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Evaluation of Anti-Bacterial Efficacy of Plant Essential Oils (Oregano Oil, Thyme Oil, Basil Oil) with Calcium Hydroxide as Intracanal Medicament Against Enterococcus Faecalis – An in Vitro Study

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Conflicts of Interest: Nil

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ABSTRACT

Introduction: Enterococcus faecalis is a gram-positive anaerobic bacteria highly virulent in nature that can cause reinfection in root canal treated tooth. Calcium hydroxide with oil-based vehicles are also used as intracanal medicament. Oils such as thyme oil, basil oil, oregano oil have proved antibacterial effect.

Aim: The aim of this study is to assess the antimicrobial efficacy of calcium hydroxide mixed with oregano oil, thyme oil, basil oil as vehicle against E.faecalis.

Materials and Methods: Thirty muller Hinton agar plates were taken as sample and were grouped into 5 groups as Group 1 - oregano oil with Calcium hydroxide, Group 2- Thyme oil with Calcium hydroxide, Group 3 - Basil oil with Calcium hydroxide, Group 4 - Calcium hydroxide with saline, Group 5 – Negative control. The agar plates were inoculated with strains of E.faecalis (ATCC29212). Agar wells were created and test intracanal medicaments were placed in agar wells in group 1-4 where group 5 was used as negative control. The agar plates were incubated at 37 ^{0}C for 72 hours. After incubating, the agar plates were examined for zone of inhibition for assessing the degree of susceptible or resistance of test organism.

Statistical analysis: Statistical analysis was done with one way anova/ kruskal wallis test and post hoc bonferroni test and was statistically significant (P <0.05) **Result:** It was shown that Thyme oil (26±0.00) was more effective in eradicating Enterococcus faecalis when compared to Basil oil (14.25 ± 0.5) and Oregano oil (23.75 ± 0.5).

Conclusion: Thyme oil with calcium hydroxide has significantly high antimicrobial efficacy against Enterococcus faecalis.

Key Messages: Enterococcus faecalis is a gram-positive anaerobic bacteria highly virulent in nature that can cause reinfection in root canal treated tooth. Calcium hydroxide with oil-based vehicles is also used as intracanal medicament. Oils such as thyme oil, basil oil, and oregano oil have proved antibacterial effect. Thyme oil with calcium hydroxide has significantly high antimicrobial efficacy against Enterococcus faecalis.

Keywords: Enterococcus faecalis , calcium hydroxide, thyme oil, basil oil, oreganeo oil, intracanal medicament , agar diffusion , invitro study

Introduction

One of the important sequelae of untreated dental caries is its apical extension into the dental pulp causing a condition termed pulpitis which further leads to another condition termed apical periodontitis causing significant morbidity and pain to the patient in concern [1]. To eliminate pulpal infection, the main treatment strategy termed endodontic therapy is performed. The most important step in endodontic therapy is debridement and biomechanical preparation of the root canal which is often accompanied by the use of chemicals and is also termed as chemomechanical preparation [2].

Complex root canal anatomy tends to retain microorganisms by maintaining an ideal environment to survive and proceed with toxin production. *Enterococcus faecalis (*E. faecalis), a predominant pathogen, is the main cause for reinfection due to its extremely resistant characteristics [3] It resists endodontic treatment by its ability to compete with other microorganisms, invades dentinal tubules, forms biofilms, resists starvation, and creates a low pH. One way of preventing bacterial infections could be by the use of essential oils, as these possess many valuable properties, bestowing antimicrobial, anti-inflammatory, and antioxidant effects [4]. A medicament that would remain within confines of the root canal and eliminate E. faecalis is hence essential for clinical success

Use of intracanal medicaments have been proposed to help achieving a complete disinfection of root canal. Calcium hydroxide , a commonly used intracanal medicament , when used alone is found to be ineffective against E.faecalis[5]. It's due to the inability to maintain an alkaline pH for the entire duration. Due to this limitation, plant essential oils can be used to maintain the alkaline pH for longer duration due to oil based vehicles being used [6]. Plant essential oils with antibiotics have shown synergistic antimicrobial efficacy against various microorganisms including E.faecalis [7]. These oils have been found to possess pharmacological & antibacterial and antifungal properties by itself.

With the available background information, the present study was performed to evaluate the in-silico and in-vitro antimicrobial efficacy of oregano oil (Origanum vulgare), basil oil (Ocimum basilicum) and thyme oil (Thymus vulgaris) with calcium hydroxide as new intracanal medicaments for eliminating E.faecalis.

Materials and Methodology:

Culture 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^{o}C 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.

Agar Diffusion Test:

Mueller Hinton Agar (MHA), being a selective medium for the growth of E. faecalis was chosen for the study. Thirty Mueller-Hinton Agar plates were prepared. A sterile cotton swab is dipped into the BHI bacterial suspension, rotated on the side of the tube to remove surplus and used to inoculate the agar plates. All the plates were uniformly inoculated by even streaking of the cotton swab in three directions. (Research Centre, Tagore Medical College and Hospital) . After the inoculums dry, with the aid of a sterile 6 mm cork borer, 4 equally spaced wells were bored in the agar plate. The agar plugs were discarded using a sterile needle.

Preparation of herbal extract:

For preparing herbal extract herbal oils such as

oregano oil (Origanum vulgare) [RV essentials PVT LTD, India] basil oil (Ocimum basilicum) [NATUROMAN, India] and thyme oil (Thymus vulgaris) [PRZ Thyme essential oil, KAZIMA perfumers, India] was used for the study. These oils were mixed with calcium hydroxide (Bhatnagar dental supply, PREVEST DENT PRO, India). Powder liquid ratio was standardized by mixing 0.2 gm of calcium hydroxide powder with 0.25cc of oil on sterile dry glass slab using the sterile cement spatula [8]. Thirty samples were grouped into 5 in which each group consists of 6 samples. The experimental groups were Group 1 - oregano oil with Calcium hydroxide, Group 2- thyme oil with Calcium hydroxide, Group 3 - basil oil with Calcium hydroxide, Group 4 - calcium hydroxide with saline, Group 5 – negative control. The experimental intracanal medicament was placed in group 1 – 4 and assessed. The wells were filled with the freshly prepared test medicaments.

Calculation of Zone Of Inhibition:

The MHA plates were examined for zones of inhibition after completion of 72 hours. 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. (figure 1)

Insilico Study:

Preparation of ligands for docking

The chemical structure of phytocompounds from Oregano oil (13 compounds), Basil oil (10 compounds), Thymus oil (24 compounds) were obtained from PubChem compound database in SDF format. By using online smiles translator all the compounds were converted in to PDB format.

Preparation of receptor

Three dimensional structure of *E. faecalis* target proteins DHFR, Glutamate racemase, Alanine racemase and Topoisomerase DNA gyrase B was retrieved from PDB database. Receptor data were downloaded and opened using Discovery Studio Visualizer to remove the natural ligand that still attached to it. Before the docking process, the non polar hydrogen atoms were added.

Docking

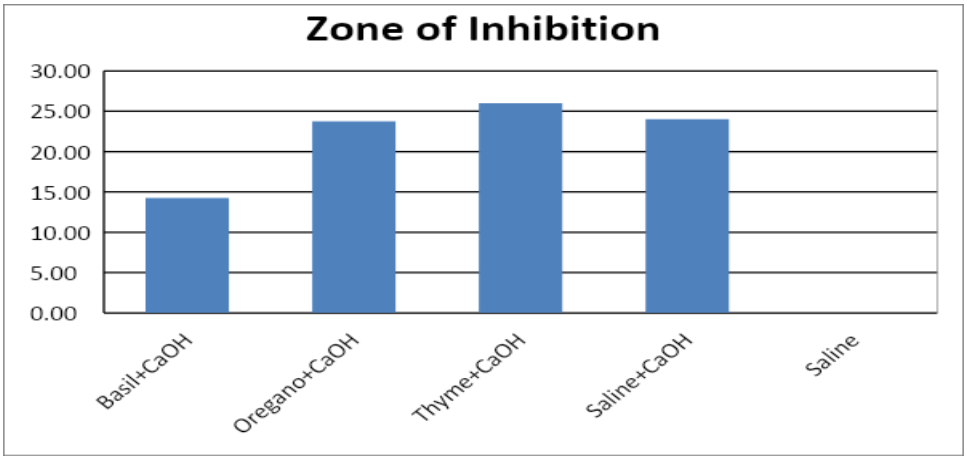
The procedure of docking of ligands with the receptor has been performed using Autodock version 0.8 of pyrx software. Docking is the process of predicting the most effectively binding ligand(s) from a library of chemicals using multiple scoring algorithms. The ligand library was created by putting all of the ligands in a pyrx Autodock (Autodock vina) folder [9]. By executing simultaneous docking of numerous ligands against the receptor, the library preparation aids in making an easier comparison research of ligands [10]. Grid batch docking was also carried out. Each docked molecule's outcome is expressed in terms of a final minimum score (Dock score interaction/ docking energy of receptor-ligand).

Result:

The mean zone of inhibition produced by four medicaments were tabulated (Table 1). All the medicaments were effective against the test microorganism. Maximum inhibition of E.faecalis was noted with Group 2 (26±0.00) followed by Group 4 (24 ± 0.8). and Group 1 (23.75 ± 0.5). Group 3 (14.25 ± 0.5) showed least action against the test microorganism. The mean difference of zone of inhibition of Group 2 was maximum with Group 4 (control group) (i.e 2 mm) implying that thyme oil in combination with calcium hydroxide had significant efficacy in eradicating E.faecalis when compared with control group ; Group 4 (control group) was least with Group 1 (i.e 0.25mm) implying that oregano oil in combination with calcium hydroxide had remarkable efficacy in inhibiting the growth of E.faecalis when compared with saline with calcium hydroxide. calcium hydroxide in combination with basil oil showed the least activity against E.faecalis inhibition.

Table 1: Descriptive Statistics

	Oregano+CaOH	Thyme+CaOH	Basil+CaOH	Saline+CaOH	Saline
Mean	23.75	26.00	14.25	24.00	0.00
SD	0.50	0.00	0.50	0.82	0.00
Min	23.00	26.00	14.00	23.00	0.00
Max	24.00	26.00	15.00	25.00	0.00



Graph 1: Representing the zone of inhibition

Graph 1 shows range and mean value of zone of inhibition in 5 groups

Zone of inhibition in Group 1 was 23.75 with 0.5 (standard deviation) SD and ranging within 23-24 mm; Group 2 was 26mm with 0 SD and ranging 26mm ; Group 3 was 14.25 with 0.5 SD ranging within 14-15mm ; Group 4 was 24 with 0.8 SD ranging within 23-25mm

Mean zone of inhibition of Group 2 > Group 4 >Group 1 >Group 3 > Group 5. Statistically more significance is seen in thyme with Calcium hydroxide medicament followed by Oregano group and saline group and least significance was seen in Basil with calcium hydroxide.

Molecular docking results show that Thyme oil, Basil oil, Oregano oil showed strong binding affinity with that of the target proteins in test microorganisms. (Table 2,3,4) In Thyme oil, 2-Aminopyrimidine-1-oxide showed strong binding affinity and also showed the good number of hydrogen bond interaction with all the target proteins.

Table 2: Molecular docking results of best four compounds from Oregano oil

S.no	Compound Name	Binding Energy kcal/mol	Hydrogen bond interaction
Alanine Racemase			
1	Trans-caryophyllene	-6	-
2	α-thujene	-6	-
3	o-cymene	-5.4	-
4	Phellandrene	-5.5	-
DHFR			
1	Trans-caryophyllene	-7	-
2	Phellandrene	-6	-
3	Thymol	-5.9	ILE-5
4	o-cymene	-5.8	
DNA gyrase			
1	Carvacrol	-6	-

2	Trans-caryophyllene	-5.9	-
3	Thymol	-5.9	-
4	Limonene	-5.7	-
Glutamate_racemase			
1	Trans-caryophyllene	-6.9	-
2	Thymol	-6	LEU-98
3	Carvacrol	-6	LYS-134
4	1,8-cineole	-6.2	

Table 3: Molecular Docking Results Of Best Four Compounds From Basil Oil

S.no	Compound Name	Binding energy kcal/mol	Hydrogen bon interaction
Alanine Racemase			
1	Methyl cinnamate	-6.9	THR-353 THR-361
2	Linalool	-5.7	GLY-229 ASN-231
3	Tau-cadinol	-5.6	MET-346
4	Epsilon-Muurolene	-5.9	-
DHFR			
1	Tau-cadinol	-6.8	-
2	Epsilon-Muurolene	-6.8	-
3	Beta-elemene	-6.9	-
4	Cubenol	-7.1	-
DNA Gyrase			
1	Alpha Bulnesene	-7.2	-
2	Beta-elemene	-6.8	-
3	Epsilon-Muurolene	-6.8	-
4	Methyl cinnamate	-6.2	GLY-80 THR-169
Glutamate Racemase			
1	Cubenol	-7.1	-
2	Epsilon-Muurolene	-6.8	-
3	Beta-elemene	-6.7	-
4	Tau-cadinol	-6.7	-

Table 4: Molecular docking results of best four compounds from Thyme oil

S.no	**Compound Name**	**Binding energy kcal/mol**	**Hydrogen bond interaction**
Alanine Racemase			
1	2-Aminopyrimidine-1-oxide	-8.2	LYS-350 GLU-352
2	Caryophyllene oxide	-6.2	THR-298
3	Caryophyllene	-6	-
4	Isoaromadendrene epoxide	-6.2	THR-298
DHFR			
1	2-Aminopyrimidine-1-oxide	-7.4	LEU-20
2	Isoaromadendrene epoxide	-7.1	-
3	(−)-Spathulenol	-6.4	-
4	α-Terpineol	-5.8	ASP-27
DNA Gyrase			

1	2-Aminopyrimidine-1-oxide	-8.2	THR-65 GLN-67
2	Caryophyllene oxide	-6.3	ASN-63
3	Cyclo propane carboxamide	-6.6	ASP-76
4	(−)-Spathulenol	-6.2	
Glutamate Racemase			
1	2-Aminopyrimidine-1-oxide	-9.2	GLN-26 ARG-102
2	Cyclo propane carboxamide	-7.2	GLN-26 ASN-29 HIS-227
3	(−)-Spathulenol	-6.8	GLN-26 SER-217
4	Isoaromadendrene epoxide	-6.9	-

Discussion:

Enterococcal strains are commonly detected in the digestive and genitourinary tracts of humans [11]. Despite the fact that enterococci are small inhabitants of the oral cavity of healthy people [12], they are commonly isolated in chronic endodontic failures [13]. Removing enterococci from infected canals is critical for preventing the loss of treated teeth. The clearance of enterococci is also hampered by their resistance to some antibiotic classes [14]. If they are resistant, they can exchange resistance genes with other species [15]. As a result, it is vital to determine their susceptibility to antibiotics, which will aid in the planning of the most appropriate medication and, secondly, will help to avoid the spread of resistance to other species. It is critical to develop novel methods of eliminating these microorganisms from infected root canals.

Essential oils have numerous beneficial features, including antibacterial, anti-inflammatory, and antioxidant actions[16]. They can be used to treat infections of the respiratory, digestive, and genitourinary systems, as well as skin diseases, and can be utilized as natural preservatives in cosmetics or pharmaceuticals[17]. Possessing a good antimicrobial activity , EOs can replace treatments with antibiotics and disinfection using antiseptics[17]. Some essential oils have been put to the test in the field of dentistry.

Out of 13 compounds (Table 2) from oregano oil , the Trans-caryophyllene showed the highest binding affinity. Most of the compounds from oregano oil does not show any hydrogen bond interaction with the target proteins but its showed the strong binding energy so these compounds may acts as potential inhibitor for Enterococcus faecalis.

Ten compounds (Table 3) from basil oil were docked with selected four target protein. Among the ten compounds best four compounds for each protein were selected based on the binding energy. Compared to all other compounds Epsilon-Muurolene showed very good binding with all the four target proteins

Out of the 24 compounds (Table 4) in thyme oil, 2-Aminopyrimidine-1-oxide showed strong binding affinity. 2-Aminopyrimidine-1-oxide also showed the good number of hydrogen bond interaction with all the target proteins. So this might act as potential inhibitor for Enterococcus faecalis after the experimental validation. E.faecalis was found to be more sensitive to the combination of thyme oil with calcium hydroxide than calcium hydroxide with saline. Oregano oil with calcium hydroxide was remarkably sensitive to test microorganisms but to a lesser extent than saline with calcium hydroxide. Basil oil showed the least activity of all groups.

In-vitro, the association of Calcium hydroxide- thyme oil, Calcium hydroxide - Basil oil and Calcium hydroxide- Oregano oil can prove to be an effective intracanal medicament in inhibiting Enterococcus faecalis and other endodontic pathogenic

microorganisms. In our study, thyme oil with calcium hydroxide shows the highest zone of inhibition about 26 mm. Being oil based, it prolongs dissolution of calcium hydroxide, thus enhancing the antimicrobial potential of calcium hydroxide[18]. Oil-based $Ca(OH)_2$ paste are more difficult to remove than $Ca(OH)_2$ powder mixed with distilled water [19]. This new combination medicament possesses additional anti-inflammatory and antioxidant effects. 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[20]. In order to use these materials in clinical practice further invivo analysis must be done.

Conclusion

Through this experiment it can be concluded that thyme oil with calcium hydroxide is effective in eradicating E.faecalis than calcium hydroxide with saline combination.

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