Conservative Dentistry Journal Vol. 11 No. 2 July-December 2021; 84-88

Research Report

White pomegranate (*Punica granatum*) peels extract bactericidal potency on *Enterococcus faecalis*

Eric Priyo Prasetyo,[1] Widya Saraswati,[1] Setyabudi Goenharto,[1] Dian Agustin Wahjuningrum,[1] Latief Mooduto,[1] Rizka Firdaus Rosidin,[2] Evelyn Tjendronegoro[3]

[1] Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
[2] Dentistry Program, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
[3] Healthcare and Research, Irvine Medical Center, University of California, California, United States of America.

ABSTRACT

Background: *Enterococcus faecalis (E. faecalis) is the most resistant bacteria in the root canals and one of the causes of recurrent endodontic treatment. Enterococcus faecalis was not the only bacteria found in failed endodontic treatment but these bacteria can thrive in unfavorable environment. White pomegranate (Punica granatum) is known for its flavonoids and tannins that function as antimicrobial agent. White pomegranate extract is potential for use as disinfection or irrigation material.* ***Purpose:*** *This aim of this study was to determine the bactericidal potency of white pomegranate (Punica granatum) extract on E. faecalis growth.* ***Methods:*** *This study was an in vitro experimental observation. E. faecalis was obtained from stock culture taken from the root canal of recurrent endodontic treatment. E. faecalis from the serial dilution were cultured in blood agar media. Antibacterial potency was determined by colony calculation of E. faecalis growth in blood agar in colony forming unit (CFU) and conducted in 6 replications for each concentration group. Statistical analysis was done using one-way analysis of variance at 5% significance level.* ***Results****: White pomegranate peels extract concentrations of 3.125%, 6.25%, 12.5% and 25% provide significant decrease in the number of E. faecalis colony compared to the control group ($p<0.05$). No bacterial growth was found on 25%, 50% and 100% concentration.* ***Conclusion:*** *The potent minimal bactericidal concentration of white pomegranate peels extract on E. faecalis was 25%.*

Keywords: *Enterococcus faecalis; white pomegranate; bactericidal; disinfection; Punica granatum.*

Correspondence: Eric Priyo Prasetyo, Department of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjend. Prof. Dr. Moestopo 47, Surabaya, 60132 Indonesia. Email: eric-p-p@fkg.unair.ac.id.

INTRODUCTION

The principles of root canal treatment include biomechanical preparation, chemo-mechanical disinfection and hermetic obturation. This principle must be supported by the successful elimination of infectious microorganisms in the root canal system.[1] There are attempts to improve the success of root canal treatment, including navigating the efficient instrumentation, effective cleansing,[2,3] antibacterial medicaments and irrigation materials.[4,5]

One of the resistant bacteria which survive disinfection is *E. faecalis*.[6] This is supported by anatomical complexity of the root canal system. *E. faecalis* may hide in dentine tubules and unaffected from biomechanical preparation and disinfection. This may cause undetected periapical abnormalities which may happen several months or years after endodontic treatment.[7]

An ideal disinfection or irrigation material for root canal treatment procedure should be able to dissolve organic and inorganic debris, low surface tension, non-carcinogenic, non-mutagenic, biocompatible, retain high antimicrobial activity, non-toxic, and economical. In the past decades, there is a trend to the use of natural products, organic wastes or herbal plants. This global 'back-to-nature' trend is a good choice because natural products are usually organic, biodegradable, easy to obtain, and economical, especially in Indonesia where the availability of such material is abundant and sustainable.

One of many natural product which has abundant bioactive substances is pomegranates (*Punica granatum*).[8] Pomegranates from different geographical regions (including edaphic characteristics, latitude, longitude, and altitude), cultivar types, cultivation and climates would affect its bioactive molecules, physiochemical and antioxidant profiles, especially its polyphenolic, anthocyanin and flavonoids content.[9] Pomegranate peel extracts have the highest antioxidant activity, flavonoid and phenolic compound compared to the seeds and fruit pulp.[10] White pomegranate cultivars contain the highest flavonoid compared to other cultivars.[8] Previous researches showed that pomegranate peel extract has antibacterial potency on cariogenic bacteria[11] and oral candidiasis.[12]

Currently there are limited study on white pomegranate from Indonesia for endodontic use. Generally, researches and analysis were done using pomegranate cultivars from Iran, India, Yemen, China, South Africa, Tunisia, Spain, Algeria, Oman, Egypt, and Turkey with known polyphenolic and flavonoid compounds.[8] Based on this introduction, the authors would like to compare the antibacterial concentration of white pomegranate (*Punica granatum*) peel extract from Indonesian cultivar on *E. faecalis* from recurrent endodontic treatment.

MATERIALS AND METHODS

This is an experimental in vitro study, conducted in Conservative Dentistry Clinic, Universitas Airlangga Dental Hospital and Microbiology Laboratory, Research Center, Faculty of Dental Medicine, Universitas Airlangga. Ethical clearance was obtained from Universitas Airlangga Faculty of Dental Medicine Ethic Committee. Materials used in this research were extracted from fresh white pomegranate from Indonesian cultivar, grown in Surabaya, East Java. *Enterococcus faecalis* bacteria used in this study were bacteria previously cultured from recurrent endodontic treatment. *E. faecalis* from stock was cultured in BHI broth media and standardized with 0.5 McFarland. The method used in this study was serial dilusion using Brain Heart Infusion (BHI) broth.

White pomegranate peels were extracted according to Balaban et al. (2021)[13] and Nozohour et al. (2018)[14] with modifications. First, the peels from white pomegranate were cleaned and dried, away from direct sunlight, and pulverized into powder and sieved. The powder was stored in a jar, mixed with 70% ethanol and stirred. Maceration was done in 72 hours, and the mixture was strained. Filtrate from the straining was dehydrated with vacuum evaporator for 3 hours to obtain the pure white pomegranate peel extract, and sterilization of this extract was done in an autoclave at 121°C for 20 minutes.

Concentrations of white pomegranate peels used in this study were 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.565%, 0.781%. Serial dilution was done by preparing 10 sterile reaction tubes containing BHI broth of 5 ml on each tube. White pomegranate peels extract of 100% concentration were poured 5 ml into the first tube. In the second reaction tube 5 ml were taken from the first tube to get 50% of white pomegranate peels extract. In the third tube, 5 ml were taken from the second tube to get 25% of white pomegranate peels extract. In the fourth tube, 5 ml were taken from the third tube to get 12.5% of white pomegranate peels extract, and these procedures were continued for the fourth until the eighth tube. In the eighth tube, 5 ml was discarded. The ninth tube was used as positive control, containing only BHI broth and *E. faecalis*. The tenth tube was used as negative control, containing only BHI broth media.

E. faecalis bacterial culture was taken 0.1 ml and poured into the first to the ninth tubes. All of the tubes were incubated for 24 hours at 37°C in anaerobic condition. After incubation period, the tubes were checked visually for any changes. The positive control was prepared using blood agar media and 0.1 ml of *E. faecalis* suspension without white pomegranate peels extract. The negative control was prepared using blood agar media and white pomegranate peels extract without *E. faecalis*.

The tubes with changes in appearance (tube with 25%, 12.5%, 6.25%, and 3.125% white pomegranate peels extracts) were taken 0.1 ml and cultured further in blood agar media for 24 hours at 37°C in anaerobic condition for confirmation on selected concentrations. Evaluation was done for colony formation on the blood agar media. The colonies grown in blood agar media were manually counted by three independent observers in colony forming units (CFU) and repeated three times. Bactericidal concentration was considered if there was 99.9% bacterial inhibition. Data of colony formation on blood agar media (in CFU) were gathered for each plate.

Bacterial colony data were analyzed statistically and significance level was set at 5%. Normality of data was analyzed using Shapiro Wilk test. Significance was tested using one-way analysis of variance.

RESULTS

The number of replications (n) for each concentration group was 6. The result data were normally distributed. Mean and standard deviation of *E. faecalis* colony growth under the influence of white pomegranate peels extract at different concentrations are shown in Table 1. The mean of *E. faecalis* colony growth under the influence of 25% white pomegranate peels extract was 0.00000 CFU, followed by white pomegranate peels extract 12.5% (8.2857 CFU), 6.25% (79.4286 CFU), and 3.125% (149.5714 CFU). The highest mean of *E. faecalis* colony growth was observed in the positive control which was 229.5714 CFU.

Analysis of variance was used in this study to check the significance among different concentrations of white pomegranate peels extract on *E. faecalis*. The significance between *E. faecalis* colony growth is shown in Table 2. We found a significant difference among different white

Table 1. Mean and standard deviation of *E. faecalis* colony growth under different concentrations of white pomegranate peels extract (WPPE)

Groups	n	*E. faecalis* colony growth (CFU) Mean ± SD
Positive Control	6	230.3333 ± 7.3394
WPPE 3.125%	6	149.6667 ± 4.5019
WPPE 6.25%	6	80.1667 ± 5.4191
WPPE 12.5%	6	8.3333 ± 1.0328
WPPE 25%	6	0.0000 ± 0.0000

Notes: n = replication; SD = standard deviation

Table 2. Significance between white pomegranate peels extract (WPPE) with different concentrations on E. faecalis colony growth

Groups	Positive Control	WPPE 3.125%	WPPE 6.25%	WPPE 12.5%	WPPE 25%
Positive Control	-	0.00000*	0.00000*	0.00000*	0.00000*
WPPE 3.125%	0.00000*	-	0.00000*	0.00000*	0.00000*
WPPE 6.25%	0.00000*	0.00000*	-	0.00000*	0.00000*
WPPE 12.5%	0.00000*	0.00000*	0.00000*	-	0.03077*
WPPE 25%	0.00000*	0.00000*	0.00000*	0.03077*	-

Note: *Statistically significant ($p<0.05$)

pomegranate peels extract concentrations groups of 25%, 12.5%, 6.25%, 3.125% and the positive control ($p < 0.05$). White pomegranate peels extract group of 25% has the highest antibacterial efficacy with 0% colony growth.

DISCUSSION

Enterococcus faecalis from failed endodontic treatment were chosen in this study because this bacterium is often found in recurrent endodontic treatment. This bacterium usually found together with other fungi and bacteria in persistent endodontic lesions.[6] These bacteria usually resistant and can survive disinfection procedure.[7]

Pomegranates consists of parts like leaf, flower, root, bark, seeds, juice and peels which contain different therapeutic properties.[15] Although both pomegranate seeds and peels have antibacterial activity, the peels have more potent effect.[14] Pomegranate peels comprise about 30-40% of the total fruit weight and contains high level of ellagic acid, ellagitannins, punicalagins, tannins, and flavonoids.[16]

White pomegranate peels extract from Indonesian cultivar, grown in Surabaya were chosen because they are economical, easily obtained and does not require lengthy transportation procedure. Limited study is reported regarding the antibacterial efficacy of white pomegranate peels extract on *E. faecalis.* Other than the cultivar selection of white pomegranate, which has excellent chemical composition (antioxidants, phenolic compounds, punicalagins, ellagic acids), in this study, extraction method with ethanol solvent was used because the extract would retain the highest antimicrobial effect.[13]

Bactericidal potency of white pomegranate peels extract on *E. faecalis* colony growth was experimentally observed with serial dilution method and inoculum of *E. faecalis* were mixed in the diluted solution of 100%, 50%, 25%, 12.5%, 6.25%, 3.125% white pomegranate peels extract concentrations, and these mixtures were grown in blood agar media. The bacterial colony growth on blood agar plates were measured in colony forming unit (CFU). Serial dilution is a laboratory calculation method to check whether a material have the ability to inhibit bacterial growth. Colony forming unit (CFU) is the gold standard method of antibacterial assessment.[17]

White pomegranate peels extraction method has a correlation on the extraction of active compounds and antibacterial efficacy, where extraction with alcohol solvent like ethanol is more optimal than water.[18] Some microorganisms are more sensitive to plant extracts than disinfection agents, in this study, *E. faecalis* is sensitive to white pomegranate peels extract in the concentration of 100%, 50% and 25%, but the lowest concentration of white pomegranate peels extract with maximal antibacterial potency is 25%.

The antibacterial efficacy of white pomegranate, as in other types of pomegranate, derived from phenolic compound such as phenolic acid (ellagic acid, chlorogenic acid, gallic acid, caffeic acid, quercetin, and ferulic acids), pro-anthocyanidins and flavonoids.[8] Pomegranate peels contain high phenolic compounds and ellagitannins.[10] Polyphenolic compound responsible for strong antioxidant potency, while the tannin content in pomegranate peel extract is responsible for antibacterial and antifungal efficacy.[8]

Polyphenols, in particular, tannins are the major components in the peel extract with a mechanism of protein precipitation to cause leakage of bacterial cell membrane, leading to cell lysis and cell death for both Gram-positive and Gram-negative bacteria.[19] Ellagic acid and punicalagin induce enlargement of bacterial cells and cell membrane thickness, destruct and change the permeability of cellular membrane, enzyme inactivation, protein precipitation, as well as disruption of bacterial communication system of quorum sensing to destroy bacteria and inhibit biofilm formation.[13]

E. faecalis can be killed with high pH level. High pH or an extreme alkaline environment would disturb the survival of most bacteria.[20] However, there are some studies stated that pH of higher than 11.5 is required for potent disinfection.[21] To be used as a root canal dressing material, white pomegranate peels extract must have positive characteristics similar to calcium hydroxide, as the gold standard of root canal dressing.[22] Calcium hydroxide has various influence on cells, from anti inflammation to apoptosis.[23,24] However previous studies reported *E. faecalis* resistance to calcium hydroxide.[25] A current study reported that pomegranate gel have similar antibacterial activity to calcium hydroxide.[17] Experiment on the effect of pomegranate peels extract on *Candida albicans* indicate an effective antifungal which approximates clotrimazole as the standard antifungal agent.[26] Current clinical trial reported the use of *Punica granatum* peels extract 5% as a complementary irrigation to non-surgical treatment of

chronic gingivitis showed improvement and no adverse effect.[27] The development of white pomegranate peels extract as a natural irrigation or root canal dressing material is expected to bring a solution to the bacterial resistance.

This study showed the higher the concentration of white pomegranate peels extract, the lower *E. faecalis* bacterial colony growth. This is caused by the high polyphenolic, flavonoid, and tannins concentration which has antimicrobial activity to inhibit *E. faecalis* colony growth. Even though this extract won't eliminate *E. faecalis* in lower concentrations of below 25%, white pomegranate peels extract can reduce the bacteria colony formation. This can be developed as a natural irrigation or root canal dressing material as an alternative to synthetic disinfectants or antibiotics to prevent multi drug-resistant bacteria in the root canal system.

Many other studies have been performed to introduce natural, biocompatible, antimicrobial and regenerating root canal irrigation materials or medicaments in order to support successful endodontic treatment and improve patient satisfaction. In conclusion, white pomegranate peels extract 25% has potent antibacterial activity on *E. faecalis*. However, further studies about the side effects on pulpal and peri-radicular cells in-vivo, characteristics and delivery method of white pomegranate peels extract should be done to prepare this extract as an alternative natural irrigation or dressing material prior to application in the root canal system.

ACKNOWLEDGEMENTS

The authors would like to thank the Publication Center, Dental Hospital, and Research Center, Faculty of Dental Medicine, Universitas Airlangga for the given facility.

REFERENCES

1. Kaiwar A, Nadig G, Hegde J, Lekha S. Assessment of antimicrobial activity of endodontic sealers on *Enterococcus faecalis*: an in vitro study. World J Dent. 2012; 3(1): 26-31. doi: 10.5005/jp-journals-10015-1123.
2. Santoso CMA, Samadi K, Prasetyo EP, Wahjuningrum DA. Root canal cleanliness between mangosteen peel extract irrigant and NaOCl 2.5%. Conservative Dentistry Journal. 2020; 10(1): 40-3. doi: 10.20473/cdj.v10i1.2020.40-43.
3. Juniarti DE, Kusumaningsih T, Soetojo A, Prasetyo EP, Sunur YK. Antibacterial activity and phytochemical analysis of ethanolic purple leaf extract (*Graptophyllum Pictum L. griff*) on *Lactobacillus acidophilus*. Mal J Med Health Sci. 2021; 17(Suppl2): 71-3.
4. Astuti RHN, Samadi K, Prasetyo EP. Antibacterial activity of *Averrhoa bilimbi Linn.* leaf extract against *Enterococcus faecalis*. Conservative Dentistry Journal. 2016; 6(2): 93-8. doi: 10.20473/cdj.v6i2.2016.93-98.
5. Harseno S, Mooduto L, Prasetyo EP. Antibacterial potency of kedondong Bangkok leaves extract (*Spondias dulcis Forst*.) against *Enterococcus faecalis* bacteria. Conservative Dentistry Journal. 2016; 6(2): 110-6. doi: 10.20473/cdj.v6i2.2016.110-116.
6. Prada I, Mico-Munoz P, Giner-Lluesma T, Mico-Martinez P, Collado-Castellano N, Manzano-Saiz A. Influence of microbiology on endodontic failure. Literature review. Med Oral Patol Oral Cir Bucal. 2019; 24(3): e364-72. doi: 10.4317/medoral.22907.
7. Del Fabbro M, Samaranayake LP, Lolato A, Weinstein T, Taschieri S. Analysis of the secondary endodontic lesions focusing on the extraradicular microorganisms: an overview. J Investig Clin Dent. 2014; 5(4): 245-54. doi: 10.1111/jicd.12045.
8. Bassiri-Jahromi S, Doostkam A. comparative evaluation of bioactive compounds of various cultivars of pomegranate (*Punica granatum*) in different world regions. AIMS Agriculture and Food. 2019; 4(1): 41-55. doi: 10.3934/agrfood.2019.1.41.
9. Hmid I, Elothmani D, Hanine H, Oukabli A, Mehinagic E. Comparative study of phenolic compounds and their antioxidant attributes of eighteen pomegranate (*Punica granatum L.*) cultivars grown in Morocco. Arabian J Chem. 2017; 10:S2675-S2684. doi: 10.1016/j.arabjc.2013.10.011.
10. Derakhshan Z, Ferrante M, Tadi M, Ansari F, Heydari A, Hosseini MS, Conti GO, Sadrabad EK. Antioxidant activity and total phenolic content of ethanolic extract of pomegranate peels, juice and seeds. Food Chem Toxicol. 2018; 114: 108-11. doi: 10.1016/j.fct.2018.02.023.
11. Ferrazzano GF, Scioscia E, Sateriale D, Pastore G, Colicchio R, Pagliuca C, Cantile T, Alcidi B, Coda M, Ingenito A, Scaglione E, Cicatiello AG, Volpe MG, Stasio MD, Salvatore P, Pagliarulo C. In vitro antibacterial activity of pomegranate juice and peel extracts on cariogenic bacteria. Biomed Res Int. 2017: 2152749. doi: 10.1155/2017/2152749.
12. Bassiri-Jahromi S, Pourshafie MR, Ardakani EM, Ehsani AH, Doostkam A, Katirae F, Mostafavi E. In vivo comparative evaluation of pomegranate (*Punica granatum*) peel extract as an alternative agent for nystatin against oral candidiasis. Iran J Med Sci. 2018; 43(3): 296-304. PMID: 29892147.
13. Balaban M, Koc C, Sar T, Akbas MY. Antibiofilm effects of pomegranate peel extracts against *B. cereus*, *B. subtilis*, and *E. faecalis*. Int J Food Sci Tech. 2021; 56: 4915-24. doi: 10.1111/ijsf.15221.
14. Nozohour Y, Golmohammadi R, Mirnejad R, Fartashvand M. Antibacterial activity of pomegranate (*Punica granatum L.*) seed and peel alcoholic extracts on *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from health centers. J Appl Biotechnol Rep. 2018; 5(1): 32-6. doi: 10.29252/jabr.01.01.06.
15. Jacob J, Rajiv P, Gopalan R, Lakhsmanapermalsamy P. An overview of phytochemical and pharmacological potentials of *Punica granatum L*. Pharmacogn J. 2019;11(5): 1167-71. doi: 10.5530/pj.2019.11.181.
16. Singh B, Singh JP, Kaur A, Singh N. Antimicrobial potential of pomegranate peel: a review. Int J Food Sci Tech. 2019; 54: 959-65. doi: 10.1111/ijfs.13964.
17. Mittal A, Tejaswi S, Mruthunjaya K, Shetty S, Ambikathanaya UK. Comparison of antibacterial activity of Calcium hydroxide, *Azadirachta indica* (neem), *Ocimum tenuiflorum* (tulsi) and *Punica granatum* (pomegranate) gels as intracanal medicaments against *Enterocuccus faecalis*: an in-vitro study. Pharmacogn J. 2021; 13(4): 988-94. doi: 10.5530/pj.2021.13.127.
18. Mathkoor MM, Oda NA, Omran ZS. Comparative study antibacterial activity of some medicinal plants extracts (leaves and peel) against some multi-drug resistant bacteria from clinical isolates. Int J Drug Delivery Tech. 2019; 39(3): 465-70. doi: 10.25258/ijddt.v9i3.25.

19. Jaisinghani RN, Makhwana S, Kanojia A. Study on antibacterial and flavonoid content of ethanolic extract of *Punica granatum* (pomegranate) peel. Microbiol Res. 2018; 9: 7480. doi: 10.4081/mr.2018.7480.
20. Jhajharia K, Parolia A, Shetty KV, Mehta LK. Biofilm in endodontics: a review. J Int Soc Prev Community Dent. 2015; 5: 1-12. doi: 10.4103/2231-0762.151956.
21. Zancan RF, Vivan RR, Lopes MRM, Weckwerth PH, Andrade FB, Ponce JB, Duarte MAH. Antimicrobial activity and physicochemical properties of calcium hydroxide pastes used as intracanal medication. J Endod. 2016; 42(12): 1822-8. doi: 101016/j.joen.2016.08.017.
22. Prasetyo EP, Widjiastuti I, Cahyani F, Kuntjoro M, Hendrijantini N, Hariyani N, Winoto ER, Nugraha AP, Goenharto S, Susilowati H, Hendrianto E, Rantam FA. Cytotoxicity of calcium hydroxide on human umbilical cord mesenchymal stem cells. Pesqui Bras Odontopediatria Clin Integr. 2020; 20: e0044. doi: 10.1590/pboci.2020.141.
23. Prasetyo EP, Kuntjoro M, Cahyani F, Goenharto S, Saraswati W, Juniarti DE, Hendrijantini N, Hariyani N, Nugraha AP, Rantam FA. Calcium hydroxide upregulates interleukin-10 expression in time dependent exposure and induces osteogenic differentiation of human umbilical cord mesenchymal stem cells. Int J Pharm Res. 2021; 13(1): 140-5. doi: 10.31838/ijpr/2021.13.01.023.
24. Prasetyo EP, Kuntjoro M, Goenharto S, Juniarti DE, Cahyani F, Hendrijantini N, Nugraha AP, Hariyani N, Rantam FA. Calcium hydroxide increases human umbilical cord mesenchymal stem cells expressions of apoptotic protease-activating factor-1, caspase-3 and caspase-9. Clinical, cosmetic and investigational dentistry. 2021;13: 59-65. doi: 10.2147/CCIDE.S284240.
25. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide. Int Endod J. 2002; 35(3): 221-8. doi: 10.1046/j.1365-2591.2002.00504.x.
26. Kumar KS, Samlin SS, Siva B, Sudharshan R, Vignesswary A, Divya K. Punica granatum as a salutiferous superfruit in the treatment of oral candidiasis – an in-vitro study. J Oral Maxillofac Pathol. 2019; 23: 188. doi: 10.4103/jomfp.JOMFP_268_19.
27. Eltay EG, Gismalla BG, Mukhtar MM, Awadelkarim MOA. *Punica granatum* peel extract as adjunct irrigation to nonsurgical treatment of chronic gingivitis. Complementary Therapies in Clinical Practice. 2021; 43: 101383. doi: 10.1016/j.ctcp.2021.101383.