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*Original Research Article*

# Comparative Evaluation of Antibacterial Activity of *Punica granatum*, *Acacia nilotica* and *Emblica officinalis* against *Enterococcus faecalis* and Their Smear Layer Removal Ability When Used as Endodontic Irrigants: an In-Vitro Study

**Dr. Payal A Jain[1], Dr. Sunil Tejaswi[2], Dr. Parinitha M.S[2], Dr. Suneeth Shetty[3], Dr. Ambikathanaya U.K[3],**

[1]Post Graduate Student, [2]Reader, [3]Lecturer,
Department of Conservative Dentistry and Endodontics, JSS Dental College and Hospital, JSS Academy of Higher Education and Research, JSS Medical Institutions Campus, Bannimantap, Sri Shivarathreeshwara Nagara, Mysuru - 570015, Karnataka, India.

Corresponding Author: Dr. Payal A Jain

## ABSTRACT

**Context:** Phytotherapeutic agents as alternatives for synthetic endodontic irrigants, due to the adverse effects of synthetic agents.
**Aims:** Aim of this *in -vitro* study is evaluate and compare antibacterial activity against E.faecalis and smear layer removal efficacy of Punica granatum, Acacia nilotica and Emblica officinalis distilled water extracts.
**Methods and Material:** Distilled water extracts of the three herbal products were prepared. Minimum inhibitory concentration was determined using Broth microdilution method. Agar well diffusion test was performed and zones of inhibition were measured to evaluate the antibacterial activity.
Sixty human maxillary incisors were decoronated, divided into six groups (n=10).
Group 1: 0.9% normal saline; Group 2: 1% sodium hypochlorite; Group 3: 17% ethylenediaminetetraacetic acid; Group 4: 6.25% Punica granatum; Group 5: 25% Acacia nilotica; Group 6: 12.5% Emblica officinalis solution. Teeth were split into two halves and observed under scanning electron microscope to analyse the amount of smear layer present.
**Results:** Minimum inhibitory concentrations of Punica granatum, Acacia nilotica and Emblica officinalis were recorded as 6.25%, 25% and 12.5% respectively, with mean inhibition zones of 21mm, 18mm and 20mm. Group 4 showed least smear layer scores, followed by Group 6 and Group 3.
**Conclusions:** Punica granatum and Emblica officinalis aqueous extracts showed effective antibacterial activity and smear layer removal efficacy in all parts of root canal.
***Keywords:*** Acacia nilotica; antibacterial activity; Emblica officinalis; Enterococcus faecalis; Punica granatum; smear layer.

## INTRODUCTION

Endodontic infections are polymicrobial in nature. *Enterococcus faecalis* is commonly isolated from persistent endodontic infections, as it resists high pH and remains within the root canal for prolonged periods, because of its capacity to survive in nutrient deprived environment. [1] Successful endodontic treatment is dependent on removal of these microorganisms and the removal of smear layer which forms during instrumentation of canal; through a thorough chemo-mechanical preparation, using proper

instrumentation along with irrigants and intracanal medicaments.

Irrigation is an essential component of endodontic therapy. Though sodium hypochlorite (NaOCl) and ethylene-diaminetetraacetic acid (EDTA) are widely used for their excellent tissue dissolution and chelation properties respectively, [2] but they have few drawbacks such as toxicity, allergic potential, disagreeable smell and taste, corrosiveness to instruments etc. [3,4] Hence, this study aimed at evaluating and comparing the antibacterial activity against *Enterococcus faecalis* and smear layer removal ability of Punica granatum, Acacia nilotica and Emblica officinalis aqueous extracts.

## MATERIALS AND METHODS

**Preparation of stock solution** – Fresh peels of Punica granatum (Pomegranate), stem barks of Acacia nilotica (Babool) and fruits of Emblica officinalis (Amla) were air dried and pulverized into fine powder. The powders were subjected to cold maceration (occasional stiring with a sterile glass rod and left undisturbed for 48 hours), followed by filtration with a sterile muslin cloth and water bath treatment at 110°C to obtain crude extracts. Distilled water extracts of each product were prepared by dissolving 10g of dried crude extract in 20g of distilled water to obtain 50% extracts (500mg/ml).

**MIC determination** - Minimum Inhibitory Concentration (MIC) of the three herbal extracts against *Enterococcus faecalis* was determined by Broth Macrodilution method; where the 50% extracts of all the three herbal products were serial diluted in Brain Heart Infusion (BHI) broth to obtain concentrations of 25, 12.5, 6.25, 3.12, 1.86, 0.78, 0.39, 0.19 and 0.09% and were cultured on Blood Agar plates to evaluate the microbial growth inhibition [Figure I(A), I(B), I(C)]. MIC was determined as the least concentration which completely inhibited the growth of the microorganism.

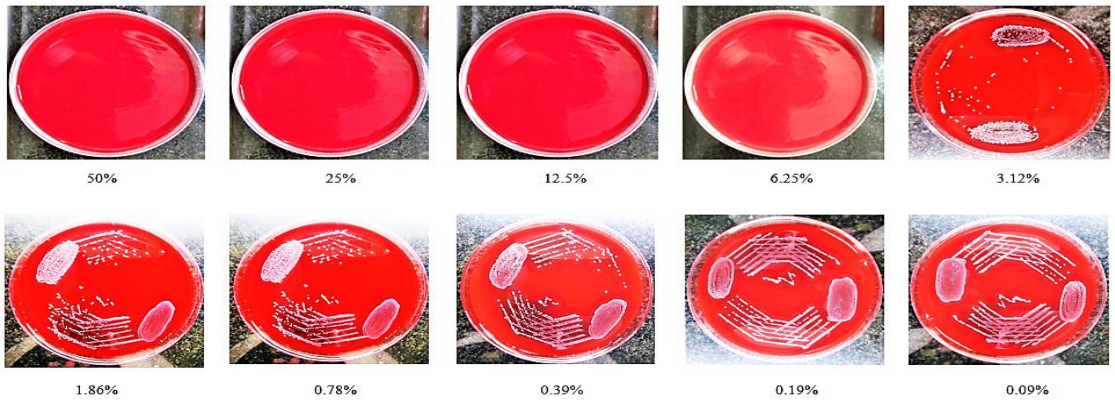



**Figure I(A): MIC of Punica granatum against *E.faecalis***

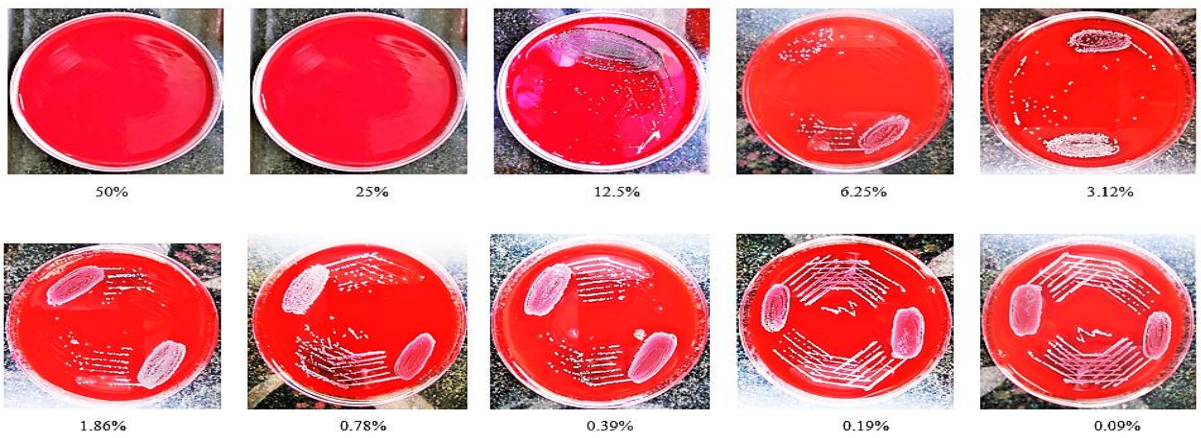



**Figure I(B): MIC of Acacia nilotica against *E.faecalis***

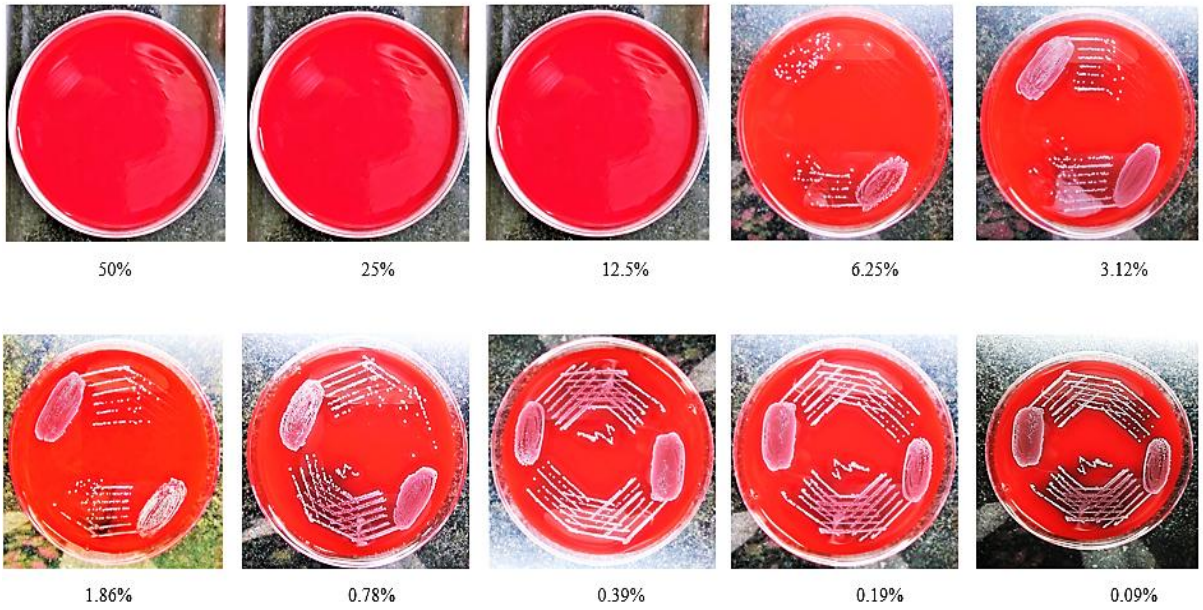



**Figure I(C): MIC of Emblica officinalis against *E.faecalis***

**Agar Well Diffusion test** - Five Mueller Hinton (MH) Agar plates were prepared and the *Enterococcus faecalis* (ATCC 29212) inoculum was spread onto the agar plates. Wells of 6mm diameter were punched using a sterile well borer and labelled accordingly as:

**Control groups**
Group A – 0.9% Saline solution (negative control)
Group B – 1% Sodium hypochlorite solution (positive control)

**Experimental groups**
Group C – 6.25% Punica granatum extract
Group D – 25% Acacia nilotica extract
Group E – 12.5% Emblica officinalis extract

The wells were filled with 250µl of each solution a zone of inhibition were measured in millimetres using a zone measuring scale [Figure II].

**Evaluation of Smear Layer removal –** Sixty extracted, human single rooted permanent maxillary incisor teeth were cleaned of all the debris and soft tissue remnants using ultrasonic scaler tips. The teeth were decoronated at CEJ using a sterile diamond disc (NTI Diamond Discs, Axis-sybronendo, Kerr Corporation, CA, USA), to obtain standardized length of 14mm and randomly divided into 6 groups (n=10):- [Figure III]

**Control Groups**
Group 1 - 0.9% Saline solution
Group 2 - 1% Sodium hypochlorite solution
(Negative controls)
Group 3 - 17% Ethylenediaminetetraacetic acid solution (DeSmear, Anabond Stedman (P) Ltd., Tsmilnsdu, India)(Positive control)

**Experimental Groups**
Group 4 – 6.25% Punica granatum solution
Group 5 – 25% Acacia nilotica solution
Group 6 – 12.5% Emblica officinalis solution.

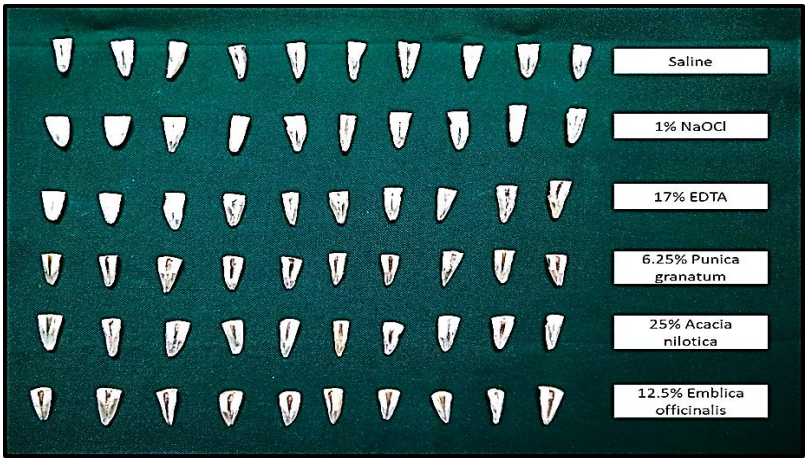



**Figure III: Specimens decoronated at CEJ with standardized length of 14mm**

Root canals were enlarged up to 60/02 hand K-file (Mani, Japan), with 2ml irrigation using respective irrigants during instrumentation. Final irrigation was done using 10ml of the respective irrigants.

All the specimens were dried using sterile absorbent paper points (PRIME Paper Points, Pearl Dent Co., Ltd., Vietnam). Non-penetrating grooves were made using a sterile diamond disc and the specimens were split into two halves and evaluated for amount of smear layer present using Scanning Electron Microscope (SEM) (Evo LS15, Carl Zeiss Microscopy GmbH, Goettingen, Germany) at x5000 magnification. [Figure IV(A), IV(B)]

The amount of smear layer remaining on the surface of the root canals and dentinal tubules was scored according to the following 5-score system. [5]

| | |
|---|---|
| Score 1 | Clean root canal having only few small debris particles |
| Score 2 | Few small agglomerations of debris covering the root canal wall |
| Score 3 | Many agglomerations of debris covering <50% of the root canal wall |
| Score 4 | Many agglomerations of debris covering >50% of the root canal wall |
| Score 5 | Complete or nearly complete root canal wall covered by debris |

## STATISTICAL METHODS:

The following methods were applied in the present study:

1. Descriptive statistics: Mean and Standard Deviation.
2. One way – ANOVA test: To compare the zones of inhibitions.
3. Kruskal-Wallis test: In the present study, mean and standard deviation of scores for smear layer removal obtained by SEM analysis between all the six groups at cervical, middle and apical thirds of the radicular dentin wall was done.
4. Mann Whitney test: In the present study, the means of two samples was done at a time for determining statistical difference between the smear layer removal scores of SEM analysis at cervical, middle and apical thirds respectively for all the six groups was done.

Significance for all statistical tests was predetermined at $p< 0.05$ (significance level $\alpha=5\%$). Hence, for all tests, P value was considered for statistical significance.

SPSS (Statistical Presentation System Software) version 22.0 for Windows was used for statistical operation.

## RESULTS

- Minimum Inhibitory Concentrations of Punica granatum, Acacia nilotica and Emblica officinalis were 6.25%, 25% and 12.5% respectively.
- Highest zone of inhibition was recorded for 6.25% Punica granatum (21mm), followed by 12.5% Emblica officinalis (20mm) and least for 25% Acacia

nilotica (18mm), when compared to positive control 1% sodium hypochlorite with zone of inhibition measuring 14mm. [Figure II], [Graph I]

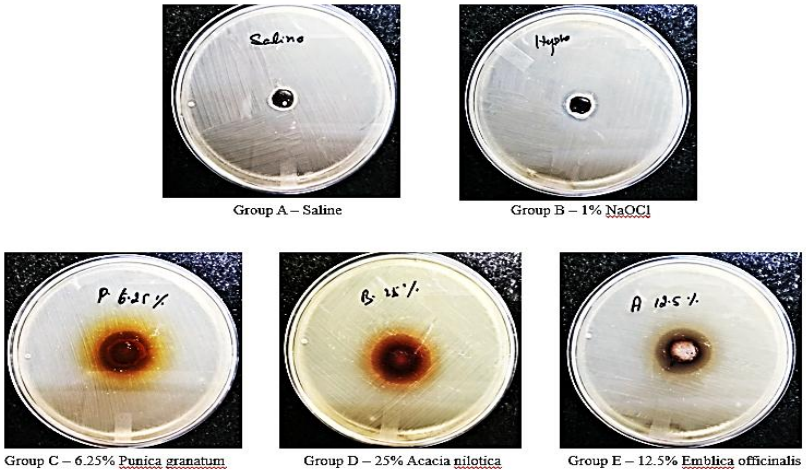
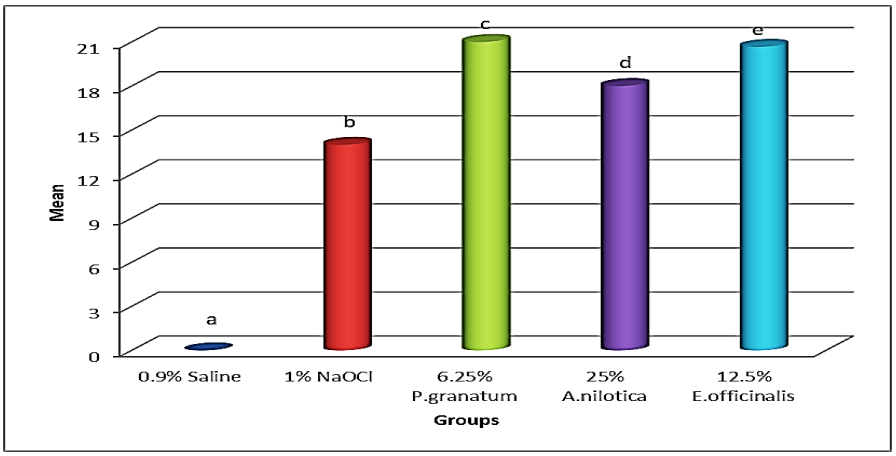



**Figure II: Agar Well Diffusion Test showing Zones of Inhibition in millimeters(mm)**




**Graph I: Graph representing zones of inhibition determined by Agar Well Diffusion test (in millimeters)**

*Mean values with different superscripts are significantly different from each (P<0.05) as indicated by One-way ANOVA (α=0.05)

**Table I: Comparison of mean smear layer scores in cervical, middle and apical thirds of root canal using Kruskal-Wallis test**

| | | N | Mean | Std. Deviation | Std. Error | Minimum | Maximum | Sig |
|---|---|---|---|---|---|---|---|---|
| Cervical | Group 1 | 10 | 4.6000 | 0.51640 | 0.16330 | 4.00 | 5.00 | |
| | Group 2 | 10 | 4.2000 | 0.42164 | 0.13333 | 4.00 | 5.00 | |
| | Group 3 | 10 | 2.0000 | 0.00000 | 0.00000 | 2.00 | 2.00 | |
| | Group 4 | 10 | 1.3000 | 0.48305 | 0.15275 | 1.00 | 2.00 | 0.00 |
| | Group 5 | 10 | 4.3000 | 0.48305 | 0.15275 | 4.00 | 5.00 | |
| | Group 6 | 10 | 1.6000 | 0.51640 | 0.16330 | 1.00 | 2.00 | |
| | Total | 60 | 3.0000 | 1.46137 | 0.18866 | 1.00 | 5.00 | |
| Middle | Group 1 | 10 | 4.9000 | 0.31623 | 0.10000 | 4.00 | 5.00 | |
| | Group 2 | 10 | 4.4000 | 0.51640 | 0.16330 | 4.00 | 5.00 | |
| | Group 3 | 10 | 2.2000 | 0.42164 | 0.13333 | 2.00 | 3.00 | |
| | Group 4 | 10 | 1.5000 | 0.52705 | 0.16667 | 1.00 | 2.00 | 0.00 |
| | Group 5 | 10 | 4.4000 | 0.51640 | 0.16330 | 4.00 | 5.00 | |
| | Group 6 | 10 | 2.0000 | 0.00000 | 0.00000 | 2.00 | 2.00 | |
| | Total | 60 | 3.2333 | 1.43050 | 0.18468 | 1.00 | 5.00 | |
| Apical | Group 1 | 10 | 5.0000 | 0.00000 | 0.00000 | 5.00 | 5.00 | |
| | Group 2 | 10 | 4.7000 | 0.48305 | 0.15275 | 4.00 | 5.00 | |
| | Group 3 | 10 | 2.7000 | 0.48305 | 0.15275 | 2.00 | 3.00 | |
| | Group 4 | 10 | 2.2000 | 0.42164 | 0.13333 | 2.00 | 3.00 | 0.00 |
| | Group 5 | 10 | 4.9000 | 0.31623 | 0.10000 | 4.00 | 5.00 | |
| | Group 6 | 10 | 2.5000 | 0.52705 | 0.16667 | 2.00 | 3.00 | |
| | Total | 60 | 3.6667 | 1.28441 | 0.16582 | 2.00 | 5.00 | |

*$P < 0.05$:* significant , *$P < 0.001$*: Highly significant

- 6.25% Punica granatum solution showed the least smear layer scores (1.3±0.483 in cervical third, 1.5±0.527 in middle third and 2.2±0.421 in apical third of root canal), followed by 12.5% Emblica officinalis solution (1.6±0.516 at cervical third, 2.00±0.00 at middle third and 2.5±0.527 at apical third of root canal), which was comparable to positive control 17% EDTA solution (2.00±0.000 at cervical third, 2.20±0.421 at middle third and 2.70±0.483 at apical third of root canal). [Table I]
- 25% Acacia nilotica solution showed the highest smear layer scores (4.30±0.483 at cervical third, 4.40±0.516 at middle third and 4.90±0.316 at apical third of root canal), which was similar to negative control groups 0.9% saline solution (4.60±0.51 6 at cervical third, 4.90±0.316 at middle third and 5.0±0.000 at apical third of root canal) and 1% sodium hypochlorite solution (4.2±0.421 at cervical third, 4.4±0.516 at middle third and 4.7±0.483 at apical third of root canal). [Table I]

A pair wise difference was found between the smear layer scores of all the groups. [Table II(A), II(B), II(C)]

**Table II(A): Mann-Whitney test for pair wise significant differences in cervical third of root canal**

| Groups | N | Mean±SD | Mean rank | *Z* | *P* (exact *P*) |
|---|---|---|---|---|---|
| Gp 1 | 10 | 4.6000 | 12.50 | -1.780 | 0.143 |
| Gp 2 | 10 | 4.2000 | 8.50 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.6000 | 15.50 | -4.119 | 0.000 |
| Gp 3 | 10 | 2.0000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.6000 | 15.50 | -3.938 | 0.000 |
| Gp 4 | 10 | 1.3000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.6000 | 12.00 | -1.314 | 0.280 |
| Gp 5 | 10 | 4.3000 | 9.00 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.6000 | 15.50 | -3.914 | 0.000 |
| Gp 6 | 10 | 1.6000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.2000 | 15.50 | -4.194 | 0.000 |
| Gp 3 | 10 | 2.0000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.2000 | 15.50 | -4.004 | 0.000 |
| Gp 4 | 10 | 1.3000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.2000 | 10.00 | -0.503 | 0.739 |
| Gp 5 | 10 | 4.3000 | 11.00 | | Not significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.2000 | 15.50 | -3.979 | 0.000 |
| Gp 6 | 10 | 1.6000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.0000 | 14.00 | -3.199 | 0.007 |
| Gp 4 | 10 | 1.3000 | 7.00 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.0000 | 5.50 | -4.147 | 0.000 |
| Gp 5 | 10 | 4.3000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.0000 | 12.50 | -2.179 | 0.143 |
| Gp 6 | 10 | 1.6000 | 8.50 | | Not significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 1.3000 | 5.50 | -3.963 | 0.000 |
| Gp 5 | 10 | 4.3000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 1.30000 | 9.00 | -1.314 | 0.280 |
| Gp 6 | 10 | 1.6000 | 12.00 | | Not significant |
| Total | 20 | | | | |
| Gp 5 | 10 | 4.3000 | 15.50 | -3.938 | 0.000 |
| Gp 6 | 10 | 1.6000 | 5.50 | | Significant |
| Total | 20 | | | | |

**Table II(B): Mann-Whitney test for pair wise significant differences in middle third of root canal**

| Groups | N | Mean±SD | Mean rank | *Z* | *P* (exact *P*) |
|---|---|---|---|---|---|
| Gp 1 | 10 | 4.9000 | 13.00 | -2.285 | 0.063 |
| Gp 2 | 10 | 4.4000 | 8.00 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.9000 | 15.50 | -4.10 | 0.000 |
| Gp 3 | 10 | 2.2000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.9000 | 15.50 | -4.030 | 0.000 |
| Gp 4 | 10 | 1.5000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.9000 | 13.00 | -2.285 | 0.063 |
| Gp 5 | 10 | 4.4000 | 8.00 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 4.9000 | 15.50 | -4.264 | 0.000 |
| Gp 6 | 10 | 2.0000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.4000 | 15.50 | -3.979 | 0.000 |
| Gp 3 | 10 | 2.2000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.4000 | 15.50 | -3.907 | 0.000 |
| Gp 4 | 10 | 1.5000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.4000 | 10.50 | -0.000 | 1.000 |
| Gp 5 | 10 | 4.4000 | 10.50 | | Not significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.4000 | 15.50 | -4.119 | 0.000 |
| Gp 6 | 10 | 2.0000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.2000 | 13.50 | -2.690 | 0.023 |
| Gp 4 | 10 | 1.5000 | 7.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.2000 | 5.50 | -3.979 | 0.000 |
| Gp 5 | 10 | 4.4000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.2000 | 11.50 | -1.453 | 0.481 |
| Gp 6 | 10 | 2.0000 | 9.50 | | Not significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 1.5000 | 5.50 | -3.907 | 0.000 |
| Gp 5 | 10 | 4.4000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 1.5000 | 8.00 | -2.517 | 0.063 |
| Gp 6 | 10 | 2.0000 | 13.00 | | Not significant |
| Total | 20 | | | | |
| Gp 5 | 10 | 4.4000 | 15.50 | -4.119 | 0.000 |
| Gp 6 | 10 | 2.0000 | 5.50 | | Significant |
| Total | 20 | | | | |

**Table II(C): Mann-Whitney test for pair wise significant differences in apical third of root canal**

| Groups | N | Mean±SD | Mean rank | *Z* | *P* (exact *P*) |
|---|---|---|---|---|---|
| Gp 1 | 10 | 5.0000 | 12.00 | -1.831 | 0.280 |
| Gp 2 | 10 | 4.7000 | 9.00 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 5.0000 | 15.50 | -4.147 | 0.000 |
| Gp 3 | 10 | 2.7000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 5.0000 | 15.50 | 4.194 | 0.000 |
| Gp 4 | 10 | 2.2000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 5.0000 | 11.00 | -1.000 | 0.739 |
| Gp 5 | 10 | 4.9000 | 10.00 | | Not significant |
| Total | 20 | | | | |
| Gp 1 | 10 | 5.0000 | 15.50 | -4.110 | 0.000 |
| Gp 6 | 10 | 2.5000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.7000 | 15.50 | -3.963 | 0.000 |
| Gp 3 | 10 | 2.7000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.7000 | 15.50 | -4.004 | 0.000 |
| Gp 4 | 10 | 2.2000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.7000 | 9.50 | -1.090 | 0.481 |
| Gp 5 | 10 | 4.9000 | 11.50 | | Not significant |
| Total | 20 | | | | |
| Gp 2 | 10 | 4.7000 | 15.50 | -3.930 | 0.000 |
| Gp 6 | 10 | 2.5000 | 5.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.7000 | 13.00 | -2.190 | 0.063 |
| Gp 4 | 10 | 2.2000 | 8.00 | | Not significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.7000 | 5.50 | -4.065 | 0.000 |
| Gp 5 | 10 | 4.9000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 3 | 10 | 2.7000 | 11.50 | -0.890 | 0.481 |
| Gp 6 | 10 | 2.5000 | 9.50 | | Not significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 2.2000 | 5.50 | -4.110 | 0.000 |
| Gp 5 | 10 | 4.9000 | 15.50 | | Significant |
| Total | 20 | | | | |
| Gp 4 | 10 | 2.2000 | 9.00 | -1.371 | 0.280 |
| Gp 6 | 10 | 2.5000 | 12.00 | | Not significant |
| Total | 20 | | | | |
| Gp 5 | 10 | 4.9000 | 15.50 | -4.030 | 0.000 |
| Gp 6 | 10 | 2.5000 | 5.50 | | Significant |
| Total | 20 | | | | |

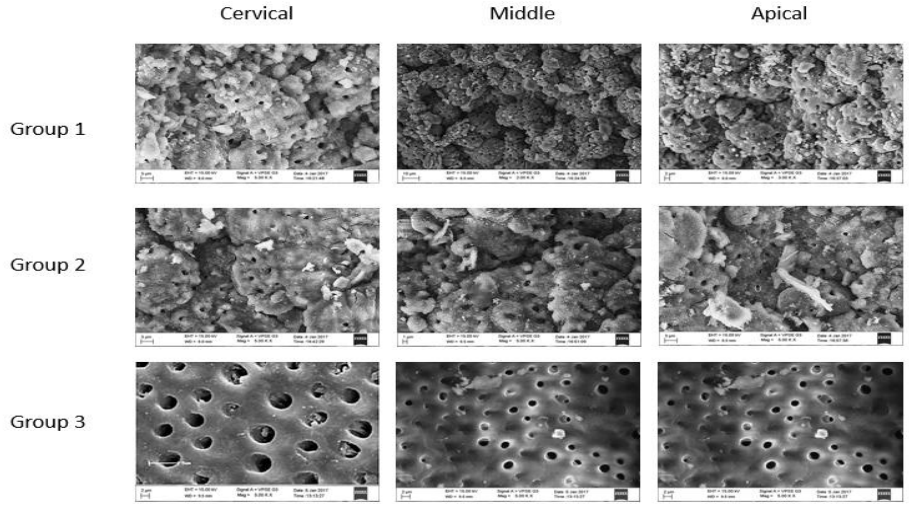



**Figure IV(A): Scanning electron microscope photomicrograph (x5000) for control groups at cervical, middle and apical thirds of root canal**

* Group 1: 0.9% saline solution; Group 2: 15 sodium hypochlorite solution; Group 3: 17% ethylenediaminetetraacetic acid solution

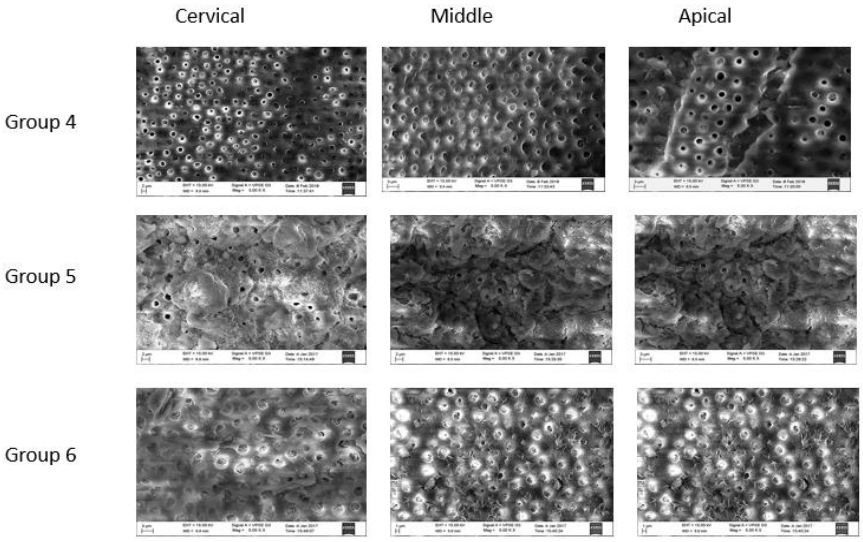



**Figure IV(B): Scanning electron microscope photomicrograph (x5000) for experimental groups at cervical, middle and apical thirds of root canal**
* Group 4: 6.25% Punica granatum solution; Group 5: 25% Acacia nilotica solution; Group 6: 12.5% Emblica officinalis solution

## DISCUSSION

The ultimate objective of endodontic treatment is eradication or significant reduction of microbiota and to flush out loose debris and remove organic and inorganic material, that is, smear layer from the root canal system.

*Enterococcus faecalis* was chosen as the test organism for the present study, as it is the most commonly isolated microorganism from the root canals of teeth with failed endodontic treatment, found in about 4-44% of primary endodontic infections and 24-74% of persistent endodontic infections. [6] *Enterococcus faecalis* survives prolonged periods of starvation and is resistant to the common intracanal irrigants and medicaments, probably because it passively maintains pH homeostasis, which occurs as a result of ions penetrating the cell membrane and cytoplasmic buffering capacity. Another reason is due to an effective proton pump mechanism that provides an additional means of maintaining pH homeostasis. This is accomplished by pumping protons into the cell to lower the internal pH. [7]

Various synthetic intracanal irrigants have been used for decades for elimination of bacterial biofilms found in the infected root canal system. However, the constant increase in antimicrobial resistance and side effects of synthetic drugs, has made phytotherapuetic compounds become popular due to their easy availability, cost effectiveness, low toxicity and lack of antimicrobial resistance. [8]

All the samples were irrigated using needle and syringe technique because it can control the volume, depth of penetration, and the flow/rate of irrigation in the root canal. To avoid confounder in the final results, any type of agitation or activation of irrigating solution was avoided. [9]

Normal saline was chosen as the negative control as it was shown that saline solution is not sufficient as an irrigant in endodontic treatment. Sodium hypochlorite (NaOCl) was chosen as the positive control for antibacterial activity evaluation, because the percentage of bacteria-free canals was increased up to 50% when NaOCl was used for irrigation. Also, 1% NaOCl has been shown to be as effective as 6% NaOCl. [10]

In this *in -vitro* study, aqueous extract solutions of Punica granatum (Pomegranate peel), Acacia nilotica (Babool stem bark) and Emblica officinalis (Amla fruit) showed potent antibacterial activity against *Enterococcus faecalis*, when compared to the control groups.

The possible reason for the antibacterial activity of Punica granatum might be due to the presence of

phytocompounds in its peel extracts like hydrolysable tannins, polyphenolics and flavonoids; specifically punicalagin, gallagic acid, catechin, quercetin, glycosides, punicalin, rutin. However, Opara et al. (2009), associated this activity with the presence of vitamin C in the pomegranate peel. [11]

The antibacterial activity of Emblica officinalis might be due to the presence of tannins present in its fruits. The fruits have 28% of the total tannins distributed in the whole plant. The fruit contains two hydrolysable tannins Emblicanin A and B, which has antioxidant properties, one on hydrolysis gives gallic acid, ellagic acid and glucose. The fruit also contains Phyllemblin. [12]

Acacia nilotica stem bark is said to be prosperous in phenolics, condensed tannins (12-20%) and phlobatannin, gallic acid, protocatechuic acid, pyrocatechol, catechin, epigallocatechin-7-gallate, epigallocatechin-5,7-gallate, epicatechin, dicatechin, quercetin, leucocyanidin gallate, sucrose and catechin-5-gallate, which might be the reason for its antimicrobial properties. [13]

The antimicrobial potential of the three herbal extract solutions has been proven to be due to the presence of secondary metabolites mainly tannins, flavonoids and polyphenols.

Tannins are polymeric phenolic substances found in nearly all plant parts. Tannins and tannic acid own their antimicrobial action to the fact that they precipitate protein and render them resistant to attack by proteolytic enzymes. [14]
Tannic acid is also reported to inhibit oxidative phosphorylation by mitochondria and inhibit electron transport system in mitochondria, resulting in antibacterial potential. [15] They are also said to cause destabilization of the cytoplasmic membrane, the permeability of the cell membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism, or deprivation of the substrates requires for microbial growth (like iron and zinc – via chelation with the metals).

Flavonoids are phenolic structures found abundantly in photosynthesizing cells. [16] They act by inhibiting the nucleic acid synthesis, inhibiting cytoplasmic membrane function by reducing its fluidity and inducing leakage of molecules from intraliposomal space. Flavonoids also act by inhibition of energy metabolism. [17]

Among polyphenols, flavan-3-ols, flavonols and tannins, have a broad spectrum higher antimicrobial activity, due to the fact that they are able to suppress a number of microbial virulence factors - such as inhibition of biofilm formation, reduction of host ligand adhesion and neutralization of bacterial toxins. [18]

Another important aspect for successful endodontic therapy is the removal of smear layer from the root canal walls and dentinal tubules. Studies have concluded that 17% EDTA removes smear layer completely from the cervical and middle thirds of the root canal system. [19] However, it is also known that EDTA has deleterious effects on dentin properties such as erosion of the peritubular and intertubular dentin and reduced microhardness. It also has limited antibacterial activity, which is because of the chelation of cations from the bacterial membrane. [4]

The results of the present study showed that Punica granatum peel and Emblica officinalis fruit extract solutions had satisfactory cleansing action, which was comparable and better than the standard 17% EDTA solution.

The SEM analysis revealed that Acacia nilotica had no smear layer removing property and its mean scores were similar to the negative control groups, i.e., 0.9% saline solution and 1% sodium hypochlorite solution.

**The limitations of this study include:**

i. Since it was an in-vitro study done on extracted teeth, the results might vary significantly in living individuals, as the body reactions

may vary. Hence, long term in-vivo studies must be conducted to evaluate their efficacy.

ii. Since it was an in-vitro study, its side effects could not be evaluated. Hence, long term clinical trials must be performed to evaluate the allergic potential of phytotherapeutic extract solutions.

iii. Another limitation is that, in the present study, the irrigants were used separately and not in combination. Hence, studies must be conducted to test the interactions of these natural extracts with other irrigants.

iv. The effects of the extract solutions on physical properties of dentin must be evaluated in future studies.

v. Due to the dark color of the Punica granatum (Pomegranate peel), Acacia nilotica (Babool stem bark) and Emblica officinalis (Amla fruit) extract solutions, there are chances of discoloration of the teeth on long term usage. Hence, long term clinical trials must be conducted to determine their discoloration potential.

vi. Toxicity of the extract solutions could not be evaluated as it was an in-vitro study. Toxicological studies must be conducted on live models in order to evaluate their toxicity on the live cells.

## CONCLUSION

It can be concluded from this in-vitro study that Punica granatum (Pomegranate peel), Acacia nilotica (Babool stem bark) and Emblica officinalis (Amla fruit) extracts have a good antibacterial property as herbal endodontic irrigants. Punica granatum (Pomegranate peel), Acacia nilotica (Babool stem bark) and Emblica officinalis (Amla fruit) extract solution were found to be equally effective in inhibiting the growth of Enterococcus faecalis.

Also, Punica granatum (Pomegranate peel) and Emblica officinalis (Amla fruit) extract solution were effective as endodontic irrigants in removing the smear layer from the cervical, middle and apical thirds of the radicular dentin wall.

Inexpensive and easy availability, acceptable taste and smell compared to the synthetic drugs, makes them a promising herbal endodontic irrigant for future use. However, as this study was an in-vitro study, long term in-vivo studies to check the efficacy might be useful to further evaluate the antimicrobial action of Punica granatum (Pomegranate peel), Acacia niotica (Babool stem bark) and Emblica officinalis (Amla fruit) extract solutions against other microorganism found in the root canals of failed endodontic treatment.

Their effects on the physical properties of radicular dentin and discoloration of the teeth must also be evaluated. Their depths of penetration into the dentinal tubules should also be evaluated using the available methods. Methods to increase the shelf life of such extract solutions must be carried out to increase their long term usage.

Research in these areas can progress further, with a long term clinical study on whether or not Punica granatum (Pomegranate peel) extract solution, Acacia nilotica (Babool stem bark) extract solution and Emblica officinalis (Amla fruit) extract solution could totally eradicate the microbial loads and smear layer from the root canal system, allowing us to develop a long term regimen for their usage.

Nonetheless, with the detailed knowledge we have gained from this study, we can use Punica granatum (Pomegranate peel), Acacia nilotica (Babool stem bark) and Emblica officinalis (Amla fruit) extract solutions effectively to reduce the microbial growth/load as herbal endodontic irrigants which can aid towards the development of potential therapeutics.

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