

Influence of Medicinal Plant Extracts on the Growth of Oral Pathogens *Streptococcus Mutans* and *Lactobacillus Acidophilus*: An *In-Vitro* Study

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Abstract

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AIM: This study investigated the antibacterial efficacy of five plant extracts, as well as the combinations of the two most effective plant, extracts either with or without commercial varnish (MI varnish) on the in vitro growth of *Streptococcus mutans* and *Lactobacillus acidophilus* in comparison to MI varnish using agar disk diffusion and broth dilution methods.

METHODS: Methanolic extractions of five plants (Cinnamon, Turmeric, Ginger, Clove and Black seed,) were tested against the growth of the two oral pathogens. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for the two most effective extracts, and their combinations with different ratios were evaluated against the growth of the two oral pathogens, followed by incorporating the two effective plants or each into commercial MI varnish to be assessed against the oral pathogens in comparison to MI varnish.

RESULTS: Only Cinnamon and Clove produced inhibition zones against *Streptococcus mutans* and *Lactobacillus acidophilus* growth. MIC for the two plants showed equal antimicrobial activity against *Streptococcus mutans*, while Cinnamon had a higher sensitivity to *Lactobacillus acidophilus* than Clove. A mixture of Cinnamon and Clove in a ratio 1:1:2 exhibited the highest antibacterial activity. Integration the mixture of both plants into MI varnish in a ratio of 1:1:1 presented the highest antibacterial activity. Meanwhile, the lowest one was recorded for the MI varnish alone.

CONCLUSION: Methanolic extract of Cinnamon and Clove has considerable antimicrobial activity against *Streptococcus mutans* and *Lactobacillus acidophilus* and a new tool for minimally invasive and adhesive dentistry avenues.

Introduction

Bacteria Dental caries is one of multifactorial infectious disease caused by acids from bacterial metabolic activity diffusing into enamel and dentine. Even as caries is a profoundly preventable disease that has seen a decline in most developed international locations these days, it stays a

noteworthy public fitness problem [1], [2]. The principal etiologic factor included in the presence of specific bacteria particularly *Streptococcus mutans* and other non-streptococcus species like *Lactobacillus acidophilus* which produce acid and bring the plaque to the critical pH [3]. Numerous preventive strategies have been attempted and tested yet none is ended up being 100% powerful for the cessation of dental caries and the journey still goes

on. Right now, Chlorhexidine is thought to be the gold standard level among chemotherapeutic agents against the most cariogenic pathogen *Streptococcus mutans*, however, the occurrence of oral side effects, for example, teeth staining, bad taste, dryness, and burning sensation debilitate patients to utilise it [4]. More critically, most antibacterial agents can also promote the development of resistant bacterial strains [5]. Therefore, it becomes a necessity for the current therapeutic research to investigate naturally available products which are safe for humans and specific for dental caries, as their side effects are insignificant, and the patient is dealt with comprehensively. Several investigations have been done to decide the utilisation of normal regular household natural basic oils including Cloves and Cinnamon as solutions for managing dental diseases like toothache and gum swelling [6], [7]. More recently, scientific research demonstrated the potential antibacterial properties of concentrates from these herbs [6], [8], [9]. Use of the improvement of such bioassays in clinical science may offer valuable learning and a way to manage oral disease. One therapeutic plant in selected group is Cinnamon (*Cinnamomum zeylanicum*) which is thought to have medical advantages [10] and has been utilised as a part of the conventional prescription for colds, flatulence, nausea and diarrhoea, also improves vitality, circulation and energy [11].

Additionally, studies have discovered that Cinnamon and Ginger (*Zingiber officinale*) may have antibacterial and antifungal properties [12], [13]. Turmeric (*Curcuma longa*) belongs to the Ginger (*Zingiberaceae*) circle of relatives. Since the time of Ayurveda (1900 BC) many therapeutic sports had been assigned to Turmeric for more than one illness and situations, like the ones of the pores and skin, pulmonary, and gastrointestinal structures, aches, pains, and liver problems [14]. Although several plants have demonstrated antibacterial activity however, to date, their antibacterial activity against cariogenic bacteria is still under research. Hence in search for novel anti-cariogenic agents, five plants which are known for their medical applications have been chosen in this study; Cinnamon, Ginger, Turmeric, Cloves and Black seed [15], [16]. Therefore, in the present study, we found it interesting to investigate the antibacterial effect of the chosen five plants in terms of bacterial growth inhibition of *Streptococcus mutans* and *Lactobacillus acidophilus*. In addition, the current study aimed to investigate the antibacterial activity of the two most effective plants in combination with commercial varnish [5% sodium fluoride varnish with Recaldent (CPP-ACP), GC America, USA] where various reports have been published on the anti-cariogenic action of casein phosphopeptide amorphous calcium phosphate (CPP-ACP) paste/solution [17], [18], [19] and the synergistic impact of CPP-ACP and fluoride [17], [18], [19], [20], meanwhile no trials have been made on medicinal plants antibacterial effect in combination with CPP-ACP.

Material and Methods

Different plants preparation

Five plants; (Cinnamon; bark) *Cinnamomum zeylanicum*, (Turmeric; rhizome) *Curcuma longa*, (Ginger; rhizomes) *Zingiber officinale*, (Clove; fruits) *Syzygium aromaticum*, and (black seed; seeds) *Nigella sativa* were tested.

Tested plants were purchased from local stores in Ismailia, Egypt. The collected plants were re-identified, and the nomenclature was rechecked and confirmed by the help of plant taxonomist. All raw substances had been washed with clean tap water, then through sterilised distilled water and air-dried. An exception for Ginger rhizomes which had been reduced wiped clean then dried in a vacuum oven at 80°C for two days. All dried plant life had been powdered the usage of the sterilised grinding system. The obtained powder was immediately subjected to the extraction procedure [21], [22].

Extracts preparation

Fifty grams of each plant powder were packed in the Soxhlet thimble then extracted successively with an organic solvent 90% methanol. Extraction carried out for each plant separately for forty-eight hours using Soxhlet extractor [23]. Each extract was filtered through filter paper (Whatman No.1) then concentrated by complete evaporation with a rotary evaporator under reduced pressure. The resulting dry extracts were re-weighed, and the percentage of the resultants were calculated from the quantity of the initial plant material (50 g). Crude extracts stock solutions were prepared by mixing dried extracts with an appropriate amount of DMSO (100%) and stored at 4°C in an airtight sterilised bottle till use [24].

Tested bacterial strains

Two bacterial strains were tested; *Streptococcus mutans* Serotype c. Carious dentin ATCC 25175 type strain which was purchase from Microbiological Resources Centre (MIRCEN), Cairo, Egypt and *Lactobacillus acidophilus* CH-2 from Chr. Hansen's Lab, Denmark. *Streptococcus mutans* was streaked on tryptic soy agar (TSA) while *Lactobacillus acidophilus* MRS agar. Both cultures were incubated for twenty-four hours at 37°C.

Antibacterial assessment

Antibacterial activities of methanolic extracts of different plants were carried out by disc diffusion assay according to the standard method [25], [26].

Disc diffusion method

The inoculum was prepared as recommended by the Clinical and Laboratory Standards Institute by direct colony suspension method [27]. Colonies of an overnight culture of both *Streptococcus mutans* and *Lactobacillus acidophilus* were suspended in Mueller-Hinton broth (Oxoid) and adjusted to 0.5 McFarland standards to reach a final inoculum corresponding to approximately 1×10^8 CFU/ml.

Total of sixty sterilised (6 mm) filter paper discs were divided into six groups which had been loaded with the different plant extracts (G1; Cinnamon, G2; Turmeric, G3; Ginger, G4; Clove, G5; Black seed, G6; positive control) ($n = 10$). Each group was further subdivided according to the type of bacterial strain that was tested on into two main subgroups ($n = 5$). The discs were saturated with 10 μ l of different plant extracts separately under aseptic conditions and 0.12% Chlorohexidine Digluconate liquid (CHX) (Sigma Aldrich, Steinheim, Germany) as a positive control. Loaded discs were dried in laminar flow for 15 minutes at room temperature and were used for the disc diffusion assay. 100 μ l from each bacterial strain was streaked separately using a sterile swab to Muller Hinton agar plates. Plates were let to dry for 15 minutes then used for the sensitivity test. All loaded discs were placed on the surface of the inoculated Mueller-Hinton agar (Figure 1). Plates were incubated for overnight at 37°C, and the antibacterial activity of each extract was expressed by measuring the diameter of the inhibition zone (mm). The experiment was done in triplicate to ensure consistency.

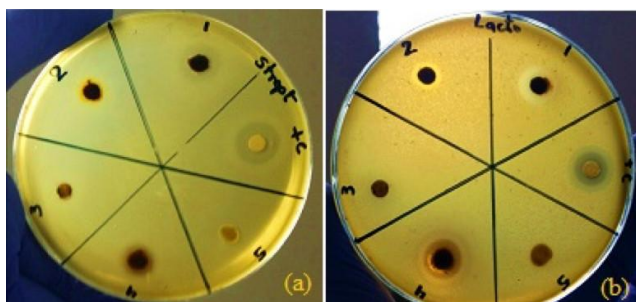


Figure 1: Antimicrobial activity of the five plant extracts against A) *Streptococcus mutans* estimated by the agar-Disc diffusion method and B) *Lactobacillus acidophilus* estimated by the agar-Disc diffusion method

Broth dilution method

The two plant extracts; Cinnamon and Clove that showed effective antimicrobial activity, were selected to demonstrate their minimum inhibitory concentrations (MICs) and Minimum Bactericidal Concentrations (MBC) according to the Clinical and Laboratory Standard Institution strategies by serial two-fold micro broth dilution technique [27]. Mueller Hinton broth (MHB) was prepared and poured into sterile test tubes. One colony of each tested bacterial

strains (*Streptococcus mutans* and *Lactobacillus acidophilus*) were inoculated separately in 2.5 ml MHB and incubated overnight at 37°C on a shaker (250 rpm). 0.5 ml of each overnight culture were inoculated into 5 ml pre-warmed MHB then incubated at 37°C on a shaking incubator for about 18 h to a final optical density (OD600) of 1. A serial dilution was done to different plant extracts in MHB medium to reach concentrations ranging from 100 to 1.563 mg/ml. 100 μ l of each prepared bacterial strain was inoculated to the tubes with different concentrations of plant extracts. A tube of MHB supplemented with different plant extracts was left uninoculated and used as a negative control for each dilution. For positive control, 100 μ l of both bacterial strains were inoculated to MHB tubes without plant extract. Tubes were incubated at 37°C for overnight and the lowest concentration that inhibits the bacterial growth was considered as the MIC value for each of the tested bacteria strain. To determine MBC value, sterilised Muller-Hinton agar (MHA) was poured into a Petri dish and was let to solidify. Samples that showed no obvious bacterial growth were streaked on the surface of the agar separately then incubated for twenty-four hours at 37°C. The lowest concentration which displayed no growth on the MHA plates was recorded as the MBC.

Combination test

The two selected plant extracts Cinnamon and Clove were combined in three different concentrations by weight: Cinnamon: Clove; C1: 1:1, C2: 1:2, and C3: 2:1 respectively. Three discs loaded with 10 μ l of each combination were tested against the growth of each tested bacterial strains (*Streptococcus mutans* and *Lactobacillus acidophilus*) using disc diffusion method as previously mentioned and utilise CHX and DMSO as a positive and negative control.

Another three different combinations and commercial MI Varnish' [5% sodium fluoride varnish with Recaldent (CPP-ACP), GC America, USA] were assessed against the growth of each tested bacterial strains using disc diffusion method. Each plant extract was incorporating into commercial MI Varnish separately by weight (V1; 1:1 = Cinnamon: MI, V2; 1:1 = Clove: MI) and a third combination was prepared by mixing two plant extracts with MI Varnish (V3; 1:1:1 = Cinnamon: Clove: MI). Ten μ l of each mixed combination was added to a sterile disc, and their antibacterial activity was tested in comparison to MI varnish (V4).

Statistics have been explored for normality the usage of Kolmogorov-Smirnov and Shapiro-Wilk assessments; statistics confirmed parametric (ordinary) distribution.

Repeated measure ANOVA changed into used to examine among extra than groups in related samples. Paired wise sample t-test turned into used to

compare among two groups in related samples. One-way ANOVA accompanied by way of Tukey post hoc test was used to compare among greater than two groups in non-related samples. The importance level became set at $P \leq 0.05$. Statistical evaluation becomes carried out with IBM® SPSS® statistics model 20 for windows.

Results

Inhibition zones results

Regarding the antibacterial activity of the tested five plant extracts in the disc-well diffusion method; the methanolic extracts of Cinnamon (G1) and Cloves (G4) showed antibacterial activity with inhibition zones diameters of 14.00 mm and 12.67 mm against *Streptococcus mutans* and 16.67 mm and 18.67 mm against *Lactobacillus acidophilus*, respectively, while the other plant extracts did not demonstrate any antibacterial activity against the two strains except methanolic extract of Turmeric showed antibacterial activity against *Lactobacillus acidophilus* with inhibition zones diameters of 9.33 mm. For the antibacterial activity against *Streptococcus mutans*, the highest inhibition mean value was found in the positive control group (G6) followed by (G1) and (G4) respectively. Meanwhile, the lowest mean value was recorded for (G2, G3 and G5) where ($P < 0.001$). While for the antibacterial activity against *Lactobacillus acidophilus*, the highest mean value was found in (G4) followed by (G6) followed by (G1) followed by (G2) respectively, meanwhile the lowest mean value was found in (G3), (G5) where ($P < 0.001$) (Figure 2).

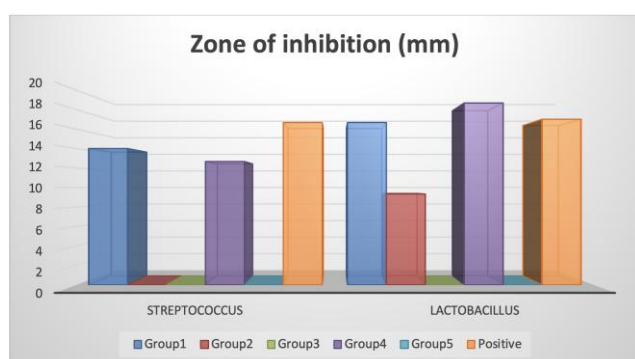


Figure 2: Bar chart representing Comparison in the mean value of inhibition zones with the different plant extracts against *Streptococcus mutans*, and *Lactobacillus acidophilus*

MIC and MBC results

The MIC and MBC were carried out for the Cinnamon (G1), and Clove (G4) extracts which were the most effective extracts according to disk diffusion test results. No statistically significant difference was

found between (G1) and (G4) in MIC with *Streptococcus mutans* where ($P = 0.364$). While the MIC for *Lactobacillus acidophilus* was exhibited a statistically significant difference between (G1) and (G4) where ($P < 0.001$). A statistically significant difference ($P < 0.001$) was found in the MBC value for both strains between (G1) and (G4). Where the highest mean value of MIC and MBC for positive inhibitory effects with *Streptococcus mutans* and *Lactobacillus acidophilus* was found in (G4) while (G1) showed the lowest mean value (Table 1).

Table 1: MIC and MBC results of Cinnamon and Clove extracts (mg/mL)

Variables	MIC				MBC			
	<i>S. mutans</i>		<i>L. acidophilus</i>		<i>S. mutans</i>		<i>L. acidophilus</i>	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group 1	13.44	4.09	5.18	0.77	23.60	1.52	16.40	2.43
Group 4	15.56	2.73	13.92	1.34	48.25	1.71	51.20	3.11
P-value	0.364ns		< 0.001*		< 0.001*		< 0.001*	

*; significant ($P \leq 0.05$) ns; nonsignificant ($P > 0.05$).

Antibacterial activity of the three different combinations between Cinnamon and Clove extracts

A statistically significant difference was recorded between the cinnamon extract mixed with Clove extract in the combination ratio of 1:1 (C1) and 2:1 (C3) where the mean inhibition zone diameter was (18.33 ± 0.58) and (19.00 ± 1.00) for C1 and (14.00 ± 1.00) and (16.33 ± 0.58) for C3 against *Streptococcus mutans* and *Lactobacillus acidophilus* respectively. Also, a statistically significant difference was found between C2 (1:2) and C3 where the mean inhibition zone diameter was (18.67 ± 0.58 , 19.67 ± 0.58) respectively. While there was no statistically significant difference between the combinations (C1) and (C2) against the two oral pathogens where ($P < 0.001$). The highest mean value was found in (C2) followed by (C1), (positive) and (C3) while the lowest mean value was found in the negative control group (Figure 3).

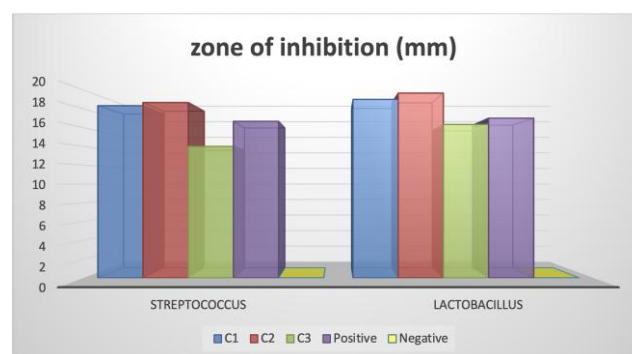


Figure 3: Bar chart representing Comparison in the mean value of inhibition zones with the three different combinations between Cinnamon and Clove extracts against *Streptococcus mutans* and *Lactobacillus acidophilus*

Antibacterial activity of the two-plant extract incorporating each/ both into MI varnish

From Figure 4 regarding the inhibition zones against *Streptococcus mutans* growth showed a statistically significant difference between the mixture of Cinnamon and Clove extract with MI Varnish in a ratio 1:1:1 (V3) and each of V1 (Cinnamon: MI = 1:1) and V2 (Clove: MI = 1:1) where ($P = 0.003$), and ($P < 0.001$). Also, a statistically significant difference was found between V3 and V4 (MI varnish) where ($P < 0.001$). While there was no statistically significant difference was found between (V1) and (V2) ($P < 0.001$). The highest mean inhibition zones value was found in (V3) followed by positive, (V1), V2 and (V4) groups while the lowest mean value was noted in the negative group. For the inhibition zones against *Lactobacillus acidophilus* growth, A statistically significant difference was found between group (V1) and each of (V2), (V3), and (V4) groups where ($P = 0.042$), ($P = 0.002$), and ($P < 0.001$). While there was no statistical significance difference between (V2) and (V3) where ($P < 0.001$).

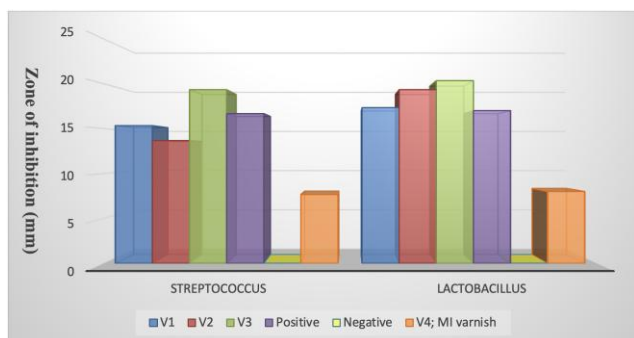


Figure 4: Bar chart representing Comparison of the zone of inhibition with one of two plant extract (Cinnamon, Clove) and their mixture each incorporating the commercial varnish separately

Discussion

Dental caries is one of the major causes of the destruction of mineralised tissue of the teeth. *Streptococcus mutans* (*S. mutans*) and *Lactobacillus acidophilus* (*L. acidophilus*) species are dominant microorganisms in the lesion of advanced caries, and these two are considered as a principle microorganism in the pathology of dental caries. *S. mutans* is the organism causing initiation of caries, whereas *L. acidophilus* causes progression of dental caries [28], [29], [30]. Thus, the existence of *S. mutans* and *L. acidophilus* in dental structure is a signal of a cariogenic biofilm and any chemical substance which can use to decrease these bacteria levels can offer additional means of stopping dental caries [31]. In recent years, researchers gave attention to the use of plant extracts against cariogenic bacteria regarding their effect on growth. For this reason, the present

study selected five plants (Cinnamon, Ginger, Turmeric, Cloves and Black seed) [15], [16] which are known for their medical applications to evaluate their effect on *S. mutans* and *L. acidophilus* bacteria. Sensitivities of these two cariogenic pathogens to the five different methanolic plant extracts in comparison to chlorhexidine gluconate (0.12%), were tested using Agar disc technique.

The results obtained from our study shows a very good antibacterial activity of two extracts; Cinnamon and Cloves extract against *S. mutans* and *L. acidophilus*. While no antibacterial activity was exhibited by Ginger and Black seeds extracts, while Turmeric has only antibacterial potential against *L. acidophilus*. According to a literature data on the effectiveness of plant extracts the results are inconsistent probably because of differences in extract preparation methods [32], therefore we assumed that the solvent used for the experiment could influence the result. In this study, we used methanol extract for each tested plant as it could allow releasing of active ingredients from Cinnamon and Cloves extract causing their antibacterial efficiency against *S. mutans* and *L. acidophilus*. The previous investigation could be supported by Cowan [33] who stated that approximately all the identified components from plants showed activity against microorganisms are saturated organic compounds or aromatic, and they are most frequently acquired through initial methanol or ethanol extraction. On the other side, using methanol solvent in our study might corrupt the effective ingredients of the Ginger and Black seed extract which masking their antibacterial potential against the two oral tested pathogens or due to the high resistance of tested strains [34]. While the methanolic extraction of Turmeric had high potential to inhibit only *L. acidophilus* growth as it might inhibit the growth in a dose-dependent manner [35] and so *L. acidophilus* could be suppressed at lower concentrations of Turmeric extraction than for *S. mutans*.

According to our study results, *L. acidophilus* was found to be most sensitive pathogen to the methanolic extract of Cinnamon, with MIC of 5.18 mg/ml followed by the methanolic extract of Clove (13.92 mg/ml). While methanolic extract for both plants showed almost equal antibacterial activity against *Streptococcus mutans*. The high antibacterial activity of the Cinnamon extract is mainly attributed to its secondary metabolites. It has been shown that Cinnamon antimicrobial properties are mainly related to its cinnamaldehyde which is highly electronegative which interferes in biological processes including electron transfer and react with nitrogen-containing components, such as nucleic acids as well as proteins, therefore inhibits the microorganism's growth [36]. Concerning the antibacterial of Clove, Shoji *et al.*, [37] presented that the methanol and aqueous extracts of this plant contain flavonoids and saponins. Though further studies are needed to identify the

active agents responsible for other biological and pharmacological activities of these plants is requisite.

In our experiment the combination, which consisted of Cinnamon to Clove at ratio 1:2 in the group (C2) was found the most active ratio compared to the other ratios in the group (C1) (1:1), and group (C3) (2:1). This latter result seemed to reflect the amount of Clove extract as the activity of combination increases by increasing Clove amount, and this could be due to the presence of active components in inadequate quantities in the Cinnamon extract to show the activity with the used dose levels [38]. Moreover, the acetonic extraction for Cinnamon has greater antimicrobial activity than water and alcohol extraction [36].

Although the high antibacterial activity of CHX (positive control) which was reported by previous studies [39], [40], the result of the present study demonstrated that incorporating Cinnamon (V1), Clove (V2) extracts separately or both (V3) into commercial varnish (5% sodium fluoride varnish with Recaldent (CPP-ACP), GC America, USA) was able to achieve a higher antibacterial activity. Also, they showed a more significant effect on the suppression of *S. mutans* and *L. acidophilus* compared with commercial varnish only (V4). Anywise, the synergistic effect between Cinnamon and Clove extract in the component of commercial varnish (V3) did not suppress the antimicrobial activity of each extract, and this could be explained the maximum inhibition zone for group V3. Thus, from the overall result, it is evident that the methanolic extract has been found to have good antimicrobial activity for Cinnamon and Clove extract against *S. mutans* and *L. acidophilus*. Also, Cinnamon and Clove extracts containing varnish can be beneficial clinically against the dental caries pathogens.

In conclusion, within the restrictions of this study, the accompanying conclusions were proposed that Cinnamon and Clove methanolic extracts would be useful compounds for the development of antibacterial agents against *S. mutans* and *L. acidophilus*, though, the latter needs higher concentration of the Clove extract to reach MIC. Although their effectiveness was less than Chlorhexidine, they may have a potential role in dental varnish for dental caries prevention.

Therefore, the present results could display; a scientific basis for the traditional use of Cinnamon and Clove on oral pathogens, contribute to the enhancement of oral health and lessen the side effects and cost of the treatment with allopathic medicine. However, additional clinical trials seem necessary to assess their safety and efficacy. Also, further studies should be carried out on the effect of both on the remineralising ability of MI varnish and enamel colour.

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