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Minimum Inhibitory Concentration Evaluation of Novel Intra-Canal Medicament: An In-Vitro Study

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ABSTRACT

Background: Root canal failure accounts on the persistence of micro-organism inside the canal. Among them E faecalis was found causative in 12-90% of cases and C albicans had been isolated from 30-45% cases. Objective of intracanal medicaments is to reduce bacterial load inside the root canal system.[1]

Aims: The aim of present in vitro study was to determine the minimal inhibitory concentration of novel medicaments like Nisin, Neem, Propolis, Platelet rich plasma used against E faecalis and C albicans.

Methods & Material: Ethanolic extract at five different concentration (100%, 50%, 25%, 12.5%, 6.25%) were prepared of Nisin, Neem, Propolis and platelet rich plasma (n=5). Five well were punched on each Mullar-Hintan agar plate and 20 µl each of the concentrations of extracts were poured in the well. Strains of E faecalis and C albicans were inoculated and incubated for 24-48 hours at 37^{o}C for observation. The diameter of the zone of inhibition was recorded in millimeters, using an inhibition zone measuring scale. The plates were duplicated in all the experiments. Statistical analysis: The results were statistically analyzed using one-way analysis of variance (ANOVA).

Results: There was significant increase in the zone of inhibition when the concentration of medicaments increases.

Conclusion: Under the limitations of present in vitro study, it can be advocated that Propolis and Nisin were effective against E faecalis while Neem was effective against C albicans. However further clinical trails are required to confirm the results of present study.

Keywords: intracanal medicaments, minimal inhibitory concentration, microflora.

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INTRODUCTION

The objective of root canal treatment is elimination of micro-flora from the root canal system.[1] This is achieved thorough bio-mechanical preparation, suitable intra-canal medicaments and copious irrigation.[2] Despite of recent advances in endodontics, the failure rate still persists. This can be attributed to persistence of micro-organism in the root canal. The endodontic environment support growth of specific micro-organism. [3] Fabricius et al showed that changes occur in root canal ecology from strict anaerobes to facultative anaerobes.[4] Among the failure cases, E faecalis was recovered in majority (12-90%).[1&5] This is due to the inherent ability of bacteria for dentinal invasion under strict anaerobic condition. Thus it can survive as a monoculture without any other bacteria. [5] Another microbe C albicans is the most commonly isolated fungal species in oral cavity (30-45%). Yeasts are seen in canals which are left open to oral cavity [6]. Hence to prevent failure of root canal therapy intracanal medicaments should be predominantly used .[1]

Calcium hydroxide is widely used as an intra-canal medicaments since 1920 ((Hermann 1920). [1] Due to release of hydroxyl ions (OH), environment becomes highly alkaline which is non-conductive to the survival of micor-organism.[7] Its has an ability of inducing tissue formation, inhibiting nutrient supply of the prevalent microflora and exhibit antibacterial effect.[8] However some strains of micro-organism are resistant to alkaline pH. [7]

The newer use as intra-canal medicaments includes Nisin which is naturally occurring antimicrobial peptide effective against intracanal pathogens.[9] Nisin was isolated by Mattick & Hirsch in 1947 from Lactococcus lactis. It basically interact with anionic lipids present in bacterial cytoplasmic membrane leading to pore formation. This results into efflux of cytoplasmic contents like adenosine triphosphate (ATP), amino acids and vital ions and ultimately cell death. [10] Nisin is commonly used as a food preservative worldwide and safer for human use.[8]

Neem is an age old medical plant with proven antibacterial properties. [2] It alters bacterial cell adhesion and inhibits the co-aggregation and colonization of the bacteria.[11] The active components like alkaloids, trepenoids, tannins etc are effective towards anaerobic infections.[2] Propolis (bee glue) is a honey bee by product with antimicrobial properties against various micro-organism. [12] It contains flavonoids which decreases bacterial cell resistance by altering its cell wall permeability.[13] Platelet rich plasma (PRP) express antibacterial properties due to release or pooled growth factor during its activation and play an important role in host defence.[14] Recent studies showed that release of thrombocidins, platelet microbicidal proteins (PMPs), platelet factor-4 (PF-4) significantly decreases bacterial load.[15]

Hence to know the antibacterial efficacy of above mentioned agents when used as an intracanal medicament, the minimal inhibitory concentration was evaluated against strains of E faecalis and C albicans in the present study.

MATERIALS AND METHODS

The medicaments used were Nisin (Bimal Pharma, Mumbai), Neem (Lab preparation), Propolis (Superbee, Singapore), Platelet rich plasma (Lab preparation) and calcium hydroxide. Preparation of calcium hydroxide mix- 1:1 ratio Calcium hydroxide powder mixed with normal saline to obtain the paste. Preparation of Neem Leaf Extract- Ripped Neem leaves, shade dried in the People's naturopathy medicinal garden were taken. Dust and debris was removed from leaves by washing with distilled water and were weighed. Fresh neem leaves of 25gms were added to 50ml of absolute ethanol and mixed for 1-2 min cautiously at 45-50 °C. Preparation of aqueous solution of propolis- 1:60 aqueous solution of Propolis was prepared by dissolving one tablet of propolis (Forever living products) in 60 ml of warm normal saline. Preparation of aqueous Nisin- Nisin was prepared by dissolving 200 mg/ ml concentration of it in distilled water. Preparation of Platelet Plasma-A 30 cc of blood is first drawn with sterile syringe from a patient and centrifuged (spun) to have components into three layers. Bottom layer composed of red blood cells, middle layer consists of platelets, white blood cells and the topmost layer consist of plasma. Preparation of Seed Inoculum:Broth cultures of the pure culture isolates of *Enterococcus faecalis* and *Candida albicans* were prepared by transferring a loop of culture into sterile nutrient broth and incubated at 37°C for 24-48 hours.

Table 1: Zone of inhibition at different concentration of intracanal medicaments.

Bacterial species	Medicaments used	Concentration of medicaments (in % or mg/ml)				
		100	50	25	12.5	7.25
		Zone of inhibition (mm ±SD)				
E. faecalis	Nisin	11 ±1.73	10 ±1.73	8 ±7	5 ±1	5 ±1
	Propolis	18 ±3	13 ±2.64	12 ±2	9 ±2	6 ±1
	Calcium hydroxide	10 ±2	6 ±1	2 ±1	0 ±0	0 ±0
	Platelet Rich Plasma	0	0	0	0	0
	Aqueous Neem Extract	5 ±1	5 ±1	3 ±1	0 ±0	0 ±0
C. albicans	Nisin	14 ±2	11 ±2	8 ±2	5 ±1	0 ±0
	Propolis	14 ±2	9 ±1.73	9 ±1	0 ±0	0 ±0
	Calcium hydroxide	12 ±1	12 ±1	10 ±1	8 ±1	0 ±0
	Platelet Rich Plasma	0	0	0	0	0
	Aqueous Neem Extract	9 ±1	9 ±1	8 ±1	6 ±1	2 ±1

Inoculation for Lawn Culture: From the prepared broth culture of pure bacterial stains, with the help of a sterile cotton swab inoculum was taken and seeded onto sterile Muller-Hinton agar plates and leaving it for 5 minutes before punching holes. After incubation this will develop into diffused heavy lawn culture in inoculated plates. Stock and Dilution of test medicaments:The dilutions of medicaments are either prepared in percentage or in mg per ml concentration. The dilution of Nisin, Propolis and PRP are prepared in percentage concentrations by dissolving them with distilled water and sterilized by passing them with 0.22 µm nitrocellulose syringe filters (Moxcare). Their dilution are 100, 50, 25, 12.5 and 7.25%.

The dilutions of Neem extract and $Ca(OH)_2$ are prepared in mg/ml concentrations by dissolving them with distilled water and sterilized by passing them with 0.22 µm nitrocellulose syringe filters (Moxcare). Their dilution are 100, 50, 25, 12.5 and 7.25 mg per ml.

Well Diffusion Method:

The well diffusion method was used to determine the microbial activity of the test medicaments using standard procedure. In this method, first the test microbial broth used to inoculate on the Muller-Hinton agar plates with the help of sterile cotton swabs to develop the lawn culture. Then to these plates 5 wells of 6 mm diameter are punched in agar plates pre-inoculated with test microorganisms in circular fashion. Undiluted over night broth cultures should never be used as an inoculum. Routine direct application of suitably diluted extracts are poured into the well. The plates were incubated at 37°C for 24 hr and then examined for clear zones of inhibition which was recorded in millimeters, using an inhibition zone measuring scale. The plates were duplicated in all the experiments.

Ethics:

Informed consent were taken from the subject to draw 30cc of blood. Patient was informed previously and the personal details were kept confidential.

Statistics:

The results were statistically analyzed using one-way analysis of variance (ANOVA) and 0.5 p value set as level of significance.

RESULTS

The zone of inhibition of all the medicaments when used against the two bacterial species are shown in table 1 and figure 1. Among all the medicaments maximum zone of inhibition was present at higher concentration (100%) except for PRP. The present

study result showed that at the highest concentration (100%) Propolis and Nisin were equally effective against E faecalis and C albicans with zone of inhibition of range from 18 ±3mm to 11 ±1.73mm. While Platelet rich plasma (PRP) showed no inhibitory effect against both bacterial species.

MIC against E faecalis of Nisin, Propolis were 7.25mg/ml, Neem and Calcium hydroxide were 25mg/ml. MIC against C albicans of Neem was 7.25mg/ml, Nisin and Calcium hydroxide were 12.5mg/ml, Propolis was 25mg/ml.

One way ANOVA showed significant variation at different concentration of Nissin against E faecalis and C albicans ($p<0.0001$ &$p=0.0026$ respectively), in Propolis significant variation ($p<0.0001$ & $p=0.037$ respectively), in Calcium hydroxide no significant variation ($p=1$ in both), Neem no significant variation ($p=1$) against E faecalis but significant variation ($p<0.0001$) against C albicans. PRP there was no significant zone of inhibition observed.

DISCUSSION

Incomplete debridement of diseased root canal system result in secondary endodontic infections.[16] The microflora prevalent in persistent periapical pathology showed 29 to 77% proportion of enterococci. [17,18,19,20] Predominantly E faecalis was found to be present in large numbers in root canals when left open. [21] This is due to the ability to suppress IL-2 and IL-4 production of lymphocytes.[22] Moreover the penetration in dentinal tubules was up to 400µm in vitro within 2-3 weeks period, [23&24] also survival at high alkaline pH of 11.5 renders it as a culprit for persistent lesion.[25] Another predominantly identified micro-organism is Candida albicans, mostly present in persistent post-treatment apical periodontitis.[18] The ability like survival as a monobloc and invasion in dentinal tubules are common like enterococci. However studies had shown that Candida species is resistant to calcium hydroxide while sodium hypochlorite is effective against it. [6] So in order to eradicate micro-organism from root canal system, intracanal medicaments is recommended. Minimum inhibitory concentration (MIC) is a research tool to determine the efficacy of any medicaments against micro-organism in vitro. [16] Agar well diffusion method is commonly used assessment method for evaluation of antibacterial activity of plants and microbial extracts.[26]

The present study was conducted to determine the MIC of Nisin, Propolis, platelet rich plasma and Neem. Since calcium hydroxide is considered as gold standard it was included in the study. The MIC of calcium hydroxide in our study was 25mg/ml for E faecalis and 12.5mg/ml for C albicans. This was in contrast to study done by Kousedghi et al which showed higher MIC against both microflora as 512mg/ml.[27] However Pallotta et al showed MIC as 16mg/ml which was akin to our result.[16]

In the present study Propolis was found to be most effective against E faecalis with maximum zone of inhibitions (18 ±3mm) and MIC was 7.25mg/ml. This was in accordance to study done by Jahromi et al and Ferreira et al though the MIC was found to be lower as 340µg/mL and 6425 µg/mL. The variations can be due to different assessment methods used or perhaps difference in the origin of propolis.[12&28] This was in contrast with the observations of Hedge et al & Tomic et al where Propolis was not found effective against E faecalis and C albicans. [11&29] According to Kousedghi et al Propolis was effective than calcium hydroxide against E faecalis while calcium hydroxide was more effective on C albicans which was akin to our results. This accounts on the lesser diffusion ability of propolis in agar medium. [27]

Nisin was second most effective medicament against E faecalis with zone of inhibition of about 11±1.73mm and MIC was 7.25mg/ml. This was in contrast with Hemadri et al which showed higher MIC as 50mg/ml, moreover Nisin showed 49% reduction and calcium hydroxide showed 31% reduction in E faecalis.[5] Furthermore MIC of Nisin against C albicans is not addressed in dental literature. According to Toaima et al when used as food preservative MIC of Nisin against C albicans was around 500ppm (0.5mg/ml) which is much lower than our results.[30]

However Neem was effective against C albicans even at lower concentration of 7.25mg/ml. This was in accordance to study done by Bohora et al which showed significant antibacterial activity against C albicans. However in our study Neem was not very effective against E faecalis at lower concentrations

which was akin to study done by Mistry et al and Kumar et al.[31&32] It could be attributed to the aqueous extract of Neem which showed better antimicrobial activity against C albicans than E faecalis.[33] Moreover our results were in contrast to study done by Hegde et al were Neem was effective against both E faecalis & C albicans.[11]

CONCLUSION

Under the limitations of present in vitro study, it can be advocated that Propolis and Nisin were effective against E faecalis while Neem was effective against C albicans. However further clinical trails are required to confirm the results of present study.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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