Propolis and Commonly Used Intracanal Irrigants.: Comparative Evaluation of Antimicrobial Potential

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The objective of endodontic therapy is not just simple cleaning and filling of root canals, but successful treatment requires the establishment of a sufficient level of disinfection. ***Aim:*** *To evaluate, in vivo, the antimicrobial and inflammatory/irritant potential of Propolis against mixed endodontic aerobic and anaerobic bacteria.* ***Method:*** *An in vivo randomized controlled trial was conducted in a group of 60 children aged 6-12 years presenting with an acute apical abscess of the maxillary primary molars. Fifteen children each were divided randomly into four groups where irrigation during pulpectomy was performed using either 2% chlorhexidine, 4% calcium hydroxide or 4% Dimethyl Sulfoxide (DMSO) extract of propolis with normal saline as the control irrigant. Microbiological samples were taken from the disto-buccal root canal before initiating the pulpectomy as well as after 3 days later and for mixed aerobic and anaerobic bacterial cultures.* ***Results:*** *In all the four groups, a significant decrease in mean aerobic colony forming units (cfu) count was seen. Maximum change in anaerobic cfu count was seen with 2% chlorhexidine.* ***Conclusions:*** *Chlorhexidine proved to be superior antimicrobial agent against both endodontic aerobes and anaerobes. Calcium hydroxide was found to be least effective.*

Keywords: *Propolis, intracanal irrigants, antimicrobial potential, endodontics, children.*

INTRODUCTION

The essential role of microorganisms in development and maintenance of pulpal and periapical diseases has clearly been demonstrated in animal models and human studies.[1] There is no solid evidence in the literature that mechanical instrumentation alone results in a bacteria-free root canal system. Considering the complex anatomy of root canal pulp space, especially in pediatric root canal system, this is not surprising. On the contrary, there is *in vitro* and clinical evidence that mechanical instrumentation leaves significant portions of the root canal walls untouched. Hence, complete elimination of bacteria from the root canal system by instrumentation alone is unlikely to be achieved.[1] Herein comes the role of disinfection of root canals. There is an array of chemical agents commercially available as irrigants, inter visit medicaments, etc, that not just eliminate persistent pathogenic endodontic microflora, but also help in dissolving out organic debris.

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The major reduction of bacteria in the root canals is achieved by the mechanical action of endodontic files and by irrigation. The need for medication increases in those cases where infection resists regular treatments and the therapy cannot be successfully completed owing to the presence of pain or continuing exudation. Until the mid-1980s, there was a preference for using strong phenolic intracanal antiseptics such as formocresol, camphorated paramonochlorophenol (CPMC), cresatin etc. CMCP proved to be one of the most toxic and irritating phenolic antiseptic followed by cresatin, formocresol and camphorated phenol (CP).[2,3] Moreover, as far as CMCP is concerned, it has a low solubility in water and a slow diffusion rate in agar. Hence, when evaluated *in vitro*, it showed a very limited antimicrobial activity against endodontic pathogens. Consequently, following their harmful effects on connective tissues and the excellent biologic and antimicrobial properties of calcium hydroxide $Ca(OH)_2$, the former aforementioned medicaments are presently not very popular in contemporary endodontic practice.[4]

Calcium hydroxide has, since its inception, proven to be an excellent therapeutic option in endodontics.[5] It has been extensively used in dentistry because of its ability to stimulate mineralization and excellent antimicrobial properties. Faria *et al* confirmed the antibacterial action of a calcium hydroxide paste as an intracanal dressing in human primary tooth root canals with pulp necrosis and apical periodontitis.[6] However; some antimicrobial studies have shown the inefficacy of even Calcium Hydroxide against certain bacterial species, e.g. *Enterococcus fecalis*. Therefore, research for new substances has always been an ongoing process.[7]

One such agent imported from holistic medicine into dentistry is Bee Propolis, a resinous mixture that honey bees collect from tree buds, sap flows, or other botanical sources. Etymologically, the Greek word *Propolis* means *'pro'* (for or in defense of) and *'polis'*

(the city), hence, *'defender of the city/hive'*. In general, propolis *in natura* is composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances with the aroma of poplar, honey and vanilla. Flavinoids present in propolis are known to impart it with antibacterial and anti-inflammatory nature.[8] Propolis also has anti-fungal effects.[9] Koo *et al* have successfully demonstrated antibacterial effect of propolis on *S. mutans*, *S. sanguis* and *A. naeslundii* in addition to the inhibition of glucosyltransferase enzyme.[10]

As far as chlorhexidine (CHX) is concerned, it has also been shown to be a potent broad-spectrum antimicrobial with the advantage of substantivity.[11] The positively charged ions released by CHX get adsorbed into dentine and prevent microbial colonization on the dentine surface for some time beyond the actual period of time of application of the medicament.[12] Furthermore, the premature loss of bond strength is one of the problems that still affects adhesive restorations and markedly reduces their durability.[13] This occurs as a result of deterioration of dentine collagen fibrils by action of endogenous matrix metalloproteinases (MMPs).[14] Dentin collagenolytic and gelatinolytic activities can be suppressed by protease inhibitors, indicating that MMP inhibition could be beneficial in the preservation of hybrid layers.[15] This was demonstrated *in vivo,* where application of CHX exerted a broad-spectrum MMP-inhibitory effect that appreciably improved the integrity of the hybrid layer in a 6-month clinical trial.[16] Considering its antimicrobial spectrum, it has been shown to be more effective against gram-positive organisms than gram-negative organisms,[17] *Vianna et al* have shown that chlorhexidine is able to inactivate many endodontic-resistant organisms in as little as 15 seconds of contact time.[18]

The first part of the current study is to assess the antimicrobial potential of a relatively novel use of propolis as a root canal irrigant in comparison with commonly used and relatively popular and conventional root canal irrigants: chlorhexidine and calcium hydroxide for use in pediatric dental patients.

MATERIALS AND METHOD

The first part of the study was conducted in the Department of Pedodontics and Preventive Dentistry in collaboration with the Department of Microbiology, Babu Banarasi Das College of Dental Sciences, Lucknow, India, after gaining clearance from Institutional Ethical Committee. It was done as a comparative assessment of 60 children aged 6-12 years with pulpally involved primary maxillary molars. A total of 15 subjects in each of four groups receiving *Sterile Physiologic Saline* (control), *2% Chlorhexidine*, *4% Calcium Hydroxide* and *4% Dimethyl Sulfoxide extract of Propolis*, as endodontic irrigants were assessed for antimicrobial efficacy for both aerobic and anaerobic microbial CFU counts.

Children with an acute apical abscess of the maxillary primary molars, children free of any systemic illness prior to initiating the endodontic procedure and children not receiving systemic antibiotics within the past 3-6 months were included in the study. However, children with abscesses were excluded if there was any evidence of a communication into the oral cavity through a sinus tract or the gingival margin. Also, children with a history of any systemic illness and/or drug history of antibiotic intake within the past 3-6 months were also excluded from the study.

Fifteen patients each with acute apical abscess in the maxillary primary molars were divided into four groups in a random manner. Group A included patients undergoing pulpectomy with only Sterile Physiologic Saline (SPS) as intracanal irrigant and served as the negative control. In Group B patients underwent pulpectomy with Chlorhexidine (2%) as intracanal irrigant. Group C comprised of patients that underwent pulpectomy with Calcium Hydroxide (4%) as intracanal irrigant and lastly Group D incorporated patients that underwent pulpectomy with Dimethyl Sulfoxide (DMSO) extract of Propolis (4%) as intracanal irrigant.

Following radiographic examination and adequate anesthesia and rubber dam isolation, an access cavity preparation was carried out. Microbiological samples were obtained from the disto-buccal root canal in all the patients or subjects during this first appointment. The sample was collected by inserting a No. 15 K-file into the distal canal followed by insertion of a number 15 paper point. Both the scrapings from the file as well as the paper point were immersed in sterile vial containing Thioglycollate Broth. Similar technique was repeated and the sample thus collected was immersed into Brain Heart Infusion Broth. Irrigation was then carried out with 2 ml of test irrigant. The biomechanical preparation of the distobuccal canal was subsequently carried out using the test irrigant only for flushing out the debris. The access cavity was finally sealed off using a temporary restorative material (Cavit G). The vial with Thioglycollate broth was sent for Anaerobic mixed bacterial Culture and the vial with Brain Heart Infusion Broth was sent for Aerobic mixed Bacterial Culture to The Department of Microbiology, Babu Banarsi Das College of Dental Sciences, Lucknow.

At the beginning of the second appointment (3 days later), the tooth was again isolated with rubber dam, temporary dressing was removed and the microbiological samples of the root canal contents were taken in the similar manner as previously described. The endodontic procedure was completed thereafter. For microbiological estimation, the vials were immediately transferred to The Department of Microbiology where further evaluation was carried out.

Each screw capped vial of Thioglycollate broth and Brain Heart Infusion Broth was shaken to disperse the sample content evenly. A 0.1 ml inoculum was taken from each vial using a sterile micropipette and inoculated on separate blood agar plates. One blood agar plate which was inoculated with Thioglycollate broth was incubated anaerobically in a Gaspack jar and the other blood agar plate which was inoculated with Brain – Heart Infusion broth was incubated under aerobic conditions. Both plates were incubated at 37°C for 24 hours. The plates were then examined; the numbers of bacterial colonies were counted using a colony counter in terms of CFU/ml of the innoculum. The statistical analysis was done using SPSS (Statistical Package for Social Sciences) Version 15.0 statistical analysis software (SPSS Inc., Chicago, IL, USA).

RESULTS

The mean age of subjects in Group A was minimum (7.87±2.13 years) followed by Group C (7.93±1.75 years) while that of Group B was maximum (8.53±1.92 years). The mean age of subjects in Group D was 8.13±1.92 years. However, on statistical evaluation, no significant difference was seen amongst the groups ($p=0.782$). In Group A, a majority of subjects were females (66.7%) while in all the other three groups the majority of subjects were males. However, on statistical evaluation, no significant intergroup difference was seen ($p>0.05$).

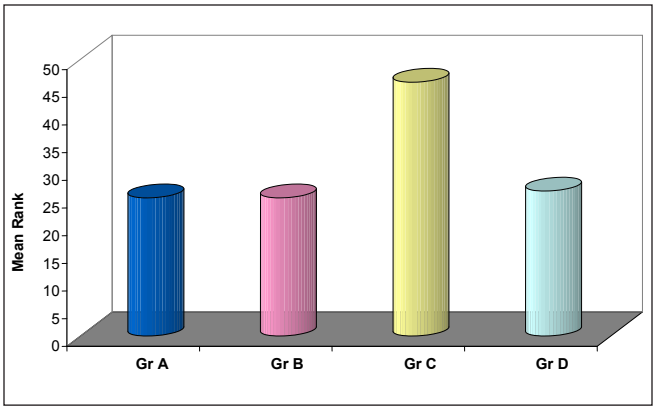

Figure 1. Analysis of variance of Mean Colony Forming units of Aerobes in different groups – after treatment (Kruskall-Wallis Test – Wilcoxon Signed Ranks)

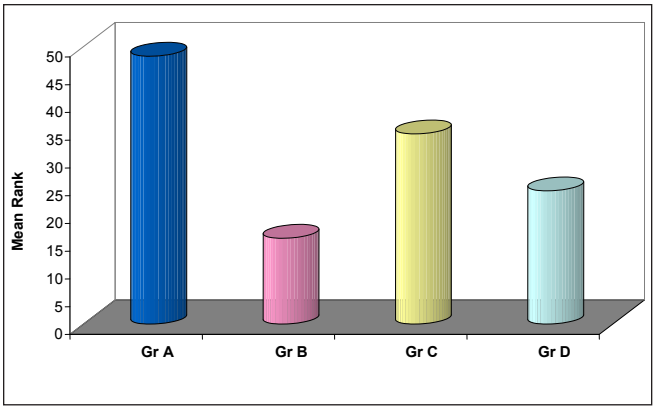

Figure 2. Analysis of variance of Mean Colony Forming Units of Anaerobes in different groups – after treatment (Kruskall-Wallis Test – Wilcoxon Signed Ranks)

Before treatment the mean exponential CFU count of aerobes in Groups A, B and C was 7.13±1.19 whereas in Group D it was 6.93±1.58. Analysis of variance using Kruskall-Wallis Test revealed no statistically significant differences in mean CFU of aerobes before treatment in the four groups (F=1.399; p=0.255).

As the differences among the groups were not found to be significant statistically on Kruskall-Wallis test, no further statistical analysis was necessary.

Before treatment the mean exponential CFU count for anaerobes was maximum in Group A (7.4±1.24) and minimum in Group C (7.13±1.19). It was found to be 7.2±1.26 in Group B and 7.13±1.19 in Group C. On comparing the data statistically by performing analysis of variance using Kruskall-Wallis test (Wilcoxon signed rank), no significant difference in mean CFU of anaerobes was seen before treatment in the four groups (χ^2=0.38; p=-0.957).

During post-treatment assessment after three days, mean aerobic CFU count in Group A and B was 3.8±1.08 which was minimum followed by Group D with 3.87±1.19 and then Group C 5.73±1.49.

Analysis of variance (Table 1, Figure 1) revealed a statistically significant intergroup difference in mean number of CFU of aerobes after treatment in four groups with Group A and Group B having minimum number while Group C had maximum number (p=0.001). As the difference among the groups was found to be significant statistically on applying Kruskall-Wallis test, multiple comparisons were performed to see the intergroup differences more clearly.

Multiple comparisons revealed that Group C had significantly higher mean CFU count for aerobes as compared to the other three groups (p=0.001). However, no statistically significant difference was observed between Group A, Group B and Group D (Table 2). On the basis of observations made above the following order of efficacy of antimicrobials against aerobes was observed:

Group C < Group A ≃ Group B ≃ Group D

During post-treatment assessment after three days, mean anaerobic CFU count in Group B was 3.73±0.88 which was minimum followed by Group A with 6.40±0.91. The mean value was observed to be 4.40±1.12 in Group D and 5.27±1.16 in Group C.

Analysis of variance (Table 3, Figure 2) revealed a statistically significant intergroup difference in mean number of CFU of anaerobes after treatment in four groups with Group B showing the minimum number while Group A had maximum number (p<0.001). As the difference among the groups was found to be significant statistically on applying Kruskall-Wallis test, multiple comparisons were performed to see the intergroup differences more clearly.

Multiple comparisons revealed that Group A had significantly higher mean CFU count for anaerobes as compared to all the other three groups (p<0.05). Group B had significantly lower mean value as compared to Group C, however, no significant difference was observed between Group B and Group D and Group C and Group D (Table 4). On the basis of observations made above the following order of efficacy of antimicrobials against aerobes was observed:

Group A > Group C > Group B ≃ Group D

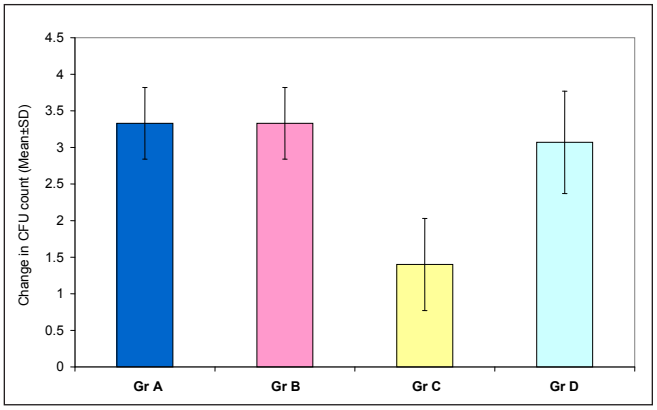

Figure 3. Comparison of change in CFU count of Aerobes after treatment in four groups

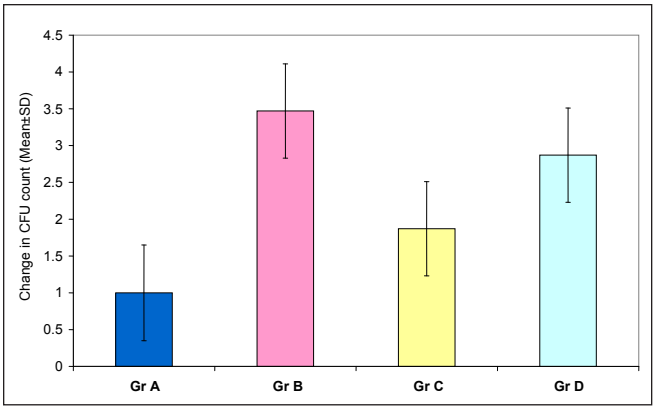

Figure 4. Comparison of change in CFU count of Anaerobes after treatment in four groups

Table 1. Analysis of variance of Mean Colony Forming Units of Aerobes in different groups – after treatment (Kruskall-Wallis Test – Wilcoxon Signed Ranks)

Group	N	Mean Rank
Group A	15	24.97
Group B	15	24.97
Group C	15	45.87
Group D	15	26.20

χ^2=16.318; p=0.001

Table 2. Multiple Intergroup Comparisons

S.No.	Comparison	"z"	'"p"
1.	Group A vs Group B	0	1
2.	Group A vs Group C	3.302	0.001
3.	Group A vs Group D	0.215	0.838
4.	Group B vs Group C	3.302	0.001
5.	Group B vs Group D	0.215	0.838
6.	Group C vs Group D	3.145	0.001

On comparison of pre-treatment and post-treatment assessment, maximum change in aerobic CFU count was seen in Group A and Group B following treatment (3.33±0.49) while minimum was seen in Group C (1.40±0.63). In all the four groups, a significant decrease in mean aerobic CFU count was seen (p<0.001) (Table 5, Figure 3). In terms of change in aerobic cfu count the order of efficacy of four groups was:

Group A ~ Group B > Group D > Group C

As far as change in mean CFU count of anaerobes was concerned, it was maximum in Group B (3.47±0.64) followed by Group D (2.87±0.64) and then Group C (1.87±0.64). Minimum change was observed in Group A (1.00±0.65). In all the four groups the change was significant statistically (Table 9, Figure 4). In terms of change in anaerobic cfu count the order of efficacy of four groups was:

Group B > Group D > Group C > Group A

DISCUSSION

Medicinal use of Propolis dates back to 3rd century B.C. in Egypt and Greece, which were perhaps the first of the civilizations to recognize its miraculous healing properties. Recent research has shown it to be a promising agent in wound healing and antimicrobial efficacy (Grange and Davey, 1990).[19] Subsequent to this, it's potential as a pulp capping agent,[20] an intracanal irrigant,[21] a mouth rinse,[22] a cariostatic agent,[23] in relieving dentinal hypersensitivity [24] and treatment of periodontitis [25] has been reported widely.

The use of 2% Chlorhexidine in the present trial is evidence based; showing bactericidal action towards pathogenic endodontic microflora.[26] As far as 4% DMSO extract of Propolis is concerned, it has well been documented in literature that 4% propolis (whether ethanolic or dimethyl sulfoxide extract) is least cytotoxic as compared to the same concentration (4%) of calcium hydroxide and resulted in long term (>50%) periodontal cell viability (even after 20 hours).[27]

Propolis has been shown to be having appreciable antimicrobial properties in numerous studies. In an *in vitro* assessment Rahman *et al*, 2010 observed Propolis to be a better antimicrobial agent against Gram positive than Gram negative microbial flora.[28] Similar observations have been made by Miorin *et al*, [29]. Gupta *et al* studied *in vitro* antibacterial efficacy of propolis, 3% sodium hypochlorite and 0.2% chlorhexidine gluconate against *Enterococcus faecalis*. They observed that as compared to 0.2% chlorhexidine-gluconate and 3.0% sodium hypochlorite solutions the efficacy of 30% propolis in DMSO and 30% propolis in ethyl alcohol was significantly lower.[30] In the present study we observed the efficacy of chlorhexidine to be higher as compared to Propolis (especially in reducing the anaerobic CFU count).

The main chemical classes present in propolis are flavonoids, phenolics and other various aromatic compounds. The antimicrobial action of propolis is generally attributed to their flavonoid content, the release of which is accentuated in the DMSO extracts rather than ethanolic extracts. Flavonoids prevent bacterial cell division, breaks down bacterial cell walls and cytoplasm.[20]

In a clinical study by Victorino *et al,*[31] the antibacterial activity of propolis based toothpastes was evaluated as an intracanal medicament. They observed that propolis based products showed good activity against aerobic bacteria, proving more effective than calcium hydroxide. However, in our present study, we observed the activity of propolis against aerobes to be lower than Calcium hydroxide.

Among the substances employed as intracanal medicaments calcium hydroxide has been the most widely used and recommended given its biological properties and antimicrobial action. In our present study, we observed it to be the most efficacious in terms of change in aerobic CFU count. The high pH of calcium hydroxide (around 12.5, resulting from the release of hydroxyl ions) exerts a deleterious effect over the bacterial cells as it damages their respective cellular membranes, denatures their proteins and alters their DNA.[32]

Chlorhexidine is a clinically important antiseptic, disinfectant and preservative. It is a potent membrane-active agent against bacteria and inhibits outgrowth, but not germination, of bacterial spores, although it is not sporicidal.[33]

In the present study, both calcium hydroxide and propolis showed to be having a better efficacy as compared to Chlorhexidine which can be attributed to their multiplicity of action at the given concentration. However, the findings of Delgado *et al*, 2010,[34] are in contrast to the findings obtained in the present study. In their *in vitro* experiment to assess whether chlorhexidine alone or in combination with $Ca(OH)_2$ could completely eliminate *E faecalis*, they observed chlorhexidine to have significantly higher antimicrobial activity against *E faecalis* as compared to calcium hydroxide.

The difference in the two studies could be attributed to the difference in design, duration of study and difference in environmental conditions of the microflora for growth. While the present study was done on a before-after design, evaluating the overall change in microbial flora and not against a particular species or strain, the focus of Delgado *et al* was on *E faecalis* alone. They adopted an *in vitro* design, where except for the medicaments in use all the conditions were controlled whereas the present study was done in human subjects themselves, where there was a change in ambient environment of the microflora being assessed.

Table 3. Analysis of variance of Mean Colony Forming Units of Anaerobes in different groups – after treatment (Kruskall-Wallis Test – Wilcoxon Signed Ranks)

Group	N	Mean Rank
Group A	15	48.27
Group B	15	15.47
Group C	15	34.27
Group D	15	24.00

χ^2=30.714; p<0.001

Table 4. Multiple Intergroup Comparisons

S.No.	Comparison	"z"	'"p"
1.	Group A vs Group B	4.615	<0.001
2.	Group A vs Group C	2.785	0.006
3.	Group A vs Group D	3.918	<0.001
4.	Group B vs Group C	3.417	0.001
5.	Group B vs Group D	1.576	0.115
6.	Group C vs Group D	1.082	0.081

The results in the present study are in agreement with the findings of Radeva *et al,*[35] who observed calcium hydroxide to be having significantly higher antimicrobial efficacy as compared to chlorhexidine in a few clinical isolates from cases with acute periodontitis.

The relative superiority of calcium hydroxide solution as compared to propolis in our study is in contrast to the findings of Awawdeh *et al,*[36] who observed Propolis to be significantly more effective than non-setting calcium hydroxide against *E faecalis* after short-term application for 1 and 2 days.

Propolis is a natural product and its composition changes from region to region. There are ample studies that have tried to explore the differences in its antimicrobial activity based on different regional sources of its procurement.[37] In the present study however, raw Brazilian propolis was obtained from Swati Enterprises®, New Delhi, India. Processing of Brazilian propolis was done at National Botanical Research Institute (NBRI), Lucknow. It involved the following steps:

1. The first step in processing was evaluation of the material on its arrival at the distillation plant. According to protocol if very waxy, it is put through a cold-water washing process where the extrinsic wax will be removed. The remaining propolis is then air-dried on stainless-steel screens. If very little extrinsic wax is found, as was the case with our sample of Brazilian Propolis, it was immediately sent for the second step.
2. The second step involved milling the solid portions of propolis into powder form followed by dissolving it in Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) to achieve the desired concentration for the research, i.e. 4%. Through a proprietary process, the remaining beeswax as well as bee parts and wood chips were removed.
3. The final step involved filtration. The propolis solution thus prepared was put through a series of filters to remove any remaining small particles of foreign material.

Such findings, as elucidated during the present trial hold more relevance in *in vivo* conditions where the continuous interaction between the microbial flora, antimicrobial agents and surrounding biological conditions influence and change the milieu interior. Under *in vivo* conditions, the subject of concern is not only to find out the strongest but also to evaluate the agent that is most sustainable within the site of action. The antimicrobial agent retaining its antimicrobial property for a longer duration of time under variable environments has the greater potential than that having the maximum antimicrobial property at a certain temperature for a particular period of time. Henceforth one can claim *in vivo* trials to be a better judge of performance of these medicaments than their mere properties. There is varying ability of different antimicrobial agents against different types of microbes. In the present study, chlorhexidine proved out to be most efficient against anaerobes. Similar observations were made by Schafer *et al,*[38] Lin *et al* [39] and Evans *et al* [40] respectively. Anaerobes can withstand the adverse environments, and it takes longer for them to be susceptible. Chlorhexidine has a unique feature in that dentine medicated with it acquires antimicrobial substantivity that may inhibit re-infection of the canal subsequent to treatment during that time period.[41] Chlorhexidine is retained in root canal dentine in levels sufficient to exert antimicrobial effects for at least 12 weeks.[6,18]

Henceforth, under such dynamic intracanal milieu, antimicrobial agents with a versatile physiology of function, e.g. propolis, whose antimicrobial property cannot be attributed to mere lowering of pH or any other single physical/ chemical property, seems to be a natural choice to obtain a suitable antimicrobial efficacy both against aerobic and anaerobic microbial colonies under varying *in vivo* conditions.

CONCLUSIONS

On the basis of observations made during the course of the present study the following conclusions were drawn. Firstly, chlorhexidine proved to be a superior antimicrobial agent as compared to dimethyl

Table 5. Comparison of change in CFU count of Aerobes after treatment in four groups

SN	Group	Pre-treatment		Post-treatment		Change		Significance of chance	
		Mean	SD	Mean	SD	Mean	SD	"z"	"p"
1.	A	7.13	1.19	3.80	1.08	3.33	0.49	3.542	<0.001
2.	B	7.13	1.19	3.80	1.08	3.33	0.49	3.542	<0.001
3.	C	7.13	1.19	5.73	1.49	1.40	0.63	3.391	0.001
4.	D	6.93	1.58	3.87	1.19	3.07	0.70	3.477	0.001

Table 6. Comparison of change in CFU count of Anaerobes after treatment in four groups

SN	Group	Pre-treatment		Post-treatment		Change		Significance of chance	
		Mean	SD	Mean	SD	Mean	SD	"z"	"p"
1.	A	7.40	1.24	6.40	0.91	1.00	0.65	3.217	0.001
2.	B	7.20	1.26	3.73	0.88	3.47	0.64	3.508	<0.001
3.	C	7.13	1.19	5.27	1.16	1.87	0.64	3.502	<0.001
4.	D	7.27	1.28	4.40	1.12	2.87	0.64	3.502	<0.001

sulfoxide (DMSO) extract of propolis against endodontic aerobes. Calcium hydroxide was found to be least effective. Secondly, chlorhexidine proved to be superior antimicrobial agent against endodontic anaerobes followed by DMSO extract of propolis. Calcium hydroxide followed by sterile physiologic saline were found to be least effective.

Despite the fact that chlorhexidine and calcium hydroxide have been time tested antimicrobials for disinfection of infected root canals, relatively novel and biogenic agents namely propolis, have opened new horizons towards a more effective elimination of endodontic pathogenic microflora.

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