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Original Research Article

Antibacterial efficacy of *Acacia nilotica, Aegle marmelos* herbal extracts against *Enterococcus faecalis*: an invitro study

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ABSTRACT

Background: Our objective was to evaluate the antibacterial efficacy of *Acacia nilotica*, *Aegle marmelos* herbal extracts against *Enterococcus faecalis*- an invitro study.
Methods: The extraction of *Acacia nilotica* bark powder and *Aegle marmelos* leaf powder was done with following three solvents (Ethanol, methanol and acetone) keeping vancomycin as a positive control. Then the study groups were assigned as follows: group I: *Acacia nilotica*, group II: *Aegle marmelos*, group III: Combination of *Acacia nilotica* and *Aegle marmelos*, group IV: vancomycin. Preparation of the *E. feacalis* inoculum with the help of Mueller Hinton Broth. Antimicrobial efficacy was evaluated by agar well diffusion assay to determine the zone of inhibition and Minimum inhibitory concentration was evaluated.
Results: Statistical analysis was performed by using one-way analysis of variance and compared by the Mann-Whitney test using the Statistical Package for the Social Sciences (SPSS) software, version 20.0. No zone of inhibition was identified for *Aegle marmelos*. Highest inhibitory zone against *E. faecalis* was seen for vancomycin (mean of 28.6 mm) followed by *Acacia nilotica*.
Conclusions: Among the test groups, vancomycin exhibited highest antimicrobial efficiency. Compared with the herbal extracts which was statistically significant. The use of herbal alternatives might prove to be advantageous considering the several undesirable characteristics of vancomycin.

Keywords: Antibacterial, *Enterococcus faecalis*, Herbal irrigants

INTRODUCTION

The main goal of the endodontic treatment is to completely eradicate the necrotic pulp tissue and their byproducts and mostly the biofilm from the root canal system and create space for subsequent root canal filling.[1] Irrespective of the instrumentation technique you use, irrespective of the obturation technique you use, if you don't perform proper irrigation, there are high chances of the recurrent or secondary endodontic infections.[2] So the irrigating solutions play a major role in prevention of reinfection.

There is no single irrigating solution that alone sufficiently covers all of the functions required from an irrigant.[3] A broad antimicrobial spectrum against anaerobic and facultative microorganisms, biofilms and ability to remove smear layer during instrumentation or dissolve it once it has formed are one among the main requirements of endodontic irrigants.[4] At present sodium hypochlorite is the most commonly used irrigant of different concentrations as it fulfills most of the ideal requirements of an endodontic irrigant. It has got excellent antimicrobial activity and tissue dissolving ability making it the irrigating solution of choice for the

endodontic treatment.[5] But it has got several undesirable characteristics such as cytotoxic effects, allergic potential and unpleasant smell and taste.[6]

The most commonly isolated microorganism in the reinfected endodontic cases is the *Enterococcus faecalis*, which is a gram-positive facultative anaerobic coccus. The biofilm formation of *E. faecalis* can impede its elimination when using conventional irrigating solutions. Enterococci possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation, cause toxin-mediated damage and withstands harsh environment.[7] Recent studies have implicated that *Enterococcus* species have become multidrug resistant including vancomycin and hence termed as vancomycin-resistant enterococci (VRE).[8] Clinical isolates of VRE isolated from primary and failed root canal cases have caused alarming concerns due to their resistance to most intracanal irrigants and medicaments.

To overcome various side effects that has been associated with currently used conventional irrigants, there has been a recent interest over the use of herbal extracts as endodontic irrigants considering cost effectiveness, increased shelf life, low toxicity. There are various herbal extracts like neem, green tea, propolis, aloe vera, miswak, chamomile etc. has been evaluated for their antimicrobial activity against *E. faecalis*.[9]

The aim of this study was to evaluate the antibacterial efficacy of *Acacia nilotica*, *Aegle marmelos* herbal extracts and vancomycin against *Enterococcus faecalis ATCC* 29212 with the help of agar well diffusion assay to determine the zone of inhibition and broth dilution assay to determine the minimum inhibitory concentration.

METHODS

This was an in vitro study which took place in the Karpaga Vinayaga institute of dental sciences from the time period of February 2022-November 2022.

Ethical approval

Got approval from the institutional ethical committee (IEC NO: KIDS/IEC/2022/11/009).

Preparation of extracts

The extraction of *Acacia nilotica* bark powder and *Aegle marmelos* leaf powder was done with following three solvents (Ethanol, methanol and acetone). Five grams of the herbal powder was immersed in 20-30 ml of the above solvents and mixed thoroughly and placed in shaker incubator for 72 hours. After 72 hours the solvents were allowed to evaporate by placing the herbal extracts in the incubator for 7 days. 200 mg of the extracted herbal products was mixed in 1ml of 10% DMSO.

Microbiological analysis

There are four study groups. Group I: Acacia nilotica, group II: *Aegle marmelos*, group III: Combination of *Acacia nilotica* and *Aegle marmelos*, group IV: vancomycin.

Bacterial strains used

Enterococcus faecalis ATCC 29212 was used in this study. Stock culture of *Enterococcus faecalis* ATCC 29212 was inoculated in Mueller-Hinton Agar (MHA, Himedia, India) and the purity was assessed by gram staining.

Preparation of inoculum

Overnight culture of *E. faecalis* ATCC 29212 was inoculated into 100ml of Mueller-Hinton broth (MHB, Himedia, India) and incubated at 37°C for 18 hours. The optical density was adjusted to match the turbidity of McFarland standard scale 0.5.

Figure 1: McFarland standards used for the standardization of number of bacteria for susceptibility testing.

A 0.5 McFarland standard is comparable to a bacterial suspension of 1.5 × 108 cfu/ml

Agar well diffusion assay

Lawn culture of *E. faecalis* ATCC 29212 was prepared on Mueller Hinton Agar (MHA) (Himedia). Wells of 8 mm diameter was punched using coke borer and 100 µl of the extracts (methanol, ethanol and acetone extracts of *Acacia nilotica* and *Aegle marmelos*) was added in the respective wells and the plates were incubated at 37°C for 24 hours (Figures 2-5).

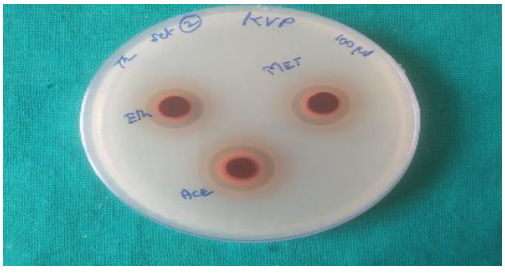

Figure 2: *Acacia nilotica.*

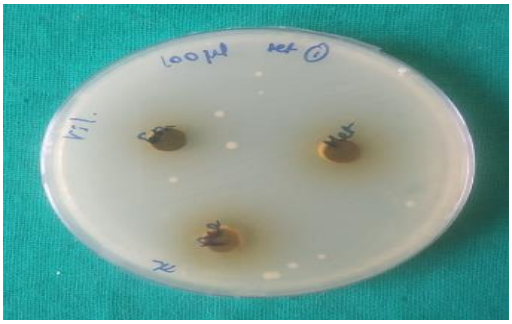

Figure 3: *Aegle marmelos.*

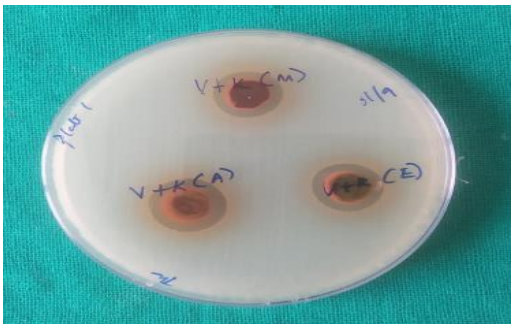

Figure 4: Combination of *Acacia nilotica* and *Aegle marmelos.*

Figure 5: Vancomycin.

The assay was done in triplicate. Following incubation period, the diameter of the zone of inhibition was measured inclusive of the diameter of the well (Table 1).

MIC determination by micro broth dilution assay

Minimum inhibitory concentration of herbal extracts (methanol, ethanol and acetone extracts of *Acacia nilotica*, *Aegle marmelos* and *Acacia nilotica* + *Aegle marmelos* combination) were determined as follows:

Gradually increasing dilutions of the test solutions were prepared on the respective labelled wells of a microtitre plate. The last well of each row served as the culture control (no test solution was added). 10 µl of *E. faecalis* ATCC 29212 suspension was added to all the wells including the culture control. The plates were incubated overnight for 37°C (Figures 6-7).

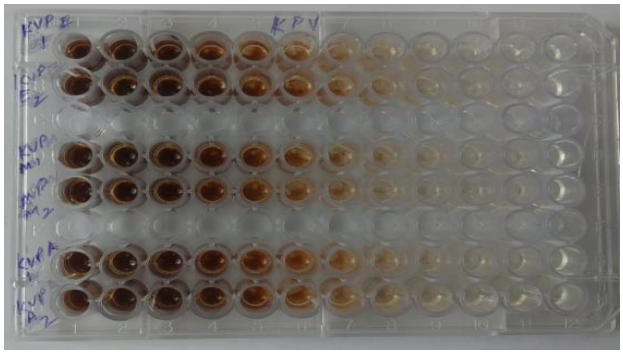

Figure *6: Acacia nilotica.*

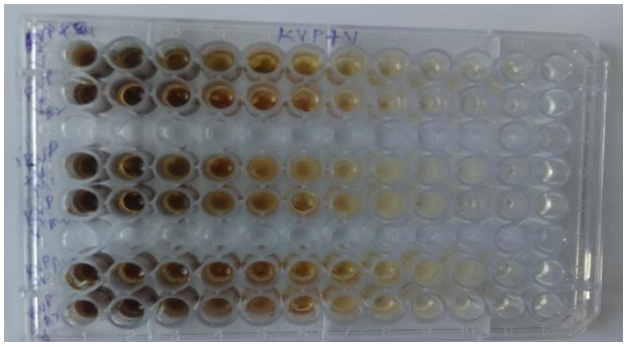

Figure 7: Combination of *Acacia nilotica* and *Aegle marmelos.*

Minimal bactericidal concentration (MBC)

The MIC was recorded as the lowest concentration of the test solution which did not permit the growth (visible turbidity) of *E. faecalis* ATCC 29212. As the MIC value could not be exactly interpreted based on visible turbidity. The MIC was determined by performing MBC.

Minimal bactericidal concentration of the test solutions were determined by spot inoculation of 5 µl of the culture from each well of the microtitre plate onto a MHA plate for *E. faecalis*. Minimum concentration of the test solution that completely inhibited (~99%) the growth of the colonies on the MHA plate was scored as the MIC of the respective test solution.

Statistical analysis

One-way analysis of variance was used for statistical analysis and compared by the Mann-Whitney test using the Statistical Package for the Social Sciences (SPSS) software, version 23.0. The criterion for statistical significance was defined as $p<0.001$.

RESULTS

Highest inhibitory zone against *E. faecalis* was seen for vancomycin (mean of 28.6 mm) followed by *Acacia nilotica* and combination of *Acacia nilotica* and *Aegle marmelos* (Table 1).

Table 1: Agar well diffusion/disc diffusion assay in triplicate.

Test group (concentration)	Acetone	Ethanol	Methanol
Group I 100 µl (*Acacia nilotica*)	17 mm	15 mm	16 mm
	17 mm	15 mm	15 mm
	18 mm	16 mm	15 mm
Group II µl (*Aegle marmelos*)	8 mm	8 mm	8 mm
	8 mm	8 mm	8 mm
	8 mm	8 mm	8 mm
Group III µl (combination of *Acacia nilotica* and *Aegle marmelos*)	17 mm	15 mm	15 mm
	16 mm	15 mm	14 mm
	17 mm	15 mm	15 mm
Group IV 30 µg (vancomycin)	21 mm	21 mm	21 mm
	20 mm	20 mm	20 mm
	21 mm	21 mm	21 mm

Table 2: Broth dilution assay.

Groups	Minimum inhibitory concentration *E. faecalis* (ATCC 29212)
Group I (*Acacia nilotica*)	Acetone (630µg/ml)
	Ethanol (630µg/ml)
	Methanol (630µg/ml)
Group IV (vancomycin)	2 µg/ml

DISCUSSION

Enterococcus faecalis, gram-positive facultative anaerobic cocci, is rarely seen in primary infection but very commonly present in secondary infection which causes endodontic failure. Its prevalence ranges from 24% to 77% in root canal failed teeth.[10] There has been a constant interest in finding an optimal irrigant in eradicating this *E. faecalis* biofilm, since the conventional endodontic irrigants possess various drawbacks. There has been lot of studies evaluating the antimicrobial effectiveness of herbal extracts as endodontic irrigants. In this study we have studied about *Aegle marmelos* and *Acacia nilotica* herbal extracts. Antimicrobial activity of different leaf extracts such as Petroleum ether, dichloromethane, chloroform, ethanol and aqueous extract of *Aegle marmelos* leaves were tested against selected Gram positive and Gram negative bacteria. Results depict that phytochemical extracts of *Aegle marmelos* exhibited significant anti-bacterial activity. However, the inhibitory activity was found to be both organism and solvent dependent.[11] *Acacia nilotica* has got various applications in dentistry for treating bleeding gums, tooth ache and its antimicrobial efficacy were also tested against cariogenic pathogens.[12] So we tried to evaluate these two herbals for its antibacterial efficacy against *E. feacalis*. The herbal extracts were dissolved in DMSO (dimethyl sulfoxide) which is a clean, safe, highly polar, and aprotic solvent that helps in retaining the pure properties of all the components of the herbal products which are being dissolved.[13] Antibacterial inertness of 10% DMSO was confirmed with the disc diffusion test. The antimicrobial activity of vancomycin against *E. faecalis* infections were shown promising for several decades. Hence it was used as a control against herbal extracts. In this study the highest zone of inhibition was shown for vancomycin followed by *Acacia nilotica* dissolved in acetone extract followed by the combination of *Acacia nilotica* and *Aegle marmelos* dissolved in acetone extract. Since there was no zone of inhibition found with *Aegle marmelos*, the minimal inhibitory concentration was not evaluated for that. The minimum inhibitory concentration was found significant with *Acacia nilotica* than vancomycin.

Since this is an in vitro study, further studies are needed to inculcate this into clinical practice. And moreover, the herbal extracts were tested for their antibacterial activity against planktonic cells and not on the *E. feacalis* biofilm. In recent years, there's been an increase in drug-resistant *E. faecalis* strains. Today, many antibiotics don't work against infections caused by these bacteria and having this as a control can be one of the limitations of the study.

CONCLUSION

Within the limitations of the study, vancomycin showed highest antibacterial activity against *E. Feacalis* planktonic cell and *Acacia nilotica* showed statistically significant antibacterial activity. To conclude the use of herbal alternatives as a root canal irrigant might prove to be advantageous considering the several undesirable characteristics of conventional endodontical irrigants and vancomycin resistant enterococcus species. Further research is needed to conclusively recommend herbal extracts as a root canal irrigant.

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Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee (IEC NO: KIDS/IEC/2022/11/009)

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