ANTIBACTERIAL ACTIVITY OF A PLANT EXTRACT AND ITS POTENTIAL FOR DISINFECTING GUTTA-PERCHA CONES

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ABSTRACT

This study evaluated the antibacterial activity of Rosmarinus officinalis extract and its potential for disinfecting gutta-percha (GP) cones. In the first experiment, a hydro-alcoholic extract of Rosmarinus officinalis (leaves) in a dilution ratio of 10% m/v was tested against Enterococcus faecalis by using the disk diffusion method. Positive and negative controls were 70% cereal alcohol and antibiotics, respectively. The procedures were performed in triplicate, and the diameters of the zones of growth inhibition were measured with a caliper after 24 h at 37ºC. In the second experiment, the disinfection procedures were evaluated on GP cones artificially contaminated with Enterococcus faecalis. The R. officinalis extract was compared with 2% chlorhexidine digluconate and 2.5% sodium hypochlorite, using a direct exposure test (5 min treatment). Sterilized and non-disinfected cones were used as negative and positive controls, respectively. After 24 h of incubation, bacterial counts were taken. For both experiments, the data were statistically analyzed by Kruskall-Wallis and Tukey's tests ($p< 0.05$). The plant extract produced zones of inhibition comparable to those of tested antibiotics. Significant GP cone disinfection was verified with all disinfectant solutions, with no significant difference between them. R. officinalis extract showed bactericidal effect on Enterococcus faecalis and capacity to disinfect GP cones contamined with it.

Key words: plant extracts; Enterococcus faecalis; gutta-percha.

ATIVIDADE ANTIBACTERIANA E POTENCIAL DE UM EXTRATO VEGETAL PARA DESINFECÇÃO DE CONES DE GUTA-PERCHA

RESUMO

Este estudo avaliou a atividade antibacteriana do extrato de Rosmarinus officinalis e do potencial deste extrato vegetal para a desinfecção de cones de guta-percha (GP). No primeiro experimento, o extrato hidroalcoólico de Rosmarinus officinalis (folhas), em diluição de 10% m/v, foi testado contra Enterococcus faecalis, utilizando o método de disco-difusão em ágar. Os controles positivos e negativos foram o álcool de cereais 70% e antibióticos, respectivamente. Os procedimentos foram realizados em triplicata e os diâmetros de halos de inibição foram mensurados com um paquímetro, após 24 horas a 37 º C. No segundo experimento, os procedimentos de desinfecção foram avaliados em cones de GP artificialmente contaminados com Enterococcus faecalis. O extrato de R. officinalis foi comparado com o gluconato de clorexidina a 2% e com hipoclorito de sódio a 2,5% através de um teste de exposição direta por 5 minutos. Cones esterilizados e cones não desinfectados foram utilizados como controles negativo e positivo, respectivamente. Após 24h de incubação, as contagens bacterianas foram enumeradas. Em ambos os experimentos, os dados foram analisados estatisticamente pelos testes de Kruskall-Wallis e Tukey ($p < 0,05$). O extrato vegetal apresentou halos de inibição semelhantes aos antibióticos testados. Expessiva descontaminação dos cones foi verificada com todas as soluções desinfetantes, sem diferenças significativas entre elas. O extrato de R. officinalis mostrou efeito bactericida sobre Enterococcus faecalis e capacidade de desinfetar cones de GP contaminados com este microrganismo.

Palavras-chave: Extratos vegetais, Enterococcus faecalis, guta-percha.

INTRODUCTION

The prevention of the dissemination of microorganisms throughout the root canal system and periapical tissues is essential for successful endodontic therapy. During endodontic procedures, care must be taken to avoid root canal cross-contamination by the instruments or filling materials[1]. Gutta-percha (GP) cones, which are widely used to fill root canals, may become contaminated by pathogens during the handling and/or storage processes in clinics[2-4]. Sterilization by

heat may alter the mechanical properties of GP cones and compromise their sealing ability[5]. Thus, several chemical methods have been used for disinfecting filling material.

The most efficient chemical disinfectants used to sterilize GP cones are sodium hypochlorite (NaOCl) and chlorhexidine (CHX) [2,3,6-8]. However, it has been reported that these agents may also alter the mechanical properties of GP cones[3,9]. Furthermore, some studies showed that NaOCl and CHX were not effective in eliminating *Bacillus subtilis* and *Enterococcus faecalis* on GP cone surfaces[2,6]. Thus, the development of alternative agents for disinfection of GP cones has been recently reported[10].

Studies involving herbal medicines have increased over recent decades, seeking therapeutic agents with proven efficacy, safety and quality. In dentistry, phytomedicine has been used to provide anti-inflammatory, antimicrobial, analgesic and sedative agents, and also endodontic irrigants[11-14]. However, few studies have evaluated the use of medicinal plant extracts for disinfecting GP cones. *Rosmarinus officinalis (*rosemary) is a widespread plant in Brazil (known there as *alecrim*) that has antibacterial and antifungal effects[15-17]. The extract may offer an alternative for disinfection of GP cones.

Thus, the aim of this study was to evaluate: 1) the antibacterial activity of *Rosmarinus officinalis* extract against *Enterococcus faecalis*; and 2) its potential for disinfection of GP cones artificially contaminated with *Enterococcus faecalis*. It was hypothesized that the efficacy of *Rosmarinus officinalis* extract on GP cones contaminated with *Enterococcus faecalis* is similar to that of other solutions commonly used for this purpose.

MATERIALS AND METHODS

Plant material

Rosmarinus officinalis leaves were collected from an experimental field of the School of Agronomical Science, Federal University of Minas Gerais (UFMG), Montes Claros, MG, Brazil. A voucher specimen of the plant is deposited in the Herbarium of the Department of Botany (UFMG).

Screening for antibacterial activity

Initially, the antibacterial activity of *Rosmarinus officinalis* leaf extract against *Enterococcus faecalis* (ATCC 4083) was verified by using a disk diffusion method. Leaves of *Rosmarinus officinalis* were collected and transferred to the Laboratory of Epidemiology and Biocontrol of Microorganisms of State University of Montes Claros (Montes Claros, MG, Brazil). The plant was identified and the leaves were dehydrated at 40ºC for 48 h. After drying, the samples were kept in sealed plastic bags in a refrigerator.

The dried leaves were ground into powder using a Willey-type mill. Afterwards, the powdered leaves were submitted to extraction with 70% v/v cereal alcohol (hydro-alcoholic extract). A ratio of 10% m/v was used for dilution. The solution was stored at ambient temperature, protected from light exposure, and periodically agitated for 48 h, after which the samples were vacuum filtered and stored at 4ºC in airtight bottles until they were used.

The selected bacteria was plated on agar plates containing Brain Heart Infusion (BHI) (Difco Laboratories; Detroit, MI, USA) and incubated at 37ºC for 24 h. Microbial suspensions were prepared in 5 ml of sterile saline to obtain a turbidity equivalent to tube 0.5 of the Mc Farland scale, which corresponds to 1.5 x 10^8 CFU/mL.

The inocula were distributed in Petri dishes (150x20 mm) containing Mueller Hinton agar using a sterile swab and left to dry for about 15 min, after which discs of sterile filter paper (6 mm) soaked in hydro-alcoholic extract or antibiotics (negative controls) were placed in the dishes. The antibiotics used were amikacin, amoxicillin and ampicillin. A solution of 70% v/v cereal alcohol was used as positive control. All procedures were performed in triplicate. The zone of inhibition was measured with a caliper after 24 h at 37ºC.

Efficacy on disinfection of gutta-percha cones

The efficacy of *Rosmarinus officinalis* extract on GP cone disinfection was evaluated using solutions commonly used in endodontic therapy as parameters. Solutions of 2.5% sodium hypochlorite (NaOCl) and 2.0% chlorhexidine (CHX) digluconate (Nature Farm; Montes Claros, MG, Brazil) were selected for this purpose. Sixty medium tapered GP cones (Endopoints Indústria e Comércio Ltda; Rio de Janeiro, RJ, Brazil) were used in this experiment.

Initially, the cones were transferred directly from the package for sterilization in a 5% NaOCl solution (Nature Farm; Montes Claros, MG, Brazil) for 30 min[7] to eliminate any contamination and standardize the samples. Then the GP cones were

individually rinsed with sterilized distilled water in order to remove the NaOCl from their surfaces, dried with sterile gauze and immersed in a 9 cm sterile Petri plate containing 20 ml of the bacterial suspension (~10^8CFU/ml) for 30 min in order to create contamination[7]. The cones were transferred to sterile filter paper pads and dried for 10 min at room temperature under aseptic conditions using a laminar flow hood (Veco; Campinas, SP, Brazil).

The artificially contaminated cones (n=12) were transferred to Eppendorf tubes containing 1.2 ml of one of the disinfecting solutions: 2.5% NaOCl, 2.0% CHX digluconate or *Rosmarinus officinalis* extract. All disinfection procedures were performed for 5 min. Sterilized (without contamination) and non-disinfected cones were used as negative and positive controls, respectively. After the disinfection period of the experimental groups, all samples were transferred to a 10% sodium thiosulphate solution ($Na_2S_2O_3$) to neutralize the disinfectant. In the sequence, the cones were transferred aseptically to Eppendorf tubes (one cone per tube) containing 1.2 ml of sterilized distilled water.

The tubes were centrifuged at a constant speed for 1 min. Aliquots of 0.1 ml were obtained from the suspension and smeared onto BHI agar plates. All plates were incubated at 37ºC for 24 h in aerobic conditions. Bacterial growth was verified by counting colony-forming units (CFU). The scores used were: 0 – absence of CFU; 1 – lower than 300 CFU; and 2 – higher than 300 CFU.

Statistical analysis

Data from both experiments were subject to statistical analysis separately, using the Kruskal-Wallis nonparametric test (α=0.05). Multiple pairwise comparisons were performed using Tukey's post-hoc test.

RESULTS

Table 1 shows the results of the agar diffusion method. The Kruskall-Wallis test had an H-value of 13.8 and p-value of 0.008. The positive control did not show any zone of inhibition, which was statistically lower than other solutions evaluated. There was no statistical difference between the *Rosmarinus officinalis* extract and the antibiotics evaluated. All antibiotics showed similar zones of inhibition.

Table 2 shows the results for efficacy of disinfection of GP cones. The Kruskall-Wallis test had an H-value of 43.9 and p-value < 0.001. The positive control had the highest CFU values. There was no difference between the disinfectant solutions evaluated, while all solutions showed similar values to the negative control.

DISCUSSION

Antibacterial activity of *Rosmarinus officinalis* leaf extract against *Enterococcus faecalis* was confirmed by using the disk diffusion method. The measurement of zones of inhibition on agar plates has been used as the standard assay to test the antibacterial activity of plant extracts[17-19]. The bacterial recovery from all plates of the positive control group revealed that the bacteria survived the experimental period and confirms the efficiency of the methodology. *Rosmarinus officinalis* was also effective in the disinfection of contaminated GP cones, presenting an effect similar to those of disinfectant solutions commonly used for this purpose. The hypothesis of this study was thus confirmed.

The presence of contamination on GP cones taken directly from the manufacturer's sealed package[2,4] justifies their disinfection prior to experimental procedures. Since GP cones may be deformed by heat,

Table 1: Zones of inhibition, in mm.

Group	Median	25%	75%	Tukey
R. officinalis extract	12.0	12.0	14.3	A
Ampicillin	38.0	38.0	38.0	A
Amikacin	26.5	23.9	28.4	A
Amoxicillin	20.0	17.0	21.5	A
Positive control	0.0	0.0	0.0	B

Same letters mean absence of statistically significant difference at $p < 0.05$.

Table 2: Disinfection of gutta-percha cones after treatment with substance tested and controls.

Group	Median	25%	75%	Tukey
NaOCl	0.0	0.0	0.0	B
CHX	0.0	0.0	0.0	B
R. officinalis	0.0	0.0	0.0	B
Positive Control	2.0	2.0	2.0	A
Negative Control	0.0	0.0	0.0	B

0.0 = absence of colony-forming units (CFU)
2.0 = higher than 300 CFU
Same letters mean absence of statistically significant difference at $p < 0.05$.

it has been proposed that samples should be sterilized with ethylene oxide[8] or chemical solutions[7] for standardization before artificial contamination with well-defined protocols. In this study, the cones were sterilized with 5% NaOCl and rinsed with sterile saline solution to avoid any residual activity of NaOCl which might affect the results of the experiment. Conversely, the contact of GP cones with the microbial suspension for 30 min was sufficient to promote their surface contamination, as observed in all positive control samples.

Enterococcus faecalis was selected for this study because it is the main pathogen associated with persistent endodontic infections and has frequently been used in experimental studies for verification of the effectiveness of disinfecting substances on contaminated GP cones[7,8]. NaOCl and CHX are frequently used to eliminate this microorganism in endodontic procedures[2,3,13,19], justifying the choice of these agents as parameters for the action of *Rosmarinus officinalis* extract. The antibacterial activity of NaOCl is mainly related to the liberation of active chlorine and its action on bacterial essential enzymes, promoting lipid peroxidation of the bacterial cytoplasmatic membrane[20,21]. On the other hand, CHX presents a broad spectrum of antimicrobial activity, substantivity and low toxicity; it is also capable of causing irreversible metabolic damage in bacterial cells[2,21].

In the present study, 2.5% NaOCl solution for 5 min was effective for disinfecting the GP cones. The CFU counts for GP cones disinfected with NaOCl were similar to those for sterilized cones. This agrees with the findings of another laboratory study[7] that reported complete bacterial elimination using NaOCl in a lower concentration (1%) for 5 min. However, another investigation[2] showed that 2.5% NaOCl only sterilized the GP cones after 10 min contact with the bacterial cells of an *Enterococcus faecalis* strain. A 2.0% CHX digluconate solution was also able to eliminate the bacterial cells deposited on the GP cones. Other studies[2,3,7] also reported that 2.0% CHX quickly killed a number of microorganisms found on the contaminated GP cone surfaces, including *Enterococcus faecalis*. Similarly to NaOCl and CHX solutions, the use of *Rosmarinus officinalis* extract for 5 min was shown to be effective in disinfection of GP cones. *Rosmarinus officinalis* is a plant of the *Lamiaceae* family and is also used as an aromatic to extend the shelf life of foods[22]. Its mechanism of action in the bacterial cell is probably related to the presence of carnosic acid and carnosol, which can disturb the bacterial membrane[16]. Some studies showed antifungal and antibacterial activity of this plant extract against *Bacillus subtilis*, *Candida albicans*, *Listeria monocytogenes* and *Staphylococcus aureus*[17]. Another investigation[23] demonstrated the bactericidal efficacy of the essential oils from *Rosmarinus officinalis* for Gram-positive (*Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*) bacteria. The fungicidal effect against *Candida albicans* was also verified[15,23].

In summary, *Rosmarinus officinalis* extract showed bactericidal effects against *Enterococcus faecalis* and the capacity to disinfect GP cones contaminated with it. Based on these outcomes, it seems that *Rosmarinus officinalis* can potentially be used during endodontic practice for disinfection of GP cones. However, further studies are needed to verify the antimicrobial action of *Rosmarinus officinalis* extract against other microorganisms that may contaminate GP cone surfaces.

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