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ORIGINAL RESERACH

An *In Vitro* Comparative Evaluation of Antimicrobial Efficacy of Propolis, Morinda Citrifolia Juice, Sodium Hypochlorite and Chlorhexidine on *Enterococcus faecalis* and *Candida albicans*

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ABSTRACT

Aim: To evaluate the antimicrobial effectiveness of Propolis, Morinda citrifolia juice, Sodium hypochlorite and Chlorhexidine on *Enterococcus feacalis* (*E. feacalis*) and *Candida albicans* (*C. albicans*), as endodontic irrigants.

Materials and methods: Four clinical isolates and one standard ATCC sample (29212) of *E. feacalis* and ATCC sample (90028) of C. albicans were inoculated into 5ml of peptone water each and incubated at 37° C for 3 to 4 hours to attain the turbidity corresponding to 0.5 McFarland standard CFU. We followed Disc and well diffusion Kirby–Bauer method to attain the zones of inhibition.

Results: Overall comparison of reagents revealed a significant difference among zones of inhibition. The standard concentration of 5% sodium hypochlorite, 2% chlorhexidine, 10% Propolis and 100% Morinda citrifolia juice illustrated the maximum inhibition zone for both test organisms.

Conclusion: All four reagents had an antimicrobial effect on the microorganisms tested. Sodium hypochlorite and Chlorhexidine were more effectual than Propolis and Morinda citrifolia juice and there was increased antimicrobial efficacy with increasing concentrations.

Clinical significance: A study for finding safe herbal agents that can be used as endodontic irrigants revealed that Propolis, Morinda citrifolia juice has antibiotic properties and can replace routinely used agents thereby limiting the side effects.

Keywords: *Candida albicans*, Chlorhexidine, *Enterococcus feacalis*, Morinda citrifolia juice, Propolis, Sodium hypochlorite.

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INTRODUCTION

Endodontic failures are mainly ascribed to the existence of microorganisms in root canals.[1] Removal of microorganisms along with debris is a vital aspect for a good root canal therapy (RCT) outcome. Many microbial organisms like *E. faecalis, C. albicans* were found in endodontic infections with studies showing that both these microbes lead to RCT failure.[2,3]

E. faecalis, a gram-positive organism, even though present in minute quantity has a key role in causing continual periradicular infections and root canal failures, by genetic polymorphism and dentin binding property. Studies reported *E. faecalis* in 4 to 40% of root canal infections and increased incidence of about nine times in treatment failures.[4] *C. albicans* is an opportunistic dentinophillic yeast showing an increased incidence in infected canals and peri-radicular tissues.[5]

Successful RCT aims at total elimination of infected tissue by disrupting and removing all the microbes, which requires profuse, repeated irrigation with intracanal medicaments apart from mechanical debridement. Irrigants frequently used in RCT are sodium hypochlorite (0.5–5.25%), hydrogen peroxide (3%) solution, EDTA, chlorhexidine gluconate (0.2–2%) and physiologic saline solution.[1-3]

Sodium hypochlorite is most frequently used medicament and has an antimicrobial effect and is a good organic tissue solvent, without leaving any noxious residues. It has few drawbacks like bad taste, toxic in nature, instrument corrosion, inflammatory and allergic reactions.[1,6]

Two percent chlorhexidine gluconate as intracanal irrigant has broad antimicrobial activity. It is less toxic, with disadvantages of discoloration of teeth, bad taste, dry mouth and in general inferior than hypo in antimicrobial removal.[1,7]

Hence research was done to look for safer herbal agents. A literature search showed that few agents have antimicrobial properties like Propolis, Morinda citrifolia juice and whether they may be employed as endodontic irrigants.[2,3]

We carried this study to appraise the antimicrobial effectiveness of propolis, sodium hypochlorite, morinda citrifolia juice and chlorhexidine on *E. feacalis* and *C. albicans*, as endodontic irrigants.

MATERIALS AND METHODS

Four clinical isolates and one standard ATCC sample (29212) of *E. feacalis* and ATCC sample (90028) of *C. albicans* were inoculated into 5 mL of peptone water each and incubated at 37° C for 3 to 4 hours to attain the turbidity corresponding to 0.5 McFarland standard CFU. We followed disc and well diffusion Kirby–Bauer method to attain the zones of inhibition.

Plates for Muller Hinton agar (MHA) at a ph of 7.2–7.4 with a depth of 4 mm were prepared for *E. Faecalis* and similarly for *Candida albicans* plates with Muller Hinton agar +2% glucose, and 0.5 µg/mL methylene blue dye was prepared. A 50 µml of the microbial suspension was kept on an agar plate and was further evenly spread with L–spreader.

Preparation of Irrigants

Ten percent Propolis solution was made by dissolving 500 mg tablet in 5 mL of warm sterile distilled water and likewise, a range of concentrations was obtained, i.e., 2.5% and 5%.

Three percent, 1.5 % and 0.75% morinda citrifolia juice were made by diluting 100 % juice.

Three percent, 2.5% and 0.5% sodium hypochlorite was made by diluting 5% hypo.

One percent, 0.5% and 0.22% chlorhexidine was made from 2% solution.

Disc Preparation

Disc preparation was done by impregnating the disc with dissimilar concentrations of irrigants. Using sterile forceps antimicrobial impregnated disc was kept on the agar surface with minimum 20 mm distance between each disc. An anti-bacterial sensitivity disc of chlortetracycline and Voriconazole which are considered as the control group was placed in the center of the plates containing *E. faecalis* and *C. albicans* respectively. The plates were inverted and incubated at 37ºC for 16–18 hours for *E. faecalis* and 24–48 hours for *C. albicans*.

Preparation of Wells

With a sterile cork borer, evenly distributed holes were made and samples were loaded in each hole. The plates were incubated at 37ºC for 16 to 18 hours and 24 to 48 hours for *E. faecalis* and *C. albicans,* respectively.

The presence and size of the inhibitory zone for each antimicrobial agent were measured on the plates by Hi-media scale. Observations were made with unaided eye while viewing the back of the Petri dish with reflected light against a black non-reflecting background. The measurement of zones of inhibition was done at a standard concentration of various antimicrobial agents followed by zones at different concentrations.

Medians and quartiles were represented for comparing the zones of inhibition for the four standard agents across the various clinical isolates of the microorganism. The relationship between the increasing concentration of an agent and its influence on zones of inhibition was tested using Spearman's correlation coefficient. The comparisons between four standard concentrations were analyzed by the Kruskal–Wallis test. The comparison of individual standard agents was further analyzed using Mann-–Whitney test, with considering p-value of <0.05 as statistically significant.

RESULTS

Comparison of reagents against *E. faecalis* revealed a significant variation among zones of inhibition (Table 1 and Graph 1–Box plot). Among individual groups, there was the insignificant difference between 10% propolis and 100% morinda citrifolia juice and between 2% chlorhexidine and 5% hypo. Both herbal agents had significantly lesser inhibition compared to 2% chlorhexidine and 5% hypo.

Table 1: Zones of inhibition for *E. faecalis* in millimetres comparing the four standard concentration of test agents

Agent and the standard concentrations	*Median zones of inhibition for E. Faecalis*	*First quartile*	*Third quartile*	*p-value (Kruskal–Wallis test)*
10% Propolis	5 mms	1	9.75	
100% Morinda citrifolia juice	5.5 mms	0	11.75	0.002* Significant
2% Chlorhexidine	17 mms	16.25	18.5	
5% Sodium hypochlorite	17 mms	14.5	18.5	

Table 2: Zones of inhibition for *E. faecalis* in millimeters for the three clinical isolates and ATCC (29212) tested using disc diffusion method for sodium hypochlorite.

Concentrations of sodium hypochlorite	*Clinical isolates*			*ATCC 29212*	*Median zones of inhibition for E. aecalis (in mms)*	*Spearmen's correlation coefficient*	*p-value and significance*
	8	*11*	*10*				
5%	16 mm	17 mm	17 mm	19 mm	17 mm		
3%	14 mm	20 mm	14 mm	16 mm	16 mm		
2.5%	15 mm	15 mm	14 mm	14 mm	14.5 mm	0.74	0.001 Significant
0.5%	14 Mm	14 mm	11 mm	14 mm	14 mm		

Table 3: Zones of inhibition of *E. feacalis* in millimetres for the 3 clinical isolates and ATCC [29212] tested using disc diffusion method for Chlorhexidine

Concentrations of Chlorhexidine	*Clinical isolates*			*ATCC 29212*	*Median zones of inhibition for E. Faecalis (in mms)*	*Spearmen's correlation coefficient*	*p-value and significance*
	8	*11*	*10*				
2%	17 mm	15 mm	17 mm	20 mm	17 mm		
1%	16 mm	12 mm	17 mm	17 mm	16.5 mm		
0.5%	14 mm	18 mm	16 mm	15 mm	15.5 mm	0.49	0.05 Significant
0.2%	15 mm	15 mm	16 mm	16 mm	15.5 mm		

Table 4: Zones of inhibition for *Candida Albicans* in millimetres comparing the four standard concentration of test agents

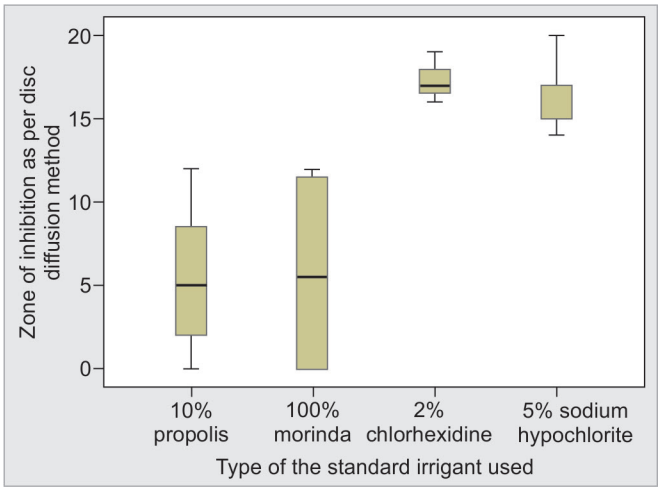
Agent and the standard Concentration	*Median zone of inhibition for C Albicans*	*First Quartile*	*Third Quartile*	*p-value and significance*
10% Propolis	3.5	3	5	
100% Morinda Citrifolia juice.	2	1.25	2	
2% Chlorhexidine	21.5	20.25	22.75	<0.001, Significant (Kruskal–Wallis test)
5% Sodium Hypochlorite	26	22.5	28	

Inhibition was absent for 2.5% propolis and a positive association between the increasing concentrations and zones of inhibition for *E. Faecalis*, indicating a dose-response relation, which was seen for all four agents. 10% propolis had a highest zone of inhibition for *E. Faecalis* and is hence the most efficient one (Graph 2–Box plot).

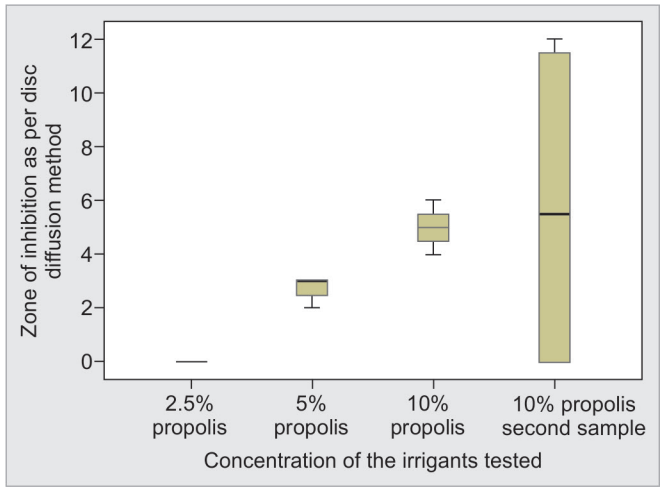
There was no inhibition for 0.75% and 1.5% Morinda citrifolia juice. 100% had the highest zone of inhibition and is hence the most efficient one (Graph 3–Box plot).

Five percent hypo and 2% chlorhexidine had the highest zone of inhibition for *E. Faecalis* and hence the most efficient ones (Tables 2 and 3).

Comparison of reagents against *C. albicans* showed a significant variation between zones of inhibition (Table 4). Among individual groups, 10% Propolis had the least zone of inhibition. Morinda citrifolia juice had significantly better zones of inhibition compared to Propolis, whereas compared to 2% chlorhexidine and 5% hypo it had significantly lesser inhibition.



Graph 1: Comparison of different reagents against *E. faecalis* demonstrating zones of inhibition



Graph 2: Zones of inhibition for various concentration gradients of propolis

Table 5: Zones of inhibition for *Candida albicans* in millimetres for the 3 clinical isolates and ATCC [90028] tested using disc diffusion method for Propolis

Concentrations of Propolis	Clinical isolates 8	11	10	ATCC 90028	Median zones of inhibition for C albicans (in mms)	Spearmen's correlation coefficient	p value and significance
10 %	5 mm	3 mm	3 mm	5 mm	4 mm	0.874	<0.001 Significant
10 %	5 mm	4 mm	3 mm	3 mm	3.5 mm		
5 %	2 mm	2 mm	2 mm	3 mm	2 mm		
2.5 %	0 mm	0 mm	0 mm	0 mm	0 mm		

Table 6: Zones of inhibition for *Candida albicans* in millimetres for the 3 clinical isolates and ATCC [90028] tested using disc diffusion method for morinda citrifolia juice.

Concentrations of Morinda Citrifolia juice	Clinical isolates 8	11	10	ATCC 90028	Median zones of inhibition for C albicans (in mms)	Spearmen's correlation coefficient	p-value and significance
100 %	2 mm	1 mm	2 mm	2 mm	2 mm	0.8	<0.001 Significant
3 %	2 mm	2 mm	2 mm	3 mm	2 mm		
1.5 %	0 mm	0 mm	0 mm	0 mm	0 mm		
0.75 %	0 mm	0 mm	0 mm	0 mm	0 mm		

Table 7: Zones of inhibition for *E. faecalis* in millimetres comparing the four standard concentration of test agents (well diffusion method)

Agent and the standard Concentration	Median zones of inhibition for E. Faecalis	First quartile	Third quartile	p-value and significance
10% Propolis	13	12.25	13.75	<0.005, Significant (Kruskal Wallis test)
100% Morinda citrifolia juice	4.5	4	5	
2% Chlorhexidine	21.5	18.75	22	
5% Sodium Hypochlorite	21.5	19.25	23	

Table 8: Well diffusion results for single sample of all the concentrations of all the four test agents for ATCC [90028] sample of *Candida albicans*

Agent and concentration		Zone of inhibition by disc diffusion
Propolis	10 %	5 mm
	5 %	3 mm
	2.5 %	0
Morinda Citrifolia juice	3 %	1 mm
	1.5 %	1 mm
	0.75 %	1 mm
Sodium hypochlorite	3 %	20 mm
	2.5 %	22 mm
	0.5 %	18 mm
Chlorhexidine	1 %	26 mm
	0.5 %	21 mm
	0.2 %	17 mm

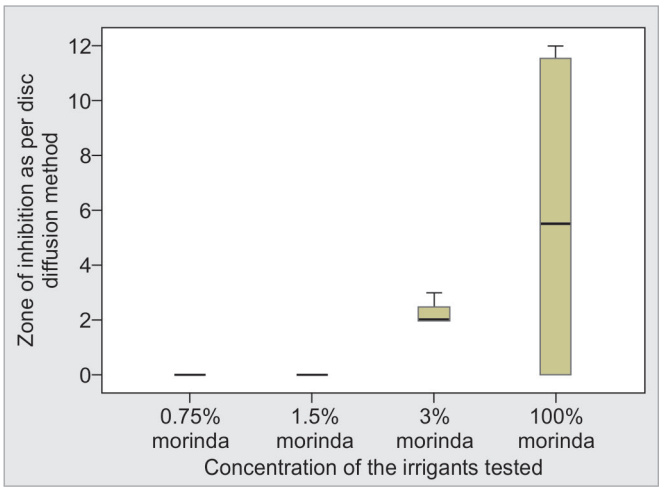
We found a positive correlation among the increasing Propolis concentrations and zones of inhibition for *C. albicans*. 10% Propolis had the highest zone of inhibition for Candida and is hence the most effective one (Table 5).

There was no inhibition for 0.75% and 1.5% concentration of Morinda citrifolia and a positive relation between the increasing concentrations and zone of inhibition. 100% Morinda citrifolia juice had the highest zone of inhibition and is hence the most effective one (Table 6).

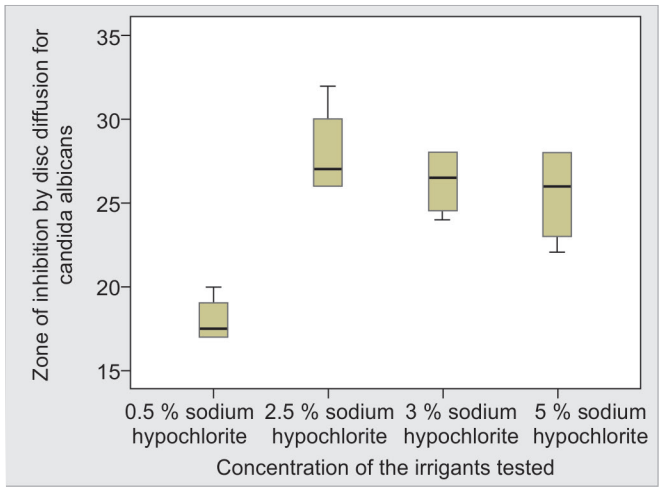
There was a positive but nonsignificant relation between the increasing concentrations and zone of inhibition *C. albicans*. There were similar zones of inhibition for 5%, 3% and 0.5% concentrations. Even the lowest concentration of 0.5% showed an adequate zone of inhibition (Graph 4–Box plot).

Two percent chlorhexidine had the highest zone of inhibition for *C. albicans* and is hence the most effective one (Graph 5–Box plot).

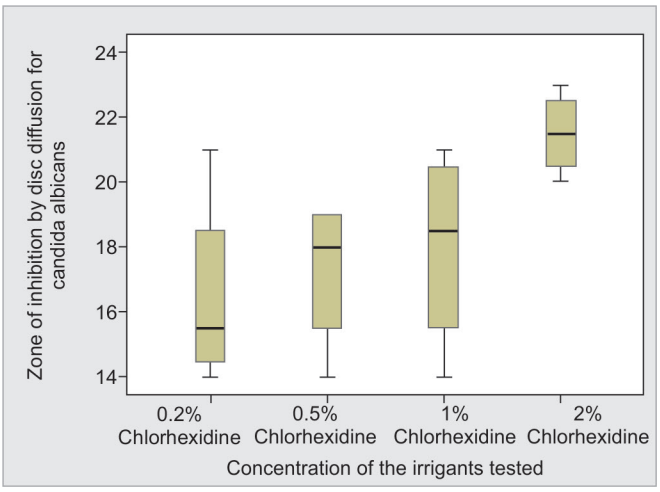
There was no significant difference between 2% chlorhexidine and 5% sodium hypochlorite's zone of



Graph 3: Zones of inhibition for different concentrations of Morinda citrifolia



Graph 4: Zones of inhibition for Candida albicans against different concentration gradients of Sodium hypochlorite



Graph 5: Zones of inhibition for *Candida albicans* against different concentrations of Chlorhexidine

inhibitions for *E. faecalis* tested by well diffusion method (Table 7).

Zone of inhibitions for *C. albicans* revealed that 1% chlorhexidine had the highest value followed by 3% sodium hypochlorite and 10% Propolis (Table 8).

Zones of inhibition for Propolis were comparatively higher than Morinda citrifolia juice against *E. faecalis*, whereas the later agent showed better zones of inhibition than the former against *C. albicans*.

DISCUSSION

Persistent microorganisms like *E. faecalis*, *C. albicans* in root canals even after therapy cause failure of treatment. Therefore, adequate treatment strategies must include conventional intracanal irrigating medicaments like sodium hypochlorite and chlorhexidine and also newer herbal products like propolos and Morinda citrifolia fruit juice.[1-3]

Flavonoids like galangin and pinocembrin and phenolic acids or their esters in propolis contribute to the antimicrobial activity.[8] Marcucci et al. showed the effective antifungal and antibacterial activity of 10% propolis.[9] Similarly, Shveta et al. found 30% solution of Propolis was effectual against *E. faecalis* and *C. albicans*, after both 48 hours and 10 days of application.[10]

Tanins present in Morinda citrifolia contributes to its antimicrobial action owing to their toxicity that causes the destruction of bacteria and fungi, the other factor being it's high pH (3.5).[11]

The comparative study revealed a significant variation among zones of inhibition of all the four tested agents. The zones of inhibition for sodium hypochlorite (5%) was comparatively higher than chlorhexidine (2%) but statistically insignificant, similar to the findings of Luddin and Ahmed.[12] Both herbal agents had significantly lesser zones of inhibition compared to conventional ones. The solvent used for propolis extraction (ethanol, chloroform, methanol or propylene glycol) influences its antimicrobial action. The stage of ripeness and processing mechanism of Morinda citrifolia juice are supposed to be reasons for lesser antimicrobial activity.[2,3,13,14]

There was no statistically significant difference between Propolis (10%) and Morinda citrifolia juice (100%) against *E. faecalis*, similar to the findings of Kandaswamy and Venkateshbabu[15] and a significant difference against *C. albicans*, with Morinda citrifolia juice showing better zones of inhibition than propolis, similar to Carbajal Mejia findings.[16]

Jainkittivong et al. found the antifungal effect of Morinda citrifolia fruit extract on *C. albicans* which varied with concentration and contact time.[17]

Propolis in its different concentrations, when tested against *C. albicans*, showed larger zones of inhibitions at higher concentration (10%). At lower concentrations, flavonoids present in the propolis might get further dissolved and be ineffective. This is similar to findings of Marcucci et al. who showed effective antifungal and antibacterial activity of 10% Propolis.[9]

Morinda citrifolia juice in its different concentrations, when tested against *C. albicans*, showed larger zones of inhibitions at higher concentration (100%). There was no inhibition for (0.75%, 1.5%). At concentration (3%), some antimicrobial effectiveness was seen. Our findings are similar to Wang et al.[11]

Hypo in various concentration against *C. albicans* demonstrated larger zones of inhibitions at higher concentration (5%), though it was not statistically significant. The efficacy of hypo at a higher concentration on both microorganisms may be due to the concentration rise is directly related to the antimicrobial efficiency and tissue dissolution capacity.[18]

Chlorhexidine in its various concentrations, when tested against *C. albicans*, revealed larger zones of inhibitions at higher concentration (2%), similar to findings of Estrella et al.[19]

We used well diffusion method to have more confirmatory and validated data and found results of higher zones of inhibition for hypo and Chlorhexidine than propolis and morinda citrifolia juice were in accordance with the disc diffusion method, without significant difference.

Further research must be concentrated to find out the antimicrobial effectiveness for propolis and morinda citrifolia juice to limit the side effects of routinely used intracanal irrigants like chlorhexidine and sodium hypochlorite.

Limitations

With three clinical isolates and one standard strain, the sample size available was four samples per group. Hence a larger study with a higher sample size may be needed to establish the significance of these findings further.

CONCLUSION

We observed that sodium hypochlorite, chlorhexidine, Propolis, and Morinda citrifolia juice have an antimicrobial effect on all the microorganisms tested, with conventional agents, sodium hypochlorite, chlorhexidine; more efficient than herbal agents Propolis and Morinda citrifolia juice.

All the four irrigants showed their increased antimicrobial efficacy with increasing concentrations.

Hypo was more effectual than chlorhexidine against both the microorganisms.

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