Evaluation of Antimicrobial Activity of Different Herbal Products against Bacillus *subtilis*, *Staphylococcus aureus*, and *Enterococcus faecalis* Using Agar Diffusion Test: An In Vitro Study

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Abstract

Introduction The main aim of this study is in vitro evaluation of the antimicrobial efficacy of different herbal products, that is, propolis, garlic, neem, aloe vera, and rosemary, against *Bacillus subtilis*, *Staphylococcus aureus,* and *Enterococcus faecalis* using agar diffusion test.

Materials and Methods In this study, total of 42 plates were prepared, 10 each of *B. subtilis*, *S. aureus*, and *E. faecalis* and 12 as test control (6 as positive and 6 as negative control). The effectiveness of five herbal products was ascertained by agar diffusion method against *B. subtilis*, *S. aureus,* and *E. faecalis*. Cultures of these test organisms were maintained on selective media slants in a test tube to collect sufficient number of microbial colonies for evaluation. The cultures were divided into three groups based upon microbes that were lawn cultured, respectively: In group A, 10 petri plates were having growth of *B. subtilis*; in group B, 10 petri plates were having growth of *S. aureus*; and in group C, 10 petri plates were having growth of *E. faecalis*. In all these 30 petri plates, five different herbal product discs were placed and these discs were designated as A (aloe vera), P (propolis), N (neem), R (rosemary), and G (garlic). Among remaining 12 petri plates, 4 petri plates were used as control (2 for positive and 2 for negative) for each of the bacteria.

Results Propolis and rosemary showed maximum zone of inhibition against *B. subtilis*. Garlic, neem, and aloe vera showed maximum zone of inhibition against *S. aureus*.

Conclusion All the herbal products showed zone of inhibition against *S. aureus*, *B. subtilis*, and *E. faecalis*.

Keywords
- *Bacillus subtilis*
- *Enterococcus faecalis*
- herbal products
- *Staphylococcus aureus*

Introduction

The success of endodontic treatment depends on the complete eradication of microbes from root canal system and prevention of reinfection. Sterilization of root canal subsequent to its debridement and prior to its obturation has been considered as a prerequisite for successful endodontic therapy.[1]

Various measures to reduce the number of microorganisms in the root canal system include the use of various instrumentation techniques, irrigation regimens, and

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intracanal medicaments in the canal. Irrigation is complementary to instrumentation in facilitating the removal of pulpal tissues and microorganisms. Sodium hypochlorite is the most commonly used irrigant in endodontics due to its tissue dissolving capability and antimicrobial properties.[2]

However, sodium hypochlorite is toxic to vital tissues, resulting in hemolysis, skin ulceration, and necrosis. In case of contact with the patient's or operator's eye, it results in immediate pain, profuse watering of eyes, intense burning, and erythema.[3]

Gutta percha cones are usually decontaminated by submerging in 1% sodium hypochlorite for 1 minute (Milton's solution) or 0.5% sodium hypochlorite for 5 minutes (Dakin's solution). But sodium hypochlorite causes crystal deposition on the surface of gutta percha, which can impede the obturation.

Owing to the potential side effects and safety concerns of conventional antimicrobial agents, interest in the preparations from medicinal plants has increased over the last few decades. Herbal products have become more popular today due to their high antimicrobial activity, biocompatibility, anti-inflammatory, and antioxidant properties. The main advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance.[4]

In endodontics, because of the cytotoxic reactions of the most of the commercial intracanal medicaments used and their inability to eliminate bacteria from dentinal tubules, there is a new trend to use biologic medication extracted from natural plants. Some of the commonly used herbal products in endodontics are propolis, garlic, neem, aloe vera, and rosemary.

Propolis(bee glue), a natural antibiotic, is a resinous substance that honey bees collect from trees of poplars. It possesses antimicrobial, anti-inflammatory, and antioxidant properties.[5,6]

Garlic (Allium sativum) is antibacterial and has immune regulatory functions. Garlic extract has shown to have a wide spectrum of antibacterial activity, including effects on *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Escherichia*, *Salmonella*, *Proteus*, *Clostridium*, *Mycobacterium*, and *Helicobacter* species.[7,8]

Neem (Azadirachta indica) is a versatile medicinal plant having a wide spectrum of biological activity. Neem leaves, seeds, and bark possess a wide spectrum of antibacterial action against gram-negative and gram-positive microorganisms. Neem leaf extract is used to treat dental plaque and gingivitis. Its powerful antibacterial action makes it a useful ingredient in mouth wash and dentifrices.[9,10]

Aloe vera (Aloe barbadensis) is a short succulent herb resembling a cactus. It has potent antibacterial, antifungal, and antiviral properties. The antimicrobial effects of aloe vera have been attributed to the plant's natural anthraquinones that have demonstrated in vitro inhibition of *Mycobacterium tuberculosis* and *Bacillus subtilis*.[11,12]

Rosemary (*Rosmarinus officinalis)* belongs to family *Labiatae*. It has antibacterial properties and is used in relieving toothache.[13]

Different species of microorganisms are commonly found in the infected root canal cases including *Enterococcus*, *Streptococcus*, and *Staphylococcus species.*

Enterococcus faecalis has been considered to be the most resistant species in the oral cavity. It is facultative anaerobic gram-positive cocci and is the most commonly implicated microorganism in asymptomatic persistent infections. The highly complex nature of the organism poses a great challenge for endodontists. *Enterococcus faecalis* is responsible for failed root canal treatment cases.[14,15]

Staphylococcus aureus is a gram-positive coccal bacterium. It causes disease and destruction of tissue through the direct invasion and by production of toxins.[16]

Certain species of *Bacillus* produce special structures called spores when growth ceases owing to the exhaustion of an essential nutrient. No growth of *B. subtilis* subspecies is used for the assessment of proper sterilization.

This in vitro study was undertaken to compare the antimicrobial efficacy of five herbal products—aloe vera (*Aloe barbadensis),* neem *(Azadirachta indica)*, garlic (*Allium sativum)*, propolis (bee glue), and rosemary (*Rosmarinus officinalis)* against *S. aureus, B. subtilis,* and *E. faecalis* using agar diffusion disc test.

Materials and Methods

Procurement of Microorganisms

The microorganisms, *E. faecalis, S. aureus,* and *B. subtilis,* used in this study were procured in freeze dried form in an air tight glass tube from Institute of Microbial Technology, Chandigarh.

Procurement of the Herbal Products

Herbal products were procured in pure dry powder form from Sirmour Herbolife Private Limited Village & PO Surla, District Sirmour, Himachal Pradesh.

Activation of Bacteria

Activation of *Enterococcus faecalis*

The freeze-dried form of *E. faecalis* was activated by dissolving *E. faecalis* in Brain Heart Infusion Broth. Ten milliliters of Brain Heart Infusion Broth were poured in each test tube. Then, five test tubes containing Brain Heart Infusion Broth and bacteria were heated on flame near open end of tube to avoid contamination. Then a tight, nonabsorbent sterile cotton plug was placed at the open end of tube to seal off the broth containing *E. faecalis* from external environment. After the inoculation, the test tubes were then kept in an incubator at 37°C for 24 hours. After 24 hours of incubation, turbidity was observed in all the tubes indicating bacterial growth.

Activation *of Staphylococcus aureus* and *Bacillus subtilis*

Staphylococcus aureus and *B. subtilis were activated by dissolving* freeze-dried forms of *these bacteria in* nutrient broth. Twenty milliliters of nutrient broth were poured in each test tube. Then, five test tubes containing *S. aureus and*

five test tubes containing *B. subtilis* were heated on flame near open end of tube to avoid contamination. Then a tight, nonabsorbent sterile cotton plug was placed at the open end of each tube to seal off the bacteria *in broth from* external environment. After the inoculation, the test tubes were then kept in an incubator at 37°C for 24 hours. After 24 hours of incubation, turbidity was observed in all the tubes indicating bacterial growth.

Preparation of Test Plates for *Enterococcus faecalis*

In this study, a total of 14 culture plates containing Brain Heart Infusion Agar media were prepared. Ten plates were selected to test the antimicrobial efficacy of different herbal products against *E. faecalis* and four plates were kept as control group. In the control group, two served as positive and two served as negative control group. In positive control group, agar plates were tested for susceptibility of *E. faecalis* to tetracycline, whereas in negative control group, distilled water was used.

Each plate was inoculated with *E. faecalis* by evenly swiping the plate using heated glass spreader via lawn culture technique. Each plate was inoculated with *E. faecalis* by evenly swiping the plate using heated glass spreader via lawn culture technique.

Preparation of Test Plates for *Staphylococcus aureus*

In this study, a total of 14 culture plates containing blood agar media were prepared. Ten plates were selected to test the antimicrobial efficacy of different herbal products against *S. aureus*. Four plates were kept as control group. In the control group, 2 served as positive and 2 served as negative control group. In positive control group, blood agar plates were used for testing the susceptibility of *S. aureus to* tetracycline, whereas in negative control group, distilled water was used.

Fourteen plates were inoculated with *S. aureus* by evenly swiping the plate using heated glass spreader via lawn technique. In each agar plate, five discs, that is, one for each herbal product were placed, at a safe distance from the edge and from each other to avoid overlapping of zones of inhibition around the discs.

In positive control group, tetracycline disc was placed in two test plates instead of herbal products. In negative control group, distilled water disc was placed in test plates instead of herbal products.

Preparation of Test Plates for *Bacillus subtilis*

In this study, a total of 14 culture plates containing blood agar medium were prepared. Ten plates were selected to test the antimicrobial efficacy of different herbal products against *B. subtilis*. Four plates were kept as control group. In the control group, two served as positive and two served as negative control group. In positive control group, blood agar plates were tested for susceptibility *B. subtilis to* tetracycline, whereas in negative control group, instead of herbal product, distilled water was used.

Fourteen plates were inoculated with *B. subtilis* by evenly swiping the plate using heated glass spreader via lawn technique.

In positive control group, tetracycline disc was placed in two test plates instead of herbal products, and in negative control group, distilled water was placed in test plates.

Introduction of Herbal Product in the Culture

In each agar plates, except control group, five discs, that is, one for each herbal product were placed, at a safe distance from the edge and from each other to avoid overlapping of zones of inhibition around the discs.

Measurement of Inhibition Zone

All the plates were kept at room temperature for 2 hours for prediffusion of materials and then incubated at 37°C for 7 days. These plates were then observed for zones of inhibition after 24 hours, 48 hours, 72 hours, and 7 days.

The zones of inhibition formed around each disc were then examined and measured in millimeters using digital Vernier caliper.

The readings thus obtained from each plate were compared and put under statistically analysis.

Data Analysis

The observations recorded in the present study were subjected to student's *t*-test and Kruskal–Wallis one-way analysis of variance. The "*p*" value was taken as significant at "*p*" < 0.05, highly significant if < 0.001.

Discussion

The present study shows that propolis had maximum mean zone of inhibition against *B. subtilis*, that is, 23.64 mm, whereas minimum mean zone of inhibition found against *S. aureus* was 11.92 mm. When compared statistically, this difference in mean zone of inhibition was found to be significant ($p < 0.05$) (►**Table 1**).

The mean zone of inhibition against *E. faecalis* was 17.63 mm. When compared statistically, this difference in mean zone of inhibition between *S. aureus*, *B. subtilis,* and *E. faecalis* was found nonsignificant ($p > 0.05$) (►**Table 1**).

Akca et al suggested that antibacterial effect of propolis against microorganisms could be complex leading to the disintegration of the cytoplasmic membrane and cell wall, partial bacteriolysis, and inhibition of protein synthesis. They claimed that the pH and the concentration of propolis might alter due to solvents and acidic propolis solutions were more effective on bacteria. In addition, bacterial cell wall and their biofilm properties were excluded as adjunct factors, which determine bactericidal effect of propolis.[17]

Table 1 Propolis

	Minimum	Maximum	Mean	± SD	SEM
S. aureus	09.50	14.67	11.92	2.52	01.26
B. subtilis	21.60	27.26	23.64	2.57	01.28
E. faecalis	13.36	26.44	17.63	5.94	02.97
Comparison		**Mean difference**		***p*-Value**	
S. aureus vs. *B. subtilis*		11.72		0.008[a]	
S. aureus vs. *E. faecalis*		05.70		0.188	
B. subtilis vs. *E. faecalis*		06.01		0.162	

Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. faecalis*, *Enterococcus faecalis*; *S. aureus, Staphylococcus aureus*; SD, standard deviation; SEM, standard error of mean.
Note: $F = 8.527$; $p = 0.008$; significant. Multiple comparisons using post-hoc Scheffe test.
[a]$p < 0.05$; significant.

Table 2 Garlic

	Minimum	Maximum	Mean	± SD	SEM
S. aureus	33.21	47.59	39.64	07.00	03.50
B. subtilis	24.63	32.74	27.98	03.51	01.75
E. faecalis	11.06	26.12	15.78	06.96	03.48
Comparison		**Mean difference**		**p-Value**	
S. aureus vs. *B. subtilis*		11.66		0.067	
S. aureus vs. *E. faecalis*		23.86		0.001[a]	
B. subtilis vs. *E. faecalis*		12.19		0.055	

Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. faecalis*, *Enterococcus faecalis*; *S. aureus, Staphylococcus aureus*; SD, standard deviation; SEM, standard error of mean.
Note: $F = 15.542$; $p = 0.001$; significant. Multiple comparisons using post-hoc Scheffe test.
[a]$p<0.05$; significant.

Table 3 Neem

	Minimum	Maximum	Mean	± SD	SEM
S. aureus	09.67	32.61	23.63	09.96	04.98
B. subtilis	15.63	22.74	18.38	03.05	01.52
E. faecalis	09.67	22.68	14.12	05.84	02.92
Comparison		**Mean difference**		***p*-Value**	
S. aureus vs. *B. subtilis*		05.25		00.58	
S. aureus vs. *E. faecalis*		09.51		00.20	
B. subtilis vs. *E. faecalis*		04.25		00.69	

Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. faecalis*, *Enterococcus faecalis*; *S. aureus, Staphylococcus aureus*; SD, standard deviation; SEM, standard error of mean.
Note: $F = 1.907$; $p = 0.204$; not significant. Multiple comparisons using post-hoc Scheffe test.
*$p<0.05$; significant.

Table 4 Aloe vera

	Minimum	Maximum	Mean	± SD	SEM
S. aureus	22.02	32.25	23.78	02.47	01.23
B. subtilis	14.63	20.87	17.27	02.64	01.32
E. faecalis	10.65	22.69	14.17	05.71	02.85
Comparison		**Mean difference**		**p-Value**	
S. aureus vs. *B. subtilis*		06.50		0.115	
S. aureus vs. *E. faecalis*		09.61		0.022[a]	
B. subtilis vs. *E. faecalis*		03.10		0.554	

Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. faecalis*, *Enterococcus faecalis*; *S. aureus, Staphylococcus aureus*; SD, standard deviation; SEM, standard error of mean.
Note: $F = 6.310$; $p = 0.019$; significant. Multiple comparisons using post-hoc Scheffe test.
[a]$p<0.05$; significant.

Garlic had maximum mean zone of inhibition against *S. aureus*, that is, 39.64 mm, whereas minimum mean zone of inhibition was observed against *E. faecalis*, that is, 15.78 mm. When compared statistically, the difference in mean zone of inhibition was found to be significant ($p < 0.05$) (►**Table 2**).

The mean zone of inhibition against *B. subtilis* was 27.98 mm. When compared statistically, the difference in mean zone of inhibition between *S. aureus*, *B. subtilis,* and *E. faecalis* was nonsignificant ($p > 0.05$) (►**Table 2**).

Jose et al suggested that allicin present in garlic inhibits both germination of spores and growth of hyphae. The antimicrobial effect of allicin is due to its chemical reaction with thiol groups of various enzymes.[18]

Neem had maximum mean zone of inhibition against *S. aureus*, that is, 23.63 mm, whereas the minimum mean zone of inhibition observed against *E. faecalis* was 14.12 mm. The mean zone of inhibition against *B. subtilis* was found to be 18.38 mm.

When compared statistically, this difference in mean zone of inhibition between *S. aureus*, *B. subtilis,* and *E. faecalis* was nonsignificant ($p > 0.05$) (►**Table 3**).

Punetha and Srinivas showed that bactericidal effect of neem is due to the presence of chemical substances such as alkaloids, glycosides, saponins, flavonoids, steroids, anthraquinones, and tannic acid. Flavonoids act as antioxidants that provide protection against free radicals that damage cells and tissues. Moreover, it prevents colonization of organisms due to its antiadherence activity.[19]

Aloe vera had maximum mean zone of inhibition against *S. aureus*, that is, 23.78 mm, whereas minimum mean zone of inhibition observed against *E. faecalis* was 14.17 mm. When compared statistically, the difference in mean zone of inhibition was found to be significant ($p < 0.05$) (►**Table 4**).

The mean zone of inhibition against *B. subtilis* was 17.27 mm. When compared statistically, the difference in mean zone of inhibition between *S. aureus*, *B. subtilis,* and *E. faecalis* was nonsignificant ($p > 0.05$) (►**Table 4**).

Table 5 Rosemary

	Minimum	Maximum	Mean	± SD	SEM
S. aureus	09.59	17.68	13.49	03.62	01.81
B. subtilis	11.49	16.55	13.80	02.10	01.05
E. faecalis	10.40	17.68	13.73	03.31	01.65

Comparison	Mean difference	*p*-Value
S. aureus vs. *B. subtilis*	0.31	0.99
S. aureus vs. *E. faecalis*	0.24	0.99
B. subtilis vs. *E. faecalis*	0.07	0.99

Abbreviations: *B. subtilis*, *Bacillus subtilis*; *E. faecalis*, *Enterococcus faecalis*; *S. aureus, Staphylococcus aureus*; SD, standard deviation; SEM, standard error of mean.
Note: $F = 0.011$; $p = 0.989$; not significant. Multiple comparisons using post-hoc Scheffe test.
*$p<0.05$; significant.

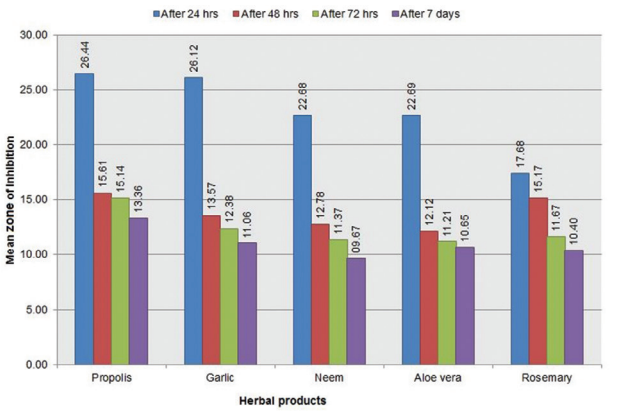

Fig. 1 Overall mean zone of inhibition of herbal products against *Enterococcus faecalis*.

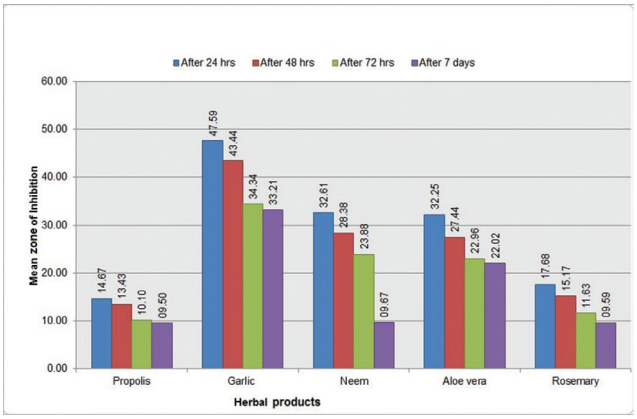

Fig. 2 Overall mean zone of inhibition of herbal products against *Staphylococcus aureus*.

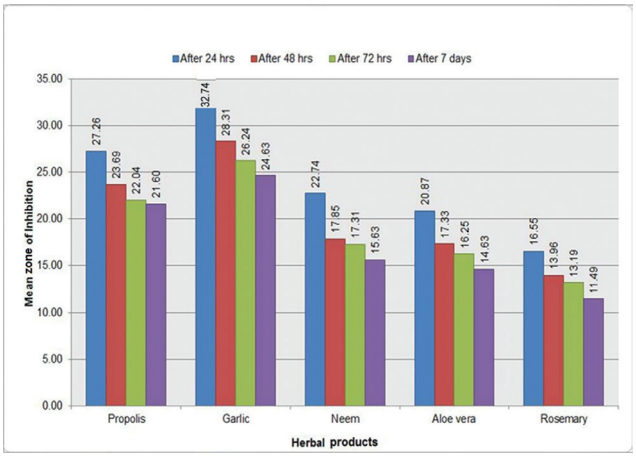

Fig. 3 Overall mean zone of inhibition of herbal products against *Bacillus subtilis*.

Athiban et al also showed that aloe vera is more effective against *S. aureus* as compared with *E. faecalis*.[20]

The results of our study found that rosemary had maximum zone of inhibition against *B. subtilis*, that is, 13.80 mm and *E. faecalis*, that is, 13.73 mm, whereas minimum zone of inhibition was observed against *S. aureus*, that is, 13.49 mm.

When compared statistically, the difference in mean zone of inhibition was found to be nonsignificant ($p > 0.05$) (►**Table 5**).

The antimicrobial effect of rosemary is due to the presence of phenolic compounds: augustic acid, benthamic acid, salvianic acid, sage coumarin, sagerinic acid, and other flavonoids.[21]

Propolis, garlic, neem, aloe vera, and rosemary showed maximum zone of inhibition after 24 hours against *E. faecalis*, *S. aureus*, and *B. subtilis*. The minimum zone of inhibition was found after 7 days (►**Figs. 1**–**3**).

Shahzad et al compared antibacterial efficacy of different aloe vera products after 48 and 96 hours. They found significant decrease in antibacterial efficacy after 48 hours.[22]

Conclusion

From the results of present study, it can be concluded that all the herbal products showed zone of inhibition against *S. aureus*, *B. subtilis*, and *E. faecalis*. Propolis showed maximum zone of inhibition against *B. subtilis* and minimum zone of inhibition against *S. aureus*. Garlic showed maximum zone of inhibition against *S. aureus* and minimum zone of inhibition against *E. faecalis*. Neem showed maximum zone of inhibition against *S. aureus* and minimum zone of inhibition against *E. faecalis*. Aloe vera showed maximum zone of inhibition against *S. aureus* and minimum zone of inhibition against *E. faecalis*.

Conflict of Interest
None declared.

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