ORIGINAL RESEARCH

In vitro assessment of antimicrobial activity of *Pothomorphe umbellata* extracts against *Enterococcus faecalis*

Emilio C Sponchiado Jr, Juliana V Pereira, André AF Marques[1], Lucas da Fonseca Roberti Garcia[2], Suzelei C França[3]

Departments of Endodontics, School of Dentistry, Federal University of Amazonas, Manaus, Amazonas, [1]State University of Amazonas, Manaus, AM, [2]Department of Dental Materials and Prosthodontics, Ribeirão Preto School of Dentistry, University of São Paulo, [3]Department of Biochemistry and Immunology, School of Medicine, University of Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil

Received : 02-03-13
Review completed : 01-05-13
Accepted : 20-12-13

ABSTRACT

Background: Due to the complex anatomy of the root canal system, biomechanical preparation is not able to completely eliminate all microorganisms present in the endodontic infections, making it necessary the use of an intracanal medication.
Aim: The aim of this study was to assess the antimicrobial activity of an intracanal medication containing the ethyl-acetate fraction of *Pothomorphe umbellata* against *Enterococcus faecalis*.
Materials and Methods: Fifty seven human maxillary canine teeth were used, of which 54 were infected with *E. faecalis* every 72 h, for 28 days, and cultured for 24 h. Contaminated teeth were randomly separated into three groups (n = 18) and treated as follows: Group I – calcium hydroxide-based medication; Group II – *P. umbellata*-based medication; Group III – contaminated teeth without medication. Three teeth were used as negative control. After 7, 14 and 28 days of treatment, six teeth from each group were assessed for the level of microbial growth after each period of treatment.
Results: The intracanal medication containing *P. umbellata* was effective against *E. faecalis* after 7, 14, and 28 days of treatment without statistically significant difference in comparison to calcium hydroxide treatment (Kruskal-Wallis test, $P > 0.05$).
Conclusion: Ethyl-acetate fraction of *P. umbellate* was efficient against *E. faecalis*, making this phytotherapy a viable option for endodontic treatment.

Key words: antimicrobial activity, calcium hydroxide, *Enterococcus faecalis*, phytotherapy, *Pothomorphe umbellata*

In endodontic infections, the purpose of the treatment procedures is to disinfect root canal systems in order to repair periapical lesions. However, the presence of some microorganisms, such as Enterococcus faecalis makes this a difficult task because it resistant to conventional treatments. This pathogen can grow and survive in a hypotonic, hypertonic medium, at a pH ranging from 2 to 10, in acid and alkaline environments and those lacking nutrients.[1,2] It also has mechanisms enabling it to adhere to the host's cells.[3,4] Furthermore, the anatomy of the root canal system makes the biomechanical preparation not effective in the complete elimination of microorganisms present in its interior, making it necessary the use of an intracanal medication.[5]

Address for correspondence:
Dr. Lucas da Fonseca Roberti Garcia
E-mail: drlucas.garcia@gmail.com

Access this article online	
Quick Response Code:	Website: www.ijdr.in
	DOI: 10.4103/0970-9290.131129

Several substances have been proposed for use as intracanal medication, among these, calcium hydroxide, the most frequently used substance, which became popular due to its antimicrobial properties and biological compatibility.[5-7] However, there have been discussions in literature about the ineffectiveness of calcium hydroxide in inhibiting *E. faecalis* growth.[1,8,9]

Thus, studies have been conducted in the field of phytotherapy with the purpose of seeking new product options that are capable of controlling these microorganisms efficiently.[10] *Pothomorphe umbellata* is a popularly used medicinal plant. In Brazil, there are two species: *P. umbellata* and *Pothomorphe peltata*, known for their choleretic, cholagogic, gastric, and anti-epileptic activities. They are used by lay persons for treating several illnesses, such as diabetes, erysipela and hepatic diseases.[11]

At present, studies are being conducted on some of their pharmacological properties, such as anti-edema, anti-platelet activating factor (platelet anti-aggregation), analgesic, anti-ulcerogenic, anti-malarial,[11] and antimicrobial activities.[12]

Thus, the aim of this study was to assess the *in vitro* antimicrobial activity of an intracanal medication containing the ethyl-acetate fraction of the leaf extracts from *P. umbellata* against E. *faecalis*, in comparison to a calcium-hydroxide-based medication.

MATERIALS AND METHODS

Preparation of *P. umbellata* extract

P. umbelatta leaves were obtained from the medicinal plant collection of the University of Ribeirão Preto in Ribeirão Preto, SP, Brazil, in March 2004.

Its aerial parts were oven-dried with forced air circulation and then ground in an ethanol/water solution (8:2) in an amber glass container for 48 h. The resulting dye was concentrated in a spinning evaporator, and later lyophilized. The fractions were acquired by liquid/liquid partitioning in a separatory funnel, by adding 100 mL of distilled water and hexane, ethyl acetate and butane solvents, successively in an increasing polarity scale: Thus, after evaporation and drying, the hexane, ethyl acetate, butane and aqueous fractions, respectively, were obtained.

Agar-diffusion test

The fractions results from the *P. umbellata* dye partitioning were submitted to the agar diffusion test. First, the samples were prepared for analysis by dissolving 30 mg of each fraction in 1 ml propylene glycol. After *E. faecalis* inoculation (ATCC 29210), the fraction was diluted in sterilized physiological solution in a ratio of 1:10 and spread on plates containing Mueller-Hinton solid medium. Sterilized 5 mm-diameter absorbent paper disks were placed on the Petri dish at equidistant points from each other, and 0.5 ml of the respective bacterial cultures were poured onto each disk. The plates were incubated at 37°C for 24 h. The control sample was embedded separately, using propylene glycol. All procedures were carried out in triplicate in a laminar flow chamber under aseptic conditions.

Tooth selection and biomechanical preparation

For the antimicrobial test, 47 human maxillary canine teeth were used. These were donated by the Tooth Bank of the School of Dentistry of Federal University of Amazonas in accordance with the ethical standards of the Helsinki Declaration. The tooth crowns were sectioned using a high speed motor MS 400 (Dabi Atlante, Ribeirão Preto, SP, Brazil) and #2135 cylindrical diamond drill (KG Soresen, São Paulo, SP, Brazil) under cooling. After this, biomechanical preparation was performed by the crown-down technique, first shaping the apical preparation with a #50 K type file (Dentsply/Maillefer, Ballaigues, Switzerland) up to 1 mm short of the root apex. Next, final irrigation was performed with 2 ml of 17% ethylenediaminetetraacetic acid (Biodinâmica, Ibiporã, PR, Brazil), allowing it to remain for 5 min, followed by 2 ml of distilled water.

Afterwards, the apical foramen were sealed with light polymerized composite resin (Master Fill, Biodinâmica, Ibiporã, PR, Brazil), and the canals were dried with absorbent paper cones and autoclaved at 121°C for 20 min.

Tooth contamination

With the aid of a syringe, 5 µl of the *E. faecalis* microbial suspension prepared with 24-h cultures adjusted to the MaCFarland scale 1 tube were inoculated into the previously autoclaved teeth. This procedure was repeated every 72 h for 28 days. During this period, the teeth were kept in an oven at 37°C. The three teeth that were not contaminated were used as negative control.[8]

In order to confirm tooth contamination after 28 days, each tooth was irrigated with 100 µl of the sterilized saline solution and a #50 sterilized absorbent paper cone was inserted into the root canal and left for 5 min. When exposure time was over, the cone was transferred to a test tube containing 1 ml of saline solution, from which four serial dilutions were made. Aliquots of 25 µl of each dilution were plated on plates containing Mueller-Hinton medium. The colony forming units (CFUs) were counted after 24 h of incubation.

Intracanal medication application

After sample collection to confirm contamination, the root canals were irrigated with 1 ml of saline solution, dried with #50 absorbent paper cones and separated into three groups (n = 18) according to the tested medication. Group I received calcium hydroxide-based medication, Group II *P. umbellate*-based medication (ethyl-acetate fraction) and Group III did not receive any medication (control) in order to assess whether the microorganism would still be viable after the tested periods, without receiving nutrients.

Medications were prepared on a sterilized glass plate, with 24 F spatula used for cements. A mixture of 0.3 ml of propylene glycol solution and 25 mg of calcium hydroxide powder was blended into a paste with a creamy consistency, similar to toothpaste. To prepare the *P. umbellata* medication, 50 mg of the ethyl-acetate fraction was required to achieve an ideal clinical consistency.

A quantity of 5 µl of the medications was placed in the root canal, using a low speed Lentulo drill #35 (Dentsply/Maillefer, Ballaigues, Switzerland). Next, the root canal orifices were sealed with pre-heated gutta-percha and kept in an oven at 37°C.

Microbiological analysis after treatment

After the dressings had been in place for 7 days, 6 teeth from each group were irrigated with 5 ml of the sterilized saline solution and a sterilized absorbent paper cone was inserted into each root canal, and allowed to remain there for 5 min. The cone was transferred to a test tube with 1 ml of saline solution, from which four serial dilutions were performed. From those solutions, 25 µl were taken out and seeded on Petri plates containing Mueller-Hinton medium at 37°C. The plates were kept at room temperature for 2 h, and then incubated at 37°C for 24 h. CFUs were counted after 24 h. The microbiological collection was also performed in one negative-control tooth. The procedures were repeated for the 14 and 28 days periods.

Data regarding the microbiological analysis in teeth were analyzed by means of the values corresponding to the decrease in the bacterial growth for each tooth after using the medications for the different periods. The absence of CFUs was considered, in percentage, as 100% of reduction in bacterial growth.

Statistical analysis

The results of measurements in millimeters of the inhibition haloes were submitted to the normal decline in adherence test, which revealed an unusual sample distribution. The Kruskal-Wallis non-parametric statistical test was performed with a level of significance of 5%, with the aid of Graphpad Prism 4.0 Software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Agar-diffusion test

The mean values of the inhibition haloes, antimicrobial activity and indicators of the dye fractions from *P. umbellata* leaves, obtained from the agar diffusion test are shown in Table 1. The data given in Table 1 demonstrated that the ethyl-acetate fraction was more effective in inhibiting *E. faecalis* growth, with statistically significant difference in relation to the other groups ($P < 0.05$).

Microbiological analysis in root canal

Table 2 shows the percentage of microbial reduction after treatment of Group I (calcium hydroxide-based medication), Group II (*P. umbellate*-ethyl-acetate fraction) and Group III (positive control).

After the period of 28 days of microbial inoculation, the microbiological analysis revealed a mean growth of 819.6×10^5 CFUs for Group I, 790.4×10^5 for Group II and 77.6×10^5 for Group III.

Analysis of the results demonstrated that calcium hydroxide (Group I) and *P. umbellate* based (Group II) intracanal medications provided microbial reduction in all the tested periods, with no statistically significant difference between them ($P > 0.05$). The material collected from the three teeth used as negative control resulted in negative cultures.

Table 1: Mean values, in millimeters, of inhibition haloes exhibited by dye fractions from *Pothomorphe umbellata* leaves

Repetitions	Samples			
	Hexane	Butane	Ethyl-acetate	Propylene glycol
1	0	0	11	0
2	0	6	12	0
3	0	0	6	0
Mean values±SD*	0±0[a]**	2±3.4[b]	9.6±3.2[c]	0±0[a]

*Different lower case letters in line indicate statistically significant difference (Kruskal-Wallis test, $P>0.05$), SD: Standard deviation **$P>0.05$, [a]=0.0004, [b]=0.0012, [c]=0.0018

Table 2: Percentage of microbial reduction in root canal after treatment

Days	Repetitions	Group I (%)	Group II (%)	Group III (%)
7	1	100.00	100.00	0.00
	2	100.00	100.00	0.00
	3	100.00	100.00	0.00
	4	100.00	100.00	0.00
	5	100.00	100.00	0.00
	6	100.00	100.00	0.00
14	7	100.00	99.99	33.00
	8	100.00	100.00	33.00
	9	100.00	100.00	33.00
	10	100.00	99.99	33.00
	11	100.00	100.00	33.00
	12	100.00	100.00	33.00
28	13	100.00	100.00	30.00
	14	100.00	100.00	30.00
	15	100.00	100.00	30.00
	16	100.00	100.00	30.00
	17	100.00	100.00	30.00
	18	100.00	100.00	30.00

DISCUSSION

The teeth were contaminated for 28 days, the time needed to allow higher penetration of microorganisms into the dentinal tubules - the factor that makes disinfecting difficult[8,13] as well as time to analyze the antimicrobial activity of the intracanal medication being formulated. A microbiological analysis was carried out before the intracanal medication was applied, in order to confirm the incidence of viable microorganisms after the long-term period of 28 days of root canal contamination. The teeth from Group III (positive control) presented viable microorganisms throughout the whole experiment. Moreover, it was observed a reduction in the bacterial population in this group because it did not receive nutrients during the treatment period. This confirmed reports that *E. faecalis* has the ability to express proteins that give it the capacity to adapt and survive under different environmental conditions such as surface proteins, adhesion factors (EfaA and Ace), aggregation substance and expression of the gelE gene, which facilitate the adherence of this microorganism to human dentin.[14]

In the agar diffusion test, it was found that the ethyl-acetate fraction presented better antimicrobial activity when compared with hexanic and butanolic fractions; therefore, it was selected as the basis for the intracanal medication being studied.

Despite of the limitations of this *in vitro* test, results obtained for *P. umbellata* (ethyl-acetate fraction) in periods of 7 and 28 days of treatment demonstrated a significant reduction of microorganisms in the main root canal. Furthermore, the amount of microorganisms in the period of 14 days of treatment presented no statistically significant difference when compared to the group in which the calcium-hydroxide-based medication was used ($P > 0.05$).

With regard to the calcium-hydroxide-based medication antimicrobial activity results, inhibition of bacterial growth was found in all the tested periods. The action of calcium hydroxide in eliminating *E.* is supported by the results obtained in similar studies, in which the antimicrobial activity of calcium hydroxide paste against seven bacterial strains, among them *E. faecalis*, were evaluated.[15,16]

The tested calcium-hydroxide pastes were efficient in inhibiting all the strains, and these findings differed from those obtained by Estrela *et al.*,[5] showing that calcium hydroxide was inefficient in inhibiting the growth of *E. faecalis*. However, this may be partly explained, considering the different vehicles used as this paste was incorporated into inert vehicles, thus damaging diffusion of the active culture medium. Moreover, the methodology used in the present study verifies the viability of bacteria in the main canal, in accordance with clinical testing of microbial cultures in endodontic practice. Such fact may be related to the non-solubilization of the paste that did not favor the dissociation of calcium and hydroxyl ions, which are responsible for the antiseptic action.[16]

It is important to keep in mind that the medication used in Group I was prepared with a pure substance, calcium hydroxide, and that in the *P. umbellate*-based medication (Group II) a crude extract fraction, ethyl-acetate, was used. The antimicrobial activity of the ethyl-acetate phase of *P. umbellata* may be related to the presence of some isolated substances, belonging to several classes of secondary metabolites. The isolation of three classes of chemical compounds from this fraction was reported, namely: Amide, C-glycosylflavones and p-coumaric acid.[17] Some of these compounds probably play a role in the growth inhibitory activity of *faecalis* found in the present study. Once these compounds are identified, it will be possible to formulate a medication with these active principals, and therefore, it may be possible to obtain even more satisfactory findings with regard to the antimicrobial activity of *P. umbellata*-based medication.

The effect of the phytotherapeutic agent as an intracanal medication, as far as the inhibitory activity against the microbiota resistant to conventional endodontic treatment is concerned, seems to be quite promising. Nevertheless, for this to be realized, further antimicrobial and biological compatibility tests are required, using longer and shorter time intervals than those used in the present study. Biological compatibility tests may also be promising since the anti-inflammatory property of *P. umbellata* has already been verified.[17-20]

CONCLUSION

Based on the methodology applied and the findings obtained it was possible to conclude that the ethyl-acetate fraction of *P. umbellata* was efficient against *E. faecalis* in the different periods of treatment, making this phytotherapy a viable option for endodontic treatment.

REFERENCES

1. Waltimo TM, Sirén EK, Orstavik D, Haapasalo MP. Susceptibility of oral *Candida* species to calcium hydroxide *in vitro*. Int Endod J 1999;32:94-8.
2. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J 2002;35:221-8.
3. Love RM. *Enterococcus faecalis* – A mechanism for its role in endodontic failure. Int Endod J 2001;34:399-405.
4. Figdor D, Davies JK, Sundqvist G. Starvation survival, growth and recovery of *Enterococcus faecalis* in human serum. Oral Microbiol Immunol 2003;18:234-9.
5. Estrela C, Estrela CR, Pécora JD. A study of the time necessary for calcium hydroxide to eliminate microorganisms in infected canals. J Appl Oral Sci 2003;11:133-7.
6. Estrela C, Sydney GB, Pesce HF, Felippe Júnior O. Dentinal diffusion of hydroxyl ions of various calcium hydroxide pastes. Braz Dent J 1995;6:5-9.
7. Lynne RE, Liewehr FR, West LA, Patton WR, Buxton TB, McPherson JC. *In vitro* antimicrobial activity of various medication preparations on *Enterococcus faecalis* in root canal dentin. J Endod 2003;29:187-90.
8. Estrela C, Pimenta FC, Ito IY, Bammann LL. Antimicrobial evaluation of calcium hydroxide in infected dentinal tubules. J Endod 1999;25:416-8.
9. Estrela C, Rodrigues de Araújo Estrela C, Bammann LL, Pecora JD. Two methods to evaluate the antimicrobial action of calcium hydroxide paste. J Endod 2001;27:720-3.
10. Gentil M, Pereira JV, Sousa YT, Pietro R, Neto MD, Vansan LP, *et al*. *In vitro* evaluation of the antibacterial activity of *Arctium lappa* as a phytotherapeutic agent used in intracanal dressings. Phytother Res 2006;20:184-6.
11. de Ferreira-da-Cruz M, Adami YL, da Espinola-Mendes E, Figueiredo MR, Daniel-Ribeiro CT. The intraperitoneal plasmodium berghei-pasteur infection of Swiss mice is not a system that is able to detect the antiplasmodial activity in the *Pothomorphe* plant extracts that are used as antimalarials in Brazilian endemic areas. Exp Parasitol 2000;94:243-7.
12. Mongelli E, Desmarchelier C, Coussio J, Ciccia G. Antimicrobial activity and interaction with DNA of medicinal plants from the Peruvian Amazon region. Rev Argent Microbiol 1995;27:199-203.
13. Gomes BP, Pinheiro ET, Sousa EL, Jacinto RC, Zaia AA, Ferraz CC, *et al*. *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:247-53.
14. Sedgley CM, Molander A, Flannagan SE, Nagel AC, Appelbe OK, Clewell DB, *et al*. Virulence, phenotype and genotype characteristics of endodontic *Enterococcus* spp. Oral Microbiol Immunol 2005;20:10-9.
15. Pereira JV, Bergamo DC, Pereira JO, França Sde C, Pietro RC, Silva-Sousa YT. Antimicrobial activity of *Arctium lappa* constituents against microorganisms commonly found in endodontic infections. Braz Dent J 2005;16:192-6.

16. Amorim Lde F, Toledo OA, Estrela CR, Decurcio Dde A, Estrela C. Antimicrobial analysis of different root canal filling pastes used in pediatric dentistry by two experimental methods. Braz Dent J 2006;17:317-22.
17. Perazzo FF, Souza GH, Lopes W, Cardoso LG, Carvalho JC, Nanayakkara NP, *et al*. Anti-inflammatory and analgesic properties of water-ethanolic extract from *Pothomorphe umbellata* (Piperaceae) aerial parts. J Ethnopharmacol 2005;99:215-20.
18. Brohem CA, Sawada TC, Massaro RR, Almeida RL, Rivelli DP, Ropke CD, *et al*. Apoptosis induction by 4-nerolidylcatechol in melanoma cell lines. Toxicol *In Vitro* 2009;23:111-9.
19. da Silva VV, Ropke CD, Miranda DV, de Almeida RL, Sawada TC, Rivelli DP, *et al*. Photoprotective effect of *Pothomorphe umbellata* on UVB radiation-induced biomarkers involved in carcinogenesis of hairless mouse epidermis. Cutan Ocul Toxicol 2009;28:54-60.
20. Marques AA, Sponchiado EC Jr, Garcia LF, Garrido AD, França SC, Lia RC. Morphological analysis of tissue reaction caused by a new endodontic paste in subcutaneous tissue of rats. J Conserv Dent 2011;14:309-13.

How to cite this article: Sponchiado EC, Pereira JV, Marques AA, Garcia LR, França SC. *In vitro* assessment of antimicrobial activity of *Pothomorphe umbellata* extracts against *Enterococcus faecalis*. Indian J Dent Res 2014;25:64-8.

Source of Support: Nil, **Conflict of Interest:** None declared.