In vitro activity of Amazon plant extracts against Enterococcus faecalis

Adriana Lígia de Castilho¹, Juliana Paola Correa da Silva¹, Cintia Helena Coury Saraceni¹, Ingrit Elida Collantes Díaz², Mateus Luís Barradas Paciencia², Antonio Drauzio Varella², Ivana Barbosa Suffredini¹,²

¹Programa de Graduação em Odontologia, Universidade Paulista, São Paulo, SP, Brazil. ²Laboratório de Extração, Núcleo de Pesquisas em Biodiversidade, São Paulo, SP, Brazil.

Submitted: November 2, 2012; Approved: March 14, 2014.

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 micro-dilution 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 Connasurus ruber var. ruber (13.13 ± 0.18 mm diameter) were active in DDA. Residues H2O 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 ¹³C 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, bio-prospection.

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 (Pastici 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 endodontology 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, tetracyclins). 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 e 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® 29212TM. 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 | MIC (µg/mL) |
|-----------------|---------------------|--------------|-------|--------|--------|---------|-------------|
| 55              | PSC 396             | 05/12/1997   | Leaves| Fabaceae|         | *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* | 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 | Piperaeae |        | *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 |        | *Moronoba coccinea* | 1,300 < MIC < 2,500 |
| 1395            | IBS 143             | 08/12/2001   | Leaves | Fabaceae |        | *Hymanaea 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 duciei* | 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 | Acanthaceae |        | *Dictamnus 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 |        | *Moronoba 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 Faboidea |        | *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 from the antibacterial activity of plant extracts and their residues, $10^6$ colony-forming units/mL (cfu/mL) 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 $10^6$ 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 $10^2$, $10^3$, $10^4$, $10^5$, $10^6$, and $10^7$ cfu/mL, were prepared in sterilized Müeller-Hinton broth medium (Oxoid Ltd, London, England) starting from the $10^8$ 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 $10^6$ 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-carotene (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 CHCl3, 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 1H NMR and 13C NMR, in CDCl3.

Fraction 13 (or UNIP147) was obtained as a crystal cream sample (30 mg). 1H NMR was obtained but it showed to be complex. So, a 13C 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, residues and standard drugs, 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; R² = 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 and 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.CHCl3, 1257.Aq, 1389.Aq, 1389.Aq, 1525.Aq and 1991.CHCl3. 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.CHCl3, 1493.BuOH, 1525.BuOH and 1991.BuOH. In relation to SH1%C, the following treatments were statistically equivalent antibacterial activity (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 1905, 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 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 Drangedorff’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...
$E. \text{faecalis}$: 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 $1.0 \times 10^5 \text{CFU/mL}$, it was possible to identify three promising extracts: EB 1493, EB 1637 and EB 1765. EB 1493 was obtained from aerial organs of $Ipomoea \text{alba}$ L. (Convolvulaceae). $I. \text{alba}$ 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 $I. \text{muricata}$ (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 $I. \text{carnea}$ (Hara-guchi 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 $Diclinanona \text{calycina}$ (Annonaceae), has shown a significant activity in the assay. Previous study made with $D. \text{calycina}$, tested against diverse microorganism in an agar-diffusion model, has shown its activity against $\text{Streptococcus oralis}$ and Myco-

| Table 2 - Results corresponding to the antioxidant activity of residues, which represent reaction to β-caroten. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Extract number  | CHCl$_3$ | BuOH | H$_2$O | Extract number  | CHCl$_3$ | BuOH | H$_2$O |
| 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            | -      | -    | -     |                 |        |      |       |

CHCl$_3$ = chloroformic residue; BuOH = butanolic residue; H$_2$O = 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.
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.

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

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. Ex-
tract 1525 was obtained from the stem of Con narus ruber
var. ruber (Con naraceae). It was observed that there is a
lack of chemical data related to the genus Con narus.
Extracts EB 1257 and EB 1991 were obtained from the same
species, S. globulifera, Clusiaceae, but plants were col-
lected in different periods of time. Reports relate biological
activity maybe related to the presence of polycyclic poly-
preneylated acylphoroglucinols and oxidized derivatives
(Marti et al., 2010). Xanthones are also present in the spe-
cies (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-

| Carbon       | luteol   | β-amyrin | 3β-hydroxihuitin-5-ene |
|--------------|----------|----------|------------------------|
| Number       | (Mahato et al., 1994) | (Mahato et al., 1994) | (Gonzalez et al., 1987) |
| 1            | 38.7     | 38.7     | 38.6                   |
| 2            | 27.4     | 27.3     | 27.38                  |
| 3            | 78.9     | 79.0     | 79.02                  |
| 4            | 38.8     | 38.8     | 38.86                  |
| 5            | 55.3     | 55.3     | 55.19                  |
| 6            | 18.3     | 18.5     | 18.42                  |
| 7            | 34.2     | 32.6     | 32.78                  |
| 8            | 40.8     | 38.8     | 38.96                  |
| 9            | 50.4     | 47.7     | 47.64                  |
| 10           | 37.1     | 37.6     | 37.84                  |
| 11           | 20.9     | 23.6     | 23.64                  |
| 12           | 25.1     | 121.8    | 121.74                 |
| 13           | 38.0     | 145.1    | 145.19                 |
| 14           | 42.8     | 41.8     | 41.72                  |
| 15           | 27.4     | 26.2     | 26.16                  |
| 16           | 35.5     | 27      | 27.19                  |
| 17           | 43      | 32.5     | 32.49                  |
| 18           | 48.2     | 47.4     | 47.43                  |
| 19           | 47.9     | 46.9     | 46.84                  |
| 20           | 150.9    | 31.1     | 31.09                  |
| 21           | 29.8     | 34.8     | 34.75                  |
| 22           | 40      | 37.2     | 37.29                  |
| 23           | 28.0     | 28.2     | 28.25                  |
| 24           | 15.4     | 15.5     | 15.55                  |
| 25           | 16.1     | 15.6     | 15.61                  |
| 26           | 15.9     | 16.9     | 16.82                  |
| 27           | 14.5     | 26      | 26.00                  |
| 28           | 18      | 28.4     | 28.41                  |
| 29           | 109.3    | 33.3     | 33.35                  |
| 30           | 19.3     | 23.7     | 23.71                  |

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

Carbon (Mahato et al., 1994) | UNIP-147 | δ (ppm) |
|-----------------------------|----------|---------|
| Carbon Number 1 | 38.7 | 38.72 | 38.7 |
| Carbon Number 2 | 27.4 | 27.46 | 27.3 |
| Carbon Number 3 | 78.9 | 79.04 | 79.0 |
| Carbon Number 4 | 38.8 | 38.93 | 38.8 |
| Carbon Number 5 | 55.3 | 55.30 | 55.3 |
| Carbon Number 6 | 18.3 | 18.32 | 18.5 |
| Carbon Number 7 | 34.2 | 34.29 | 32.6 |
| Carbon Number 8 | 40.8 | 40.80 | 38.8 |
| Carbon Number 9 | 50.4 | 50.45 | 47.7 |
| Carbon Number 10 | 37.1 | 37.17 | 37.6 |
| Carbon Number 11 | 20.9 | 20.94 | 23.6 |
| Carbon Number 12 | 25.1 | 25.15 | 121.8 |
| Carbon Number 13 | 38 | 38.07 | 145.1 |
| Carbon Number 14 | 42.8 | 42.84 | 41.8 |
| Carbon Number 15 | 27.4 | 26.95 | 26.2 |
| Carbon Number 16 | 35.5 | 35.59 | 27 |
| Carbon Number 17 | 43 | 43.01 | 32.5 |
| Carbon Number 18 | 48.2 | 48.32 | 47.4 |
| Carbon Number 19 | 47.9 | 47.99 | 46.9 |
| Carbon Number 20 | 150.9 | 150.96 | 31.1 |
| Carbon Number 21 | 29.8 | 29.69 | 34.8 |
| Carbon Number 22 | 40 | 40.01 | 37.2 |
| Carbon Number 23 | 28.0 | 28.06 | 28.2 |
| Carbon Number 24 | 15.4 | 15.39 | 15.5 |
| Carbon Number 25 | 16.1 | 16.12 | 15.6 |
| Carbon Number 26 | 15.9 | 15.94 | 16.9 |
| Carbon Number 27 | 14.5 | 14.57 | 26 |
| Carbon Number 28 | 18 | 18.01 | 28.4 |
| Carbon Number 29 | 109.3 | 109.34 | 33.3 |
| Carbon Number 30 | 19.3 | 19.31 | 23.7 |
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: CHCl₃, BuOH and H₂O. It was observed that after extracts 321 and 352 were fractionated, a significant improvement of the antibacterial activity could be observed in their H₂O 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 (guava 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.H₂O, 1637.CHCl₃, 1905.BuOH, 1905.H₂O, 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**

Authors thank FAPESP for their financial support.

**References**

Alviano WS, Alviano DS, Diniz CG, Antoniolli AR, Alviano CS, Farias LM, Carvalho MA, Souza MM, Bolognese AM (2008) *In vitro* antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine. Arch Oral Biol 53:545-552.

Aslangul E, Ruimy R, Chau F, Garry L, Andremont A, Fantin B (2005) Relationship between the level of acquired resistance
to gentamicin and synergism with amoxicillin in *Enterococcus faecalis*. Antimicrob. Agents Chemother 49:4144-4148.

Barrella GE, Suffredini IB, Ribeiro FV, Cirano FR, Pimentel SP (2012) Evaluation of the effect of an organic extract obtained from *Ipomoea alba* L. on experimental periodontitis in rats. Braz Oral Res 26:158-164.

Carneiro AL, Teiceira MF, Oliveira VM, Fernandes OC, Cauper GS, Pohlit AM (2008) Screening of Amazonian plants from Adolpho Ducke forest reserve, Manaus, state of Amazonas, Brazil, for antimicrobial activity. Mem Inst Oswaldo Cruz 103:31-38.

Cowan MM (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12:564-582.

Duggan JM, Sedgley CM (2007) Biofilm formation of oral and endodontic *Enterococcus faecalis*. J Endod 33:815-818.

Estrela C, Silva JA, de Alencar AH, Leles CR, Decúrcio DL, Pimentel SP, Suffredini IB, Ribeiro FV, Cirano FR, Pimentel SP, Barrella GE, Gutierrez RM, Mitchell S, Solis RV (2008) *Hancock HH 3rd, Sigurdsson A, Trope M, Moiseiwitsch J* (2001) *Cowan MM* (1999) Plant products as antimicrobial agents. Clin Microbiol Rev 12:564-582.

Gutierrez RM, Mitchell S, Solis RV (2008) *Psidium guajava*: a review of its traditional uses, phytochemistry and pharmacology. J Ethnopharmacol 117:1-27.

Hancock HH 3rd, Sigurdsson A, Trope M, Moiseiwitsch J (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol and Endod 91:579-586.

Haraguchi M, Gorniak SL, Ikeda K, Minami Y, Kato A, Watson AA, Nash RJ, Molyneux RJ, Asano N (2003). Alkaloidal components in the poisonous plant, *Ipomoea carnea* (Convolvulaceae). J Agric Food Chem 51:4995-5000.

Ho R, Violette A, Cressend D, Raharivelomanana P, Corrupt PA, Hostettmann K (2012) Antioxidant potential and radical-scavenging effects of flavonoids from the leaves of *Psidium cattleianum* grown in French Polynesia. Nat Prod Res 26:274-277.

Hosler D, Burket SL, Tarkanian MJ (1999) Prehistoric polymers: rubber processing in ancient Mesoamerica. Science 284:1988-1991.

Horner R, Liscano MG, Maraschin MM, Salla A, Meneghetti B, Dal Forno NLF, Righi RA (2005) Susceptibildade antimicrobiana entre amostras de *Enterococcus* isoladas no Hospital Universitário de Santa Maria. J Bras Patol Med Lab 41391-41395.

Jiang LM, Hoozenkamp MA, van der Sluis LWM, Wesselink PR, Crieelra W, Deng DM (2011) Resazurin metabolism assay for root canal disinfectant evaluation on dual-species biofilms. J Endod 37:31-35.

Jayaoglu G, Örtstävik D (2004) Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. Crit Rev Oral Biol Med 15:308-320.

Kayaoglu G, Ömrüllü H, Akca G, Gürel M, Gençay Ö, Sorkun K, Sahil B (2011) Antibacterial activity of propolis *vs.* conventional endodontic disinfectants against *Enterococcus faecalis* in infected dentinal tubules J Endod 37:376-381.

Kristich AJ, Li YH, Cvtikvitch DG, Dunny GM (2004) Esp-independent biofilm formation by *Enterococcus faecalis*. J Bacteriol 186:154-163.

Kurata R, Adachi M, Yamakawa O, Yoshimoto M (2007) Growth suppression of human cancer cells by polyphenolics from sweetpotato (*Ipomoea batatas* L.) leaves. J Agric Food Chem 55:185-190.

Love MR (2001) *Enterococcus faecalis* - a mechanism for its role in endodontic failure. Int Endod J 34:399-405.

Marti G, Eparvier V, Moretti C, Prado S, Grellier P, Hue N, Thoison O, Delpech B, Guérin F, Litaudon M (2010) Antiplasmonic benzophenone derivatives from the root barks of *Symphonia globulifera* (Clusiaceae). Phytochemistry 71:964-974.

Maurya A, Srivastava SK (2009) Large-scale separation of clavine alkaloids from *Ipomoea muricata* by pH-zone-refining centrifugal partition chromatography. J Chromatogr B Analyst Technol Biomed Life Sci 877:1732-1736.

McLaughlin JL (2008) Paw paw and cancer: annonaceous acetogenins from discovery to commercial products. J Nat Prod 71:1311-1321.

Mkounga P, Fomum ZT, Meyer M, Bodo B, Nkengfack AE (2009) Globulixanthone F, a new polyoxygenated xanthone with an isoprenoid group and two antimicrobial biflavonoids from the stem bark of *Symphonia globulifera*. Nat Prod Commun 4:803-808.

Pana E, Cao S, Brodie PJ, Miller JS, Rakotondrajaona R, Rato-voson F, Birkinshaw C, Andriantsiferana R, Rasamison VE, Kingston DG (2010) An antiproliferative xanthone of *Symphonia pacciflora* from Madagascar rainforest. Nat Prod Commun 5:751-754.

Pastiici MB, Mencacci A, Moretti A, Palladino N, Maria Lapo-lorca L, Bistoni F, Baldelli F (2008) *In vitro* antimicrobial activity of ampicillin-ceftriaxone and ampicillin-ertapenem combinations against clinical isolates of *Enterococcus faecalis* with levels of aminoglycoside resistance. Open Microbiol J 2:79-84.

Pereira EM, Gomes RT, Freire NR, Aguier EG, Brandão MG, Santos VR (2011) *In vitro* antimicrobial activity of Brazilian medicinal plant extracts against pathogenic microorganisms of interest in Dentistry. Planta Med 77:401-404.

Pérez EG, Cassels BK (2010) Alkaloids from the genus *Duguetia*. Alkaloids Chem Biol 68:83-156.

Pinheiro ET, Gomes BP, Ferraz CC, Sousa EL, Teixeira FB, Souza-Filho FJ (2003) Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J 36:1-11.

Pinheiro ET, Gomes BPA, Drucker DB, Zaat AA, Ferraz CC, Souza-Filho FJ (2004) Antimicrobial susceptibility of *Enterococcus faecalis* isolated from canals of root filled teeth with periapical lesions. Int Endod J 37:756-763.

Pratt DE, Miller EE (1984) A flavonoid antioxidant in Spanish peanuts (*Arachis hypogoea*). JAOCs 61:1064-1067.

Pinto SC, Costa WF, Ribeiro JP, Guimarães DO, Ferri PH, Feirreira HD, Seraphin JC (2002) Tannin composition of *Ipomoea barbatima* species. Fitoterapia 73:292-299.

Souto R, Andrade AFB, Uzeda M, Colombo APV (2006) Prevalence of “non-oral” pathogenic bacteria in subgingival biofilm of subjects with chronic periodontitis. Braz J Microbiol 37:208-215.
Steed LE, Truong VD (2008) Anthocyanin content, antioxidant activity, and selected physical properties of flowable purple-fleshed sweetpotato purees. J Food Sci 73:S215-S21.

Suffredini IB, Varella AD, Oliveira AA, Younes RN (2002) In vitro anti-HIV and antitumor evaluation of Amazonian plants belonging to the Apocynaceae family. Phytomedicine 9:175.

Suffredini IB, Sader HS, Gonçalves AG, Reis AO, Gales AC, Varella AD, Younes RN (2004) Screening of antibacterial active extracts obtained from plants native to Brazilian Amazon rain forest and Atlantic forest. Braz J Med Biol Res 37:379-384.

Suffredini IB, Paciencia MLB, Nepomuceno DC, Younes RN, Varella AD (2006) Antibacterial and cytotoxic activity of Brazilian plant extracts Clusiaceae. Mem Inst Oswaldo Cruz 101:287-290.

Suffredini IB, Paciencia MLB, Varella AD, Younes RN (2006) Antibacterial activity of Brazilian Amazon plant extracts. Braz J Infect Dis 10:400-402.

Wagner H, Bladt S (1996) Plant Drug Analysis. A thin layer chromatography atlas. 2 ed. Springer, Berlin.

Younes RN, Varella AD, Suffredini IB (2007) Discovery of new antitumoral and antibacterial drugs from Brazilian plant extracts using high throughput screening. Clinics 62:763-768.

Younes RN, Varella AD, Suffredini IB (2000) Extração e rastreamento de novas drogas em plantas brasileiras. Acta Oncol Bras 20:15-19.

All the content of the journal, except where otherwise noted, is licensed under a Creative Commons License CC BY-NC.