IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 17, Issue 6 Ver. 3 (June. 2018), PP 19-24
www.iosrjournals.org

Antimicrobial efficacy of commercially available plant essential oils with calcium hydroxide as intracanal medicaments against Enterococcus faecalis: An in-vitro study

Garima Tiwari[1], Sudha Patil[2], Prashant Bondarde[3], Swapnil Khadke[4], Rupali Gakhare[5]

[1, 4, 5] (PG student, Department of Pedodontics, ACPM Dental College, Dhule, India)
[2](Professor, Department of Pedodontics, ACPM Dental College, Dhule, India)
[3](Professor and Head, Department of Pedodontics, ACPM Dental College, Dhule, India)
Corresponding author: Garima Tiwari

Abstract: ***Introduction:*** *Persistent root canal infection by E. faecalis is the greatest hurdle to successful endodontic treatment. Plant essential oils possess strong antimicrobial activity that can be harnessed to eliminate this bacterium from the root canal environment.* ***Methods:*** *E. faecalis (ATCC 29212) was cultivated in BHI broth and incubated at 37°C for 24 hours. Using cotton swabs, the bacterial suspension was inoculated on 30 Mueller Hinton Agar plates. Four wells were made in each agar plate and were filled with the test medicaments and incubated at 37°C for 72 hours. Group A: Aniba rosaeodora and calcium hydroxide; Group B: Origanum vulgare and calcium hydroxide; Group C: Calcium hydroxide alone and Group D: Triple antibiotic paste. The zones of inhibition were obtained using Kirby-Bauer well diffusion method.* ***Results:*** *All the medicaments were found to be effective in inhibiting E. faecalis. Maximum inhibition was noted with Group D (27.4 ± 1.5 mm) followed by Group B (25.0 ± 1.0 mm) and Group C (22.2 ±0.9 mm). Group A showed least inhibitory activity.* ***Conclusion:*** *Essential oils in combination with calcium hydroxide can be used as an effective intracanal medicament against E. faecalis to prevent reinfection and improve longevity of root canal treatment.*

Date of Submission: 23-05-2018 Date of acceptance: 05-06-2018

I. Introduction

Complete debridement and disinfection of the root canals from pathogenic microorganisms and their toxins are pre-requisites for successful endodontic treatment.[1] However, complex root canal anatomy tends to shelter microorganisms by providing an ideal environment to survive and continue their pathological process. The inherent antibacterial limitations of endodontic materials add to the challenge of obtaining a disinfected state in the root canal system.

Enterococcus faecalis is an extremely resistant strain of microorganism that persists even after treatment, frequently causing reinfections. 24% to 77% of endodontic failures are attributed to this microorganism.[2] It resists endodontic treatment by virtue of various survival and virulence factors, viz its ability to compete with other microorganisms, invade dentinal tubules, form biofilms and resist nutritional deprivation. E. faecalis can gain entry into the root canal system during treatment, between appointments, or even after the treatment has been completed.[2] This makes it necessary to consider treatment regimens that aim at eliminating or preventing the infection of E. faecalis at each phase.[3] A medicament that would remain within the confines of the root canals and eliminate E. faecalis is thus essential for clinical success.

Calcium hydroxide, a commonly used intracanal medicament, when used alone, has been proven ineffective at eliminating E. faecalis. This is due to its inability to maintain an alkaline pH for the entire duration of the dressing.[2] Triple antibiotic paste, a combination of three potent antimicrobials has proven its efficacy against E. faecalis.[4] However, the risk of development of resistant bacterial strains remains.

Plant essential oils have been found to possess excellent pharmacological- antibacterial and antifungal activity, thus, are a promising source of new natural drugs.[5] Oregano oil and rosewood oil have shown remarkable activity against E. faecalis when tested in vitro.[5-7] The biological profile of these oils is due to a combination of component molecules hence, resistance has not yet been reported.[1] Plant essential oils in combination with antibiotics have shown synergistic antimicrobial efficacy against various micro-organisms including E. faecalis.[6]

This study aimed to evaluate the in-vitro antibacterial efficacy of rosewood oil (Aniba rosaeodora) and oregano oil (Origanum vulgare) in combination with calcium hydroxide as a new intracanal medicament to eliminate E. faecalis. Triple antibiotic paste routinely used for canal sterilization can be regarded as a standard to compare antimicrobial activity of test medicaments.

II. Material And Methods

Preparation of the test medicaments:

Rosewood oil- Aniba rosaeodora (Dr. Jain's Forest Herbals Pvt. Ltd.) and Oregano oil- Origanum vulgare (Dève herbes) were procured from the retail market. Calcium hydroxide powder (Deepashree products) was used in combination with the essential oils for preparation of the medicament. Calcium hydroxide powder was mixed separately with each of the essential oils on a sterile glass slab using a sterile mixing spatula in 1:1 (w/v) ratio.

Calcium hydroxide powder was mixed with normal saline (Sodium chloride 0.9% w/v) to prepare the routinely used intracanal medicament. Triple antibiotic paste- Ciprofloxacin + Metronidazole + Minocycline (1:1:1) was freshly prepared by mixing with normal saline to obtain a thick paste. Standard strain of Enterococcus faecalis (ATCC 29212) was used to test the antimicrobial efficacy of these medicaments.

Cultivation of E. faecalis:

Standard strain of Enterococcus faecalis (ATCC 29212) (HiMedia, Mumbai) spores were grown and maintained in 25 ml of Brain Heart Infusion (BHI) broth (HiMedia Laboratories, Mumbai) by incubating at 37^oC for 24 hours, as per manufacturer's instructions. Viable bacterial growth was indicated by a change in turbidity of the solution. The broth culture suspension of bacteria was adjusted at a turbidity equivalent to the barium sulfate standard of 0.5 McFarland units (equivalent to $1.5x10^8$ CFU/ml), with sterile BHI taken as standard. (Figure 1)

Preparation of the media:

Mueller Hinton Agar (MHA), being a selective medium for the growth of E. faecalis was chosen. Thirty Mueller-Hinton Agar plates were prepared. A sterile cotton swab was dipped into the BHI bacterial suspension, rotated on the side of the tube to remove surplus and used to inoculate the agar plates. (Figure 2) All the plates were uniformly inoculated by even streaking of the cotton swab in three directions.

Agar well diffusion:

After the inoculums dried, with the aid of a sterile 6 mm cork borer, 4 equally spaced wells were bored in the agar plate.(Figure 3) The agar plugs were discarded using a sterile needle. The wells were filled with the freshly prepared test medicaments. (Figure 4)The experimental groups were:
Group A: Calcium hydroxide + Rosewood oil
Group B: Calcium hydroxide + Oregano oil
Group C: Calcium hydroxide + Normal saline
Group D: Triple Antibiotic Paste

The MHA plates were incubated at 37^oC for 72 hours in an upright position.

Calculation of the Zone of Inhibition:

The MHA plates were examined for zones of inhibition after completion of 72 hours. (Figure 5) Zone of inhibition or clear zone indicated the degree of susceptibility or resistance of the test organism to the antibacterial agent. The point of abrupt diminution of growth which corresponded to the complete growth inhibition was taken as the zone edge. Inhibition zones were measured with the aid of a ruler (mm) in each of the thirty plates.

Statistical analysis

Data was analyzed using SPSS version 20 (SPSS Inc., Chicago, IL). The mean values of the zone of inhibition of the four different groups were compared using ANOVA test. Pair-wise inter group comparison of the mean zone of inhibition values between the four groups was done using Post-hoc Tukey HSD test. The level $P < 0.05$ was considered as the cutoff value or significance.

III. Result

The mean zones of inhibition produced by the four medicaments were tabulated (Table 1). All the medicaments were effective in inhibiting the test organism. Maximum inhibition of E. faecalis was noted with Group D (27.4 ± 1.5 mm), followed by Group B (25.0 ± 1.0 mm) and Group C (22.2 ± 0.9 mm). Group A showed least inhibitory activity. The mean difference of zone of inhibition in between the control group D was least with group B i.e. 2.43 mm; (Table 2) implying that oregano oil in combination with calcium hydroxide had remarkable efficacy in inhibiting growth of E. faecalis as compared to Triple Antibiotic Paste. Also, calcium hydroxide alone and in combination with rosewood oil inhibited E. faecalis but to a relatively lesser extent.

Table no.1 shows the range and the mean values of zone of inhibition (mm) in the four groups. Zone of inhibition in Group A was 17.8 mm with 0.8 mm SD and ranging within 16 to 20 mm. Group B caused zone of inhibition within 23 to 26 mm with 25 mm mean and 1 SD. Group C caused 22.2 mm mean and 0.9 mm SD and the values were within 21 to 24 mm range. Group D which was positive control had mean zone of inhibition of 27.4 mm with 1.5 mm SD and was within range of 24 to 30 mm.

Table 1: Descriptive finding of Zone of inhibition (mm) in the four groups

	N	Mean± S.D	95% Confidence Interval for Mean (Lower- Upper)		Minimum	Maximum
Group A	30	17.8±0.8	17.5	18.1	16	20
Group B	30	25.0±1.0	24.6	25.4	23	26
Group C	30	22.2±0.9	21.8	22.5	21	24
Group D	30	27.4±1.5	26.8	28.0	24	30

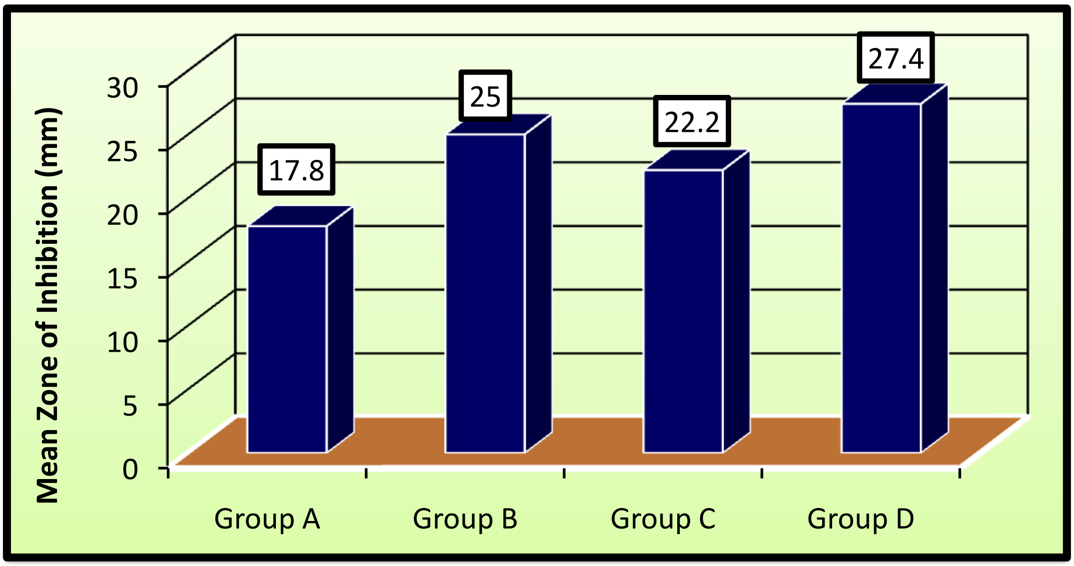
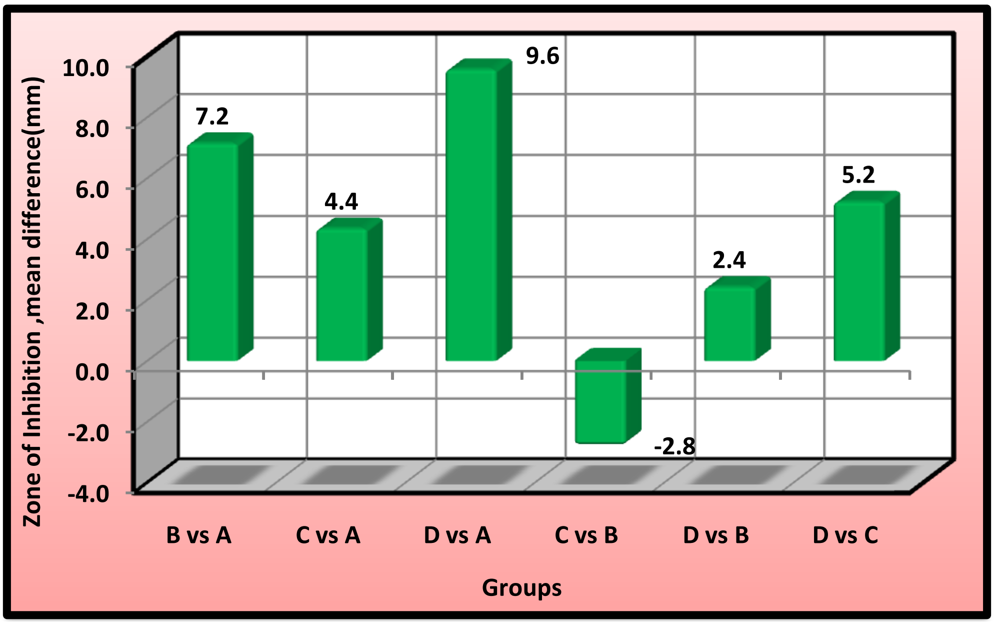


Table no. 2 shows that mean difference of zone of inhibition in between the control group D was least with Group B i.e. 2.43 mm, medium with Group C and maximum with Group A and the mean difference was statistically very highly significant ($p<0.05$). Mean zone of inhibition of Group D > Group B> Group C> Group A and the difference was statistically significant ($p<0.05$)

Table No. 2 Pairwise inter group comparison of zone of inhibition in four groups using multiple comparison with Post-hoc Tukey HSD test.

Group (1)	Group (2)	Mean Difference (1-2)
A	B	-7.15*
A	C	-4.35*
A	D	-9.58*
B	C	2.80*
B	D	-2.43*
C	D	-5.23*

* The mean difference is significant at the .05 level.



IV. Discussion

Enterococcus faecalis has an inherent ability to undergo multiple genetic polymorphisms. After invading the dentinal tubules it binds to dentin with the aid of serine protease, gelatinase, and collagen-binding protein. This bacterium can endure prolonged periods of starvation and utilize serum for nutrition. Serum from alveolar bone and periodontal ligament helps E. faecalis bind to type I collagen.[7] These properties bestow a remarkable ecological resistance to the organism against harsh conditions. The biofilm state found in the root canals, makes the organism more tolerant to phagocytosis, antibodies, and antimicrobials.[8]

At a pH of 11.5 or greater, E. faecalis is unable to survive. However, the buffering capacity of dentin within the root canals does not allow this high pH to be maintained for longer periods. Therefore, high alkalinity of calcium hydroxide fails to render its antimicrobial effect with present techniques.[9-12] In addition; E. faecalis maintains pH homeostasis passively to stabilize the pH. Studies state that intracanal dressings of calcium hydroxide left in place for over 10 days were ineffective against E. faecalis in dentinal tubules. Triple antibiotic paste (TAP) containing metronidazole, ciprofloxacin, and minocycline has been reported to be a successful regimen in controlling the root canal pathogens.[4,13] However, E. faecalis is multi drug resistant; and is reported to have developed resistance to well known antibiotics like vancomycin, gentamicin, etc. and may soon develop one against Triple Antibiotic Paste.

In view of the increasing emergence of bacterial resistance to antibiotic therapy and newly emerging resistant pathogens, it is necessary to find newer compounds with potential antimicrobial activity.

Essential oils extracted from plants have been found to be effective medicinal alternatives to antibiotics and antiseptics without showing the same secondary effects.[7]They possess good antimicrobial, anti inflammatory, anti oxidant & immune system response stimulating activities. Plant essential oils are complex natural mixtures of 20-60 components at different concentrations. Essential Oils are reported to be more effective than the major component antimicrobial compound itself.[15] Hence, development of resistance among microorganisms is not reported.

Amongst various plant essential oils, Rosewood oil (Aniba rosaeodora) and Oregano oil (Origanum vulgare) have been reported to possess significant inhibitory antimicrobial activity against Enterococcus faecalis species. Origanum vulgare was tested to be inhibitory against E. faecalis in both planktonic and biofilm state.

The antimicrobial activity of Aniba rosaeodora is attributed to the high percentage of terpene alcohols-linalool (88.6%) in the oil; whereas oxygenated monoterpenes- Thymol (41 %), Carvacrol (2.15%) & linalool (0.6%) form the basis of antimicrobial activity of Origanum vulgare essential oil.[16] Lipophilic terpenols, mono- & sesquiterpenoid compounds disrupt bacterial cytoplasmic membrane causing inhibition of enzymes due to leakage of ions with coagulation of cell contents and resultant bacterial inhibition.

With several advantageous properties of essential oils, it is desirable to develop a new highly active antimicrobial therapeutic combination of essential oils and conventionally used medicament to deliver improved efficacy in combating persistent infections and drug resistance.

Enterococcus faecalis was found to be remarkably sensitive to the combination of oregano oil and calcium hydroxide, although to a lesser extent than combination antibiotics. This was in accordance to the study by Rosato et al. The combination of Rosewood oil and calcium hydroxide showed lesser activity against the test organism than calcium hydroxide alone; which indicates that the antimicrobial activity of rosewood oil and calcium hydroxide did not act in synergism. Test results were similar to that obtained by Fethi Benbelaïd et al (2014)[7] who reported inhibition zones larger than 27 mm with O. vulgare against various strains of E. faecalis.

In vitro, the association of Calcium hydroxide- Rosewood oil and Calcium hydroxide- Oregano oil can prove to be an effective intracanal medicament in inhibiting Enterococcus faecalis and other endodontic pathogenic microorganisms. The new medicament comprising of Origanum vulgare and calcium hydroxide exhibits combined antibacterial effect of EO and calcium hydroxide. Being oil based, it prolongs dissolution of calcium hydroxide, thus enhancing the antimicrobial potential of calcium hydroxide. This new combination medicament possesses additional anti-inflammatory and antioxidant effects.[17] Also, resistance to the medicament among microorganisms is less likely to develop owing to multiple constituents.

The usefulness of agar well diffusion method to determine the effectiveness of these test medicaments is limited. The hydrophobic nature of these essential oils prevents the uniform diffusion of the medicaments through the agar medium, eventually not expressing its full effective potential. To confirm the validity of these materials, further in vivo studies are needed.

V. Conclusion

The increased resurgence of interest in natural therapies is a result of increasing consumer demand for effective, safe and natural products. Oregano oil and rosewood oil in combination with calcium hydroxide can be used as herbal alternatives to conventional intracanal medicaments to improve antimicrobial effect and retention within the root canal. However, in vivo studies are required to confirm the validity of use of this new medicament. In the rapid and methods of its elimination from the tooth may well define the future of this specialty**.**

References

[1]. Sue C. Chao, D. Gary Young and Craig J- Oberg. Screening for Inhibitory Activity of Essential Oils on Selected Bacteria, Fungi and Viruses. J. Essent. Oil Hes. Sep/Oct 2000;12:639-49.

[2]. Charles H. Stuart, Scott A. Schwartz, Thomas J. Beeson and Christopher B. Owatz. Enterococcus faecalis: Its Role in Root Canal Treatment Failure and Current Concepts in Retreatment. JOE Feb 2006;32(2):93-98.

[3]. Norhayati Luddin, Hany Mohamed Aly Ahmed. The antibacterial activity of sodium hypochlorite and chlorhexidine against Enterococcus faecalis: A review on agar diffusion and direct contact methods. Journal of Conservative Dentistry Jan-Feb 2013;16(1):9-16.

[4]. Rangasamy Vijayaraghavan, Veerabathran Mahesh Mathian, Alagappan Meenakshi Sundaram, Ramachandran Karunakaran, Selvaraj Vinodh. Triple antibiotic paste in root canal therapy. J Pharm Bioall Sci 2012;4:230-33.

[5]. Hammer K. A., Carson C.F., Riley T.V. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 1999;86:985–90.

[6]. A. Rosato, M. Piarulli, F. Corbo, M. Muraglia, A. Carone, M.E. Vitali and C. Vitali. In Vitro Synergistic Action of Certain Combinations of Gentamicin and Essential Oils. Current Medicinal Chemistry 2010;17:3289-95.

[7]. Fethi Benbelaïd, Abdelmounaïm Khadir, Mohamed Amine Abdoune, Mourad Bendahou, Alain Muselli, Jean Costa. Antimicrobial activity of some essential oils against oral multidrugresistant Enterococcus faecalis in both planktonic and biofilm state. Asian Pac J Trop Biomed 2014;4(6):463-72.

[8]. Hegde V, Kesaria D. Comparative evaluation of antimicrobial activity of neem, propolis, turmeric, liquorice and sodium hypochlorite as root canal irrigants against E. Faecalis and C. Albicans - An in vitro study. Endodontology. 2013; 25(2):38-45

[9]. Sjogren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. International Endodontic Journal 1991;24:119-25.

[10]. Turk TB, Sen BH and Ozturk,T, Turkey I. In vitro antimicrobial activity of calcium hydroxide mixed with different vehicles against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;108:297-301.

[11]. Neelakantan P, Sanjeev K and Subbarao CV. Duration-dependent susceptibility of endodontic pathogens to calcium hydroxide and chlorhexidene gel used as intracanal medicament: an in vitro evaluation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;104:e138-e41.

[12]. Estrela C, Bammann LL, Pimenta FC & Pécora JD. Control of microorganisms in vitro by calcium hydroxide pastes. International Endodontic Journal 2001;34,341–5.

[13]. Kusgoz A, Ozcan E, Arslan I, Inci M. Antibacterial activity of calcium hydroxide combined with triple antibiotic paste against Enterococcus faecalis; an in vitro study. Cumhuriyet Dent J 2013;16(1):25-30.

[14]. Maria Lis-Balchin , S. Hart , S. G. Deans & E. Eaglesham. Comparison of the Pharmacological and Antimicrobial Action of Commercial Plant Essential Oils, Journal of Herbs, Spices & Medicinal Plants 1996;4(2):69-86.

[15]. Sandra Laise F. Sarrazin, Ricardo B. Oliveira, José Guilherme S. Maia and Rosa Helena V. Mourão. Antibacterial Activity of the Rosewood (Aniba rosaeodora and A. parviflora) Linalool-rich Oils from the Amazon. EJMP 2016;12(2) Article no.EJMP.22901:1-9.

[16]. Carlos Estrela, Rosane Galhardo Ribeiro, Cyntia R.A. Estrela, Jesus Djalma Pécora, Manoel Damião Sousa-Neto. Antimicrobial Effect of 2% Sodium Hypochlorite and 2% Chlorhexidine Tested by Different Methods. 5B8raz Dent J 2003;14(1):58-62.

[17]. Loä Pez P, Saä Nchez C, Batlle R, Neriän C. Solid- and Vapor-Phase Antimicrobial Activities of Six Essential Oils: Susceptibility of Selected Foodborne Bacterial and Fungal Strains. J. Agric. Food Chem. 2005;53:6939-46.

FIGURE LEGENDS:

Figure 1: Brain Heart Infusion broth showing turbidity equivalent to barium sulfate standard of 0.5 McFarland units indicating growth of Enterococcus faecalis ($1.5x10^8$ CFU/ml) with sterile Brain Heart Infusion taken as standard.

Figure 2: Streaking of Mueller Hinton Agar plate with Enterococcus faecalis bacterial suspension using cotton swab

Figure 3: Preparation of four equally spaced wells of 6mm diameter on Mueller Hinton Agar plate using cork borer

Figure 4: Test medicaments placed in wells of Mueller Hinton Agar plate

Figure 5: Zones of inhibition against E. faecalis observed after incubation of Mueller Hinton Agar plate with test medicaments at 37 degree Celsius for 72 hours.

Garima Tiwari "Antimicrobial efficacy of commercially available plant essential oils with calcium hydroxide as intracanal medicaments against Enterococcus faecalis: An in-vitro study.”."IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), vol. 17, no. 6, 2018, pp 19-24.