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RESEARCH ARTICLE

CHEMICAL COMPOSITION AND ANTIMICROBIAL EFFICACY OF CALCIUM HYDROXIDE WITH PEPPERMINT OIL AND TO COMPARE ITS EFFECT WITH CALCIUM HYDROXIDE WITH SALINE AGAINST ROOT CANAL PATHOGENS OF DECIDUOUS TEETH

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ABSTRACT

Background: For endodontic treatment in deciduous teeth, thorough instrumentation and cleaning is required which may not be possible due to difficult anatomic configuration of deciduous teeth. So root canal filling material at least should possess antimicrobial efficacy. Therefore present study was planned to find out the bioactive ingredients and antimicrobial efficacy of calcium hydroxide mixed with peppermint oil (CaOH+P) and its effect was compared with routinely used calcium hydroxide with saline (CaOH+S) in the field of pediatric dentistry.

Materials and methods: To find out the bioactive ingredients in peppermint oil, gas chromatography mass spectrophotometry was performed and for evaluating antimicrobial efficacy, agar diffusion method was employed in which, Muller Hincton agar was used. In MH agar plates, punching was done at two equidistant points and test materials were filled and kept in incubator at 37ºC for 24 hours. Zone of inhibition was measured in millimeter. Six times repetitions of the procedure was performed. Statistically data was analyzed by using ANOVA and Tukey's post-hoc comparison test. P-value <0.05 was used for level of significance.

Results: It was observed from the results that bioactive ingredients in peppermint oil were octanol (48.17%) followed by menthol (20.45%) and other ingredients were very less in quantity. Zone of inhibition obtained in CaOH+P oil paste against root canal pathogens in decreasing order were against Staph.aureus>E.coli>E.faecalis=P.aeruginosa with statistically significant difference (0.0001, $p<0.05$) while in CaOH+S paste, antimicrobial efficacy in decreasing order was E.Coli>Staph.aureus=E.faecalis>P.aeruginosa which was not significant statistically (0.373, $p>0.05$)

Conclusion: Zone of inhibition obtained in CaOH+P oil paste for Staph.aureus were higher as compared to CaOH+S paste, while for other root canal pathogens i.e; E.coli, E.faecalis and P.aeruginosa, CaOH+S paste showed larger zones as compared to CaOH+P oil paste.

INTRODUCTION

Root canal treatment in deciduous teeth is challenging because the root canal configuration of deciduous molars shows that roots are very thin, tortuous and curved with ribbon like canals along with numerous accessory and lateral canals [I]. Therefore instrumentation and cleaning of root canals become difficult. There should be root canal filling material which can fill the accessory and lateral canals of deciduous molars and cause antimicrobial efficacy [II, III].

Calcium hydroxide has been used as root canal filling material in pediatric dentistry as root canal filling material for deciduous teeth because of its properties like biological properties associated with release of Ca^{+} ions and antibacterial properties associated with release of OH^{-} ions due to its high pH [IV].

Various vehicles have also been tried. Out of which, use of calcium hydroxide mixed with saline or distilled water was more. But it said that with water based vehicle, calcium hydroxide dissociate faster into Ca^{+} and OH^{-} ions and remains only for short duration of time for its action [V].

Therefore in the present study, peppermint oil has been used as it is oily vehicle and also its known antimicrobial action available in the literature. Expecting that, after mixing calcium hydroxide with peppermint oil, it will remain in the area of action for longer duration to show its antimicrobial efficacy.

In the literature, menthol and cavacrol are the two main ingredients which have been thought to be in higher conc. in peppermint oil. So to understand the composition of peppermint oil which was used for the present study, gas chromatography and mass spectrophotometry was done. Studies by use of peppermint oil have been found in medical literature. But use of peppermint oil is only available with respect to its use in mouthwash. Considering the beneficial properties associated with peppermint oil, present study was carried out to first to find out the composition of peppermint oil which is used for the present research work and secondly to find out the antimicrobial efficacy of calcium hydroxide mixed with peppermint oil against root canal pathogens of deciduous teeth and to compare its effect with the routinely used calcium hydroxide mixed with saline.

MATRIALS AND METHODS

Present study was an *in vitro* study. It was approved by institutional ethical committee. Peppermint oil which was used in the present study was procured from Aromatantra, Mumbai. Calcium hydroxide powder (Prevest Denpro Limited, Jammu, India) was mixed with peppermint oil and was compared with calcium hydroxide powder mixed with saline paste.

Gas Chromatography/ Mass Spectrometry (GC/MS):

200µl of peppermint oil was mixed in 10ml methanol. It was vortexed and allowed to stand for 5 minutes. Syringe of 0.45µm was filtered and pepprmint oil then injected on GC/MS for analysis.

Column used was of fused silica capillary column DB 5-MS (15m x 0.25mm i.d., 0.25um) with column temperature, 40^{0} (2 min)-8^{0}C/min-150^{0}C (0 min), 10^{0}C/ minute-300 ^{0}C (10 minutes).

Injector temperature was 250^0C. Injector method was split, injection with amount of 2µl. Carrier gas was helium with flow of 1.2ml/ minute. Transfer line temperature was 280^0C.

Ionization method used was EI. Ionization current was 50µA with ionization voltage, 70eV, Ion source temperature, 220^0C and detection method used was full scan.

Antimicrobial efficacy:

Microbial strains used for the study were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212) and *Psudomonas Aeruginosa* (ATCC 27853). These strains were obtained from Microbiologics, USA, Hi Media, Pvt., Ltd. and were procured from the department of Microbiology, Jawaharlal Nehru Medical College, Wardha, Maharashtra, India.

Powder liquid ratio was standardized by mixing one scoop of calcium hydroxide powder equivalent to 0.2 g and 10 drops of peppermint oil equivalent to 0.1 cc. Powder and liquid was mixed on a dry sterile glass slab using sterile cement spatula.

Stock cultures of microorganisms were used for the study. Microorganisms were cultivated in Brain Heart Infusion Broth. Growth of microorganisms was carried out in blood agar paltes. Mueller Hinton Agar was used to find out the susceptibility of microorganisms.

Method: Stock cultures of microbial strains were added to 5ml BHI broth and incubated at 37°C for 24 hrs. Blood agar plate was used for subculturing of strains. Colonies of microorganisms were then inoculated in nutrient broth for 4-6 hours. Its turbidity was adjusted to 0.5 standard of McFarland opacity scale. Whole procedure was performed in Biosafety Cabinet II. Mueller-Hinton agar plates with 90 millimeter diameter and 4 millimeter thick agar medium were used. Bacterial dilutions were then taken with the help of swab and Lawn technique of culture was employed in which bacterial colonies were swabbed uniformly onto agar plates. Punching in Muller Hincton agar plates was done by using the open end of 6mm diameter micropipette. Freshly mixed pastes were then filled in the punched holes of agar medium. Whole experiment was repeated 6 times. All the Muller Hincton agar plates after the procedure were incubated at 37ºC for 24 hrs.

Zones of inhibition around the paste were measured in millimeters using HiAntibiotic Zone Scale (HiMedia). Zones with larger diameters were interpreted as having greater antimicrobial efficacy.

For statistical analysis, descriptive and inferential statistics was used. Statistical tests used in the present study were One way ANOVA and multiple comparison: Tukey test . Software used for statistical analysis was SPSS 17.0 version, Graph pad prism 4 and $p<0.05$ was considered as level of significance.

RESULTS

Table 1 shows the composition of peppermint oil obtained by using gas chromatography mass specrophotometry analysis. Total 26 ingredients were obtained in peppermint oil, out of which major conc. was that of octanol (48.17%) followed by menthol (20.45%) and D-Limonene (13.66%). Other ingredients which were less in conc. were Glutaconic acid (2.95%), Methanone (2%), Estragole (0.97%), Carenol (0.99%), Caranone

(1.95%), Menthol acetate (2.59%), Anisic acid 4, dinitrophenyl ester (0.36%), Propanedioic acid (0.27%), P-Methane-3,8 did,cis 1,3, trans (0.27%), 4-Isopeny-1 methyl 1,2 cyclohexadiol (0.63%), Cyclohexanemethaniol,2 hydoxy, 4 trimethyl (0.76%), a-boubonene (0.61%), P-Methane-3,8 did,cis 1,3, trans (1.30%), Caryophyllene (1.26%), Cubebene (0.28%), Mourolene (0.15%), Ylangene (0.75%), Cadinal (10) 4- diene (0.14%), 3- Carene (0.18%), Spiro(azetidin, 2-one 42 tricyclodecane) (0.11%), 2,6- Dimethyl 1,3,6 heptatriene (0.29%), Silabenzene-1- methyl (0.05%).

Table 2 and Graph 1 shows zones of inhibition in mm of CaOH+P paste against root canal pathogens. Zones of inhibition in mm for *Staph.aureus* were larger i.e; 25.66±0.51 followed by for *E.coli* in which it was 16.33±1.36 and equal zones of inhibition for *E.faecalis* and *P.Aeruginosa* i.e; 12.66±1.03 respectively with statistically significant difference (p-value: 0.0001, $p<0.05$).

Table 3 and Graph 2 shows zones of bacterial growth inhibition in mm of CaOH+S paste against root canal pathogens. Zones of inhibition shown by CaOH+S paste for *Staph.aureus* was 17.33±3.26, for *E.coli* it was 19.33±1.63, for *E.faecalis*, it was 17.33±3.01 and for *P.aeruginosa*, it was 17.00±3.52 respectively. One way analysis of variance showed that the difference was not statistically significant (0.373, $p>0.05$) between and within groups.

DISCUSSION

Success of endodontic treatment is dependent upon the reduction or elimination of the infecting bacteria [VI]. Complex anatomy of deciduous teeth prevents complete elimination of microorganisms from the root canals [VII]. Various materials as an intracanal antimicrobials have been used in dentistry [VIII].

Calcium hydroxide has been used for a variety of purposes since its introduction into dentistry in the early part of the twentieth century. Calcium hydroxide has a high pH and it is chiefly used in dentistry because of its ability to stimulate mineralization and antibacterial properties [IX].

Peppermint oil and its constituents have been found to be used commercially in food, cosmetics and pharmaceutical industries. Menthol is used in the form of raw material in toothpaste, toothpowder, chewing gums, mouth fresheners, candies, confectionary, cough drops, analgesic balms and perfumes. The fresh or dried leaves are the source of mint and are used in breath fresheners, antiseptic mouth rinses, toothpaste, chewing gum, mint chocolate teas, drinks, beverages, jellies, syrups, candies, ice creams. Menthol is the substance which is responsible to give the peppermint oil their characteristic aromas and flavors [X].

The antimicrobial activity of peppermint oil is due to the presence of terpenoides menthol, menthone, 1-8-cineole, methyl acetate, menthofuran, isomenthone, limonene, b-pinene, germacerene-d, trans-sabinene hydrate and pulegone [XI]. In the present study, 26 ingredients were obtained by GS/MS analysis in peppermint oil. Octanol was found to be in higher conc. (48.17%) followed by menthol (20.45%) and D-Limonene (13.66%). Other ingredients were very less in conc. which included Glutaconic acid, Methanone, Estragole, Carenol, Caranone, Menthol acetate, Anisic acid 4, dinitrophenyl ester, Propanedioic acid, P-

Methane-3,8 did,cis 1,3, trans, 4-Isopeny-1 methyl 1,2 cyclohexadiol, Cyclohexanemethaniol,2 hydoxy, 4 trimethyl, a-boubonene, P-Methane-3,8 did,cis 1,3, trans, Caryophyllene, Cubebene, Mourolene, Ylangene, Cadinal (10) 4- diene, 3-Carene, Spiro(azetidin, 2-one 42 tricyclodecane), 2,6- Dimethyl 1,3,6 heptatriene, Silabenzene-1-methyl.

Very few studies are available in the dental literature with respect to use of peppermint oil. In the study of Shahdad *et al.*, 2007 [XII], efficacy of food-simulating solvents like water, heptanes and peppermint on the hardness of denture teeth after varying storage times was evaluated and observed that there were minor fluctuations in hardness which was not found to be statistically significant. In the study carried out by Thosar *et al.*, 2016 [XIII], antimicrobial efficacy of zinc oxide with peppermint oil and zinc oxide with eugenol oil was assessed and compared. Results of their study showed that the larger zones of inhibition were obtained in ZO+P oil paste against four bacterial strains following the decreasing order in sequence as: *E.coli* (20.66±2.06)> *Staph.Aureus* (18±0.00) > *E.faecalis* (8.00±0.00)= *P.Aeruginosa* (8.00±0.00). Zones of inhibition obtained by using ZOE paste also followed the same pattern in decreasing order for microorganisms as that of ZOP paste. But the highest values for zones of inhibition in mm were obtained in case of ZOP paste when compared with ZOE paste against all the microorganisms. Thosar *et al*., 2013 [XIV] carried out study to find out the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of peppermint essential oil against oral pathogens like Staphylococcus aureus ATCC 25923, Enterococcus fecalis ATCC 29212, Escherichia coli ATCC 25922. Results showed that mean MIC and MBC values of peppermint oil were 0.62 ± 0.45 and 9.75 ± 14.88 respectively. It was concluded that peppermint oil can be used effectively as an intracanal antiseptic solution against oral pathogens.

In present study, antimicrobial effect of calcium hydroxide mixed with peppermint oil (CaOH+P) was evaluated and compared with calcium hydroxide with saline (CaOH+S). It was observed that largest zones of inhibition were obtained in CaOH+P oil paste against *Staph.aureus* (25.66±0.51) Zones of inhibition in decreasing order were *Staph.aureus* (25.66±0.51)> *E.coli (*16.33±1.36)> *E.faecalis* (12.66±1.03)= *P.Aeruginosa* (12.66±1.03) Difference was found to be significant statistically (p-value: 0.0001, $p<0.05$). But in CaOH+S paste, highest values were obtained for the zones of inhibition against *E.coli* (19.33±1.63), almost equal values for zones of inhibition against *Staph.aureus* (17.33±3.26) and *E.faecalis* (17.33±3.01) and least values for zones of inhibition against *P.aeruginosa* (17.00±3.52). When CaOH+P paste was compared with CaOH+S paste, zones of inhibition against Staph.aureus were larger in CaOH+P paste while for rest of other microorganisms like E.coli, E.faecalis and P.aeruginosa, zones of inhibition values were more in CaOH+S paste with the difference which was not significant statistically (0.373, $p>0.05$).

Both the paste had shown their effect against the microorganisms studied. So eventhough, CaOH+S paste is routinely used in dentistry, CaOH+P paste

can also be used effectively in dentistry with added advantage of its oily base which will stay in the area of action for longer duration to show the antimicrobial effect of calcium hydroxide powder.

CONCLUSION

CaOH+P paste is oily based material. Calcium hydroxide also has its own advantages in terms of antimicrobial effect and mineralization. When calcium hydroxide is mixed with peppermint oil with combined effect of both the ingredients, antimicrobial efficacy shown will be beneficial against the root canal pathogens of deciduous teeth.

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Table 1: Chemical composition of Peppermint oil

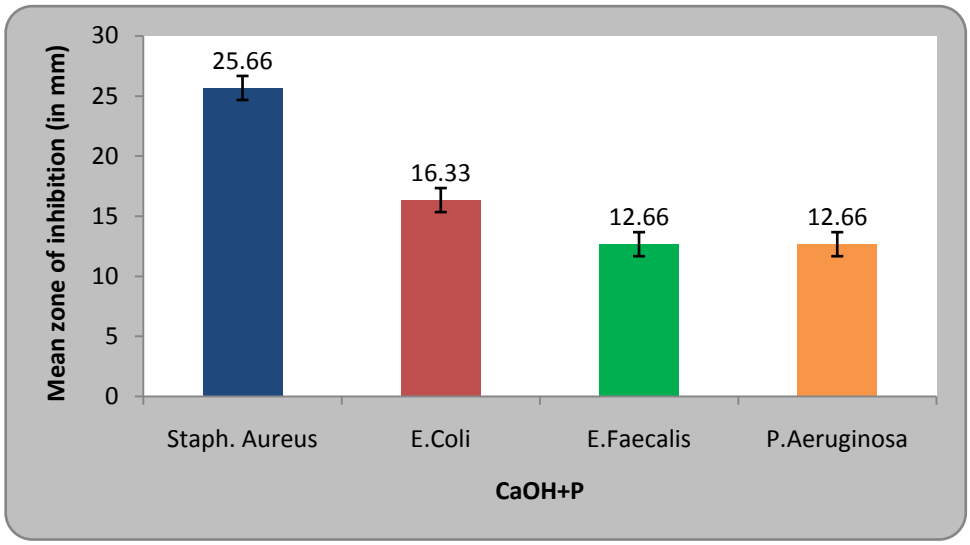
Sr no	Identified Compound	% area
1	Glutaconic acid	2.95
2	Octanol	48.17
3	D-Limonene	13.66
4	Methanone	2.
5	Menthol	20.45
6	Estragole	0.97
7	Carenol	0.99
8	Caranone	1.95
9	Menthol acetate	2.59
10	Anisic acid 4, dinitrophenyl ester	0.36
11	Propanedioic acid	0.27
12	P-Methane-3,8 did,cis 1,3, trans	0.27
13	4-Isopeny-1 methyl 1,2 cyclohexadiol	0.63
14	Cyclohexanemethaniol,2 hydoxy, 4 trimethyl	0.76
15	a-boubonene	0.61
16	P-Methane-3,8 did,cis 1,3, trans	1.30
17	Caryophyllene	1.26
18	Cubebene	0.28
19	Cadinene	0.73
20	Mourolene	0.15
21	Ylangene	0.75
22	Cadinal (10) 4- diene	0.14
23	3- Carene	0.18
24	Spiro(azetidin, 2-one 42 tricyclodecane)	0.11
25	2,6- Dimethyl 1,3,6 heptatriene	0.29
26	Silabenzene-1- methyl	0.05

Table 2: Zones of bacterial growth inhibition in mm of CaOH+P oil paste against root canal pathogens

Microorganisms	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	P-value
					Lower Bound	Upper Bound			
Staph.aureus	6	25.66	0.51	0.21	25.12	26.20	25.00	26.00	0.0001, **S**, p<0.05
E.coli	6	16.33	1.36	0.55	14.89	17.76	15.00	18.00	
E.faecalis	6	12.66	1.03	0.42	11.58	13.75	12.00	14.00	
P.aeruginosa	6	12.66	1.03	0.42	11.58	13.75	12.00	14.00	

S: Significant

Graph 1: Zones of bacterial growth inhibition in mm of CaOH+P oil paste against root canal pathogens



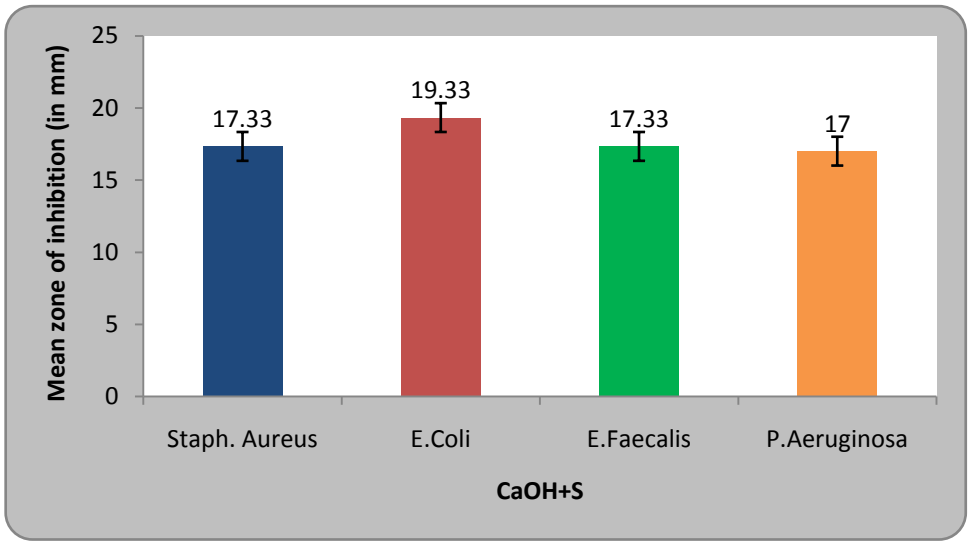
(0.0001, S, p<0.05) S: Significant

Table 3: Zones of bacterial growth inhibition in mm of CaOH+S paste against root canal pathogens

Microorganisms	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	p-value
					Lower Bound	Upper Bound			
Staph Aureus	6	17.33	3.26	1.33	13.90	20.76	12.00	20.00	0.373 **NS**, p>0.05
E.Coli	6	19.33	1.63	0.66	17.61	21.04	18.00	22.00	
E.faecalis	6	17.33	3.01	1.22	14.17	20.49	14.00	20.00	
P.Aeruginosa	6	17.00	3.52	1.43	13.30	20.69	12.00	20.00	

NS: Not Significant

Graph 2: Zones of bacterial growth inhibition in mm of CaOH+S paste against root canal pathogens



(0.373, NS, p>0.05) NS: Not significant