were isolated from *M. coccinea*, as well as xanthone derivatives and flavonoids (Mkounga *et al.*, 2009).

sulted in lupeol, α-amyrin and 3β-hydroxyglutin-5-ene as isolated compounds, widely known for their biological and pharmacological activities.

All 25 extracts were submitted to an initial fractionation, resulting in three residues to each extracts: $CHCl_3$, BuOH and H_2O. It was observed that after extracts 321 and 352 were fractionated, a significant improvement of the antibacterial activity could be observed in their H_2O residue. For that reason, both extracts were also chosen to be studied in a near future. Extract 321, obtained from the leaves of *Psidium densicomum* (Myrtaceae), belongs to a group of plants (guajava family) well known to contain terpenes, triterpenoids and flavonoids (Ho *et al.*, 2012; Gutierrez *et al.*, 2008). Extract 352 was obtained from aerial parts of *Stryphnodendron pulcherrimum* (Fabaceae), species taxonomically related to a medicinal plant known in Brazil as *barbatimao*, that was already studied against oral pathogens (Pereira *et al.*, 2011) to contain tannins as the main chemical class of compounds (Santos *et al.*, 2002).

As fractionation of the 25 extracts was done exactly in the same way to obtain residues, added to the fact that all residues were tested at the same time, in both biological assays, it is possible to suggest a comparative analysis based on the results obtained from the biological assays of residues. Antibacterial results obtained from the residues when compared to their original extracts may take different courses: (a) biological activity has been missing during fractionation, and synergism was lost; (b) biological activity is concentrated in one fraction and its antibacterial activity may be improved or not, in relation to the original extract; (c) biological activity appears in two or more fractions and the antibacterial activity may have improved or not, in relation to the original extract. Based on that premises, a large number of extracts can be considered for further analyses, which is profitable but time-consuming. For that reason, it is primordial to establish a method according to which the extracts and their residues are going to be compared among each other, and the more adequate way is to rank MIC's and MBC's values of treatments when MDBA is used, and to compare growth inhibition zones of treatments by analysis of variance ANOVA, as applied in the present work when DDA is used. If that idea is applied, it is possible to create a ranking establishing which extract and/or residue is best to be prioritized in the studies.

As the current study aims the identification of a new compound to be used in root canal treatment, it is important to compare the efficacy of extracts treatments to SH, which is one of the compounds of choice, for this application. SH was prepared at the concentration of 1% to be used as standard drug, despite its wide range of concentrations found in literature, ranging from 1 to 5.25% that are available in the marketing. Studies reporting the efficacy of SH in biofilms obtained from *E. faecalis* and/or *S. mutans* were done and this compound showed better efficacy when used against biofilms obtained from planktonic cultures (Jiang *et al.*,

2011). Although a direct comparison between SH and plant extracts is not feasible, since SH is an isolated compound and plant extracts are highly complex mixtures of compounds of various origins, the results obtained for SH provide an important reference for a comparative, albeit indirect, analysis of the antibacterial activity of extracts. Moreover, it is highly expected that when active substances are isolated from the extracts, they may become more active than the original extract, which is going to be further done.

Antioxidant activity was also evaluated, and resulted that 55.BuOH, 841.BuOH, 1257.BuOH, 1373.BuOH, 1389.BuOH, 1525.BuOH, 1525.H_2O, 1637.$CHCl_3$, 1905.BuOH, 1905.H_2O, 1991.BuOH, 1999.BuOH showed activity in the β-caroten TLC assay. Further evaluation of isolated compounds shall be done in the near future. Chemical data found in the literature is consistent with results we obtained in the TLC analysis of the main chemical classes as phenolic compounds, triterpenes and alkaloids, and will support future isolation procedures to be done with all seven active extracts.

Results related to the efficacy of the extracts against planktonic cultures do not assure that the extracts are going to be as effective against biofilms, and for that reason, further *in vitro* studies regarding the activity of extracts against biofilms are being developed today as well as the evaluation of the extracts in toxicological assays, in order to gather information to support *in vivo* studies (Barrella *et al.*, 2012) with the extracts.

Conclusions

Nature is a potential source of new drugs to be used in Dentistry. In the present work, seven plant extracts obtained from species native to the Amazon rain forest showed significant activity against *E. faecalis* and showed that antioxidant compounds may be present as well, indicating that possibly phenolic compounds may be involved in antibacterial activity. Also, statistical design here proposed also contributes to optimize the decision-making of which extract shall be prioritized for chemical studies so as to isolate active compounds that might be introduced in endodontic therapy.

Acknowledgments

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