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In-vitro anti-bacterial effectiveness test of 2% chlorhexidine digluconate and ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) on the growth of Enterococcus faecalis bacterial colony

Ni Kadek Eka Widiadnyani[1*], Putu Ratna Kusumadewi Giri[1],
I Gusti Ayu Fienna Novianthi Sidiartha[1], I Ketut Tunas[2]

[1]Bachelor of Dentistry and Dentistry Profession Program, Faculty of Medicine, Universitas Udayana, Bali, Indonesia
[2]Management of Health Information Department, Universitas Bali Internasional, Bali, Indonesia

*Corresponding to:
Ni Kadek Eka Widiadnyani; Bachelor of Dentistry and Dentistry Profession Program, Faculty of Medicine, Universitas Udayana, Bali, Indonesia;
eka.widiadnyani@unud.ac.id

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ABSTRACT

Background: Necrotic teeth with periapical lesions often experience root canal treatment failure. Enterococcus faecalis bacteria cause 85-90% of root canal infections. 2% Chlorhexidine digluconate (CHX) can eliminate Enterococcus faecalis bacteria but cannot dissolve necrotic tissue, while green meniran phytochemical compounds (Phyllantus niruri Linn) have anti-bacterial properties. This study aims to determine the effectiveness, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) between green meniran leaf extract with 2% chlorhexidine digluconate and meniran extract only against the growth of Enterococcus faecalis.

Method: This is an in vitro experimental study with a broth microdilution method consisting of two treatment groups. The negative control contained BHIB media and 0.05 ml of bacteria; the positive control had 0.05 ml of bacteria with 2% chlorhexidine digluconate, the group one consisted of BHIB media, bacteria and various concentrations of meniran extract. In contrast, group two consisted of BHIB media, bacteria, various concentrations of green meniran with 2% chlorhexidine digluconate. Data were analyzed using the One Way Anova test with LSD test.

Results: Compared with negative control, in group one, we found the MIC of meniran extract was 3.125% with 94% anti-bacterial power, and the MBC was 6.25% with no bacterial growth. In group 2, we found the MIC of meniran extract and the 2% chlorhexidine digluconate was 0.78% with 9.3% anti-bacterial power, while the MBC was 1.56%.

Conclusion: The increasing concentration of meniran extract also increases the anti-bacterial effect. A combination of meniran extract and 2% chlorhexidine digluconate give better anti-bacterial effects than meniran extract only.

Keywords: 2% Chlorhexidine Digluconate, Enterococcus faecalis, Meniran Leaf, Phyllanthus niruri Linn, Root Canal Treatment
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INTRODUCTION

Infection of dental pulp tissue begins as a result of caries, dental operative procedures and trauma involving gram-negative anaerobic bacteria in the oral cavity that can cause apical periodontitis requiring root canal treatment.[1] Failure in root canal treatment is very common. The main cause of failure in root canal treatment is *Enterococcus faecalis*. It is a facultative gram-positive anaerobic that is dominantly found in persistent endodontic infections with a prevalence of 24% – 77% despite root canal treatment.[2-4]

The three basic principles of root canal treatment are the endodontic triad: preparation (cleaning and shaping), sterilization (medicaments), and obturation.[5] However, many studies have revealed that, even with the advent of rotary instrumentation systems and chemical irrigation, which can reduce instrumentation time and contact time of chemical additives in root canals, they are not effective enough to rid root canal bacteria.[6] Root canal sterilization materials should have antiseptic properties that can inhibit the reproduction and metabolism of microorganisms with low toxicity.[5,7]

Several substances have been recommended as adjuncts for root canal sterilization; the most commonly used is 2% chlorhexidine digluconate (CHX). The CHX cannot dissolve microorganisms and root canal tissue, but it has a strong antimicrobial effect against gram-negative and gram-positive bacteria and lower cytotoxicity.[8] In addition, CHX cannot eliminate root

canal bacterial biofilms.[9] According to Athanassiadis et al., chlorhexidine can remain in root canal dentin for up to 12 weeks for an antimicrobial effect.[10,11] Despite the indicated properties of each irrigating agent, none is sufficiently capable of completely sterilizing the root canal system.[11] Thus, another effective antimicrobial agent is needed to sterilize and overcome the resistance problem efficiently.

Ingredients from herbal sources can replace the chemical one; one of the herbal plants that can be used is the Meniran plant (*Phyllanthus niruri Linn*). Based on empirical and clinical research, green meniran leaf has several benefits such as anti-inflammatory, analgesic, antipyretic, and other treatments that provide antihistamines.[11,12] Based on research, green meniran phytochemicals (*Phyllanthus niruri Linn*) contain secondary metabolites of flavonoids, terpenoids, alkaloids, steroids, tannins, and saponins. Several research results show that terpenoid compounds have anti-bacterial activity.[12-14] Research by Cushnie and Lamb and Shoaib et al. stated that many flavonoids had been reported to have properties such as anti-bacterial, antiviral, antifungal, anti-inflammatory and several other activities. The mechanism of flavonoids that work as anti-bacterial by increasing cell membrane permeability. Flavonoids work by damaging cell walls and cytoplasmic membranes; in addition, flavonoids can also prevent bacterial cell division so that bacteria cannot develop properly and cannot form complex compounds, as extracellular proteins that make up bacterial cell membranes.[15,16] This mechanism follows the results of research by Diana et al., which states that meniran plant extract works actively as an anti-bacterial against Enterococcus faecalis.[17] Green meniran leaf extract (*Phyllanthus niruri Linn*) is expected to play an important role in the eradication of bacteria in the root canal so that it is expected to have a very important impact on increasing the success of subsequent treatments.[13] Based on the background above, this study aims to know the difference in effectiveness, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) between meniran extract with 2% CHX meniran extract only against the growth of Enterococcus faecalis bacteria.

MATERIALS AND METHODS

This is an in-vitro experimental study with a post-test-only control group design. This research was carried out at the Research Center Laboratory of the Faculty of Dentistry, Airlangga University, Surabaya, in July 2020. The study sample was *Enterococcus faecalis* bacteria ATCC 29212. The green meniran leaf extract preparations were macerated with 96% ethanol solutions and conducted in the Pharmacognosis Laboratory, Faculty of Mathematics and Natural Sciences, Udayana University. Meniran extract was prepared in 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78 % concentrations.

The *Enterococcus faecalis* bacteria were prepared by placing it in BHIB media until the turbidity was equivalent to the standard 0.5 *McFarland* (1.5 x 108 CFU/ml). in this study, we divided the sample into four groups; the first group consisted of 0.05 ml of standardized bacterial suspension with 0.5 *McFarland* was grown in a tube containing BHIB and meniran extract with various concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78%). The second group consisted of 0.05 ml of standardized bacterial suspension with 0.5 *McFarland* was grown in a tube containing BHIB, meniran extract with various concentrations (100%, 50%, 25%, 12.5%, 6.25). %, 3.12%, 1.56%, 0.78%) and 2% CHX. The third group is a negative control, which contains BHIB media and *Enterococcus faecalis* bacteria to ensure bacterial contamination in the media. The last is positive control group contained BHIB media, 0.05 ml of Enterococcus faecalis bacterial suspension and 2% CHX.

The difference in the effectiveness of anti-bacterial growth, MIC, and MBC was determined by counting the number of bacterial colonies on blood agar media, which was calculated manually and expressed by CFU (Colony Forming Unit), and compared with positive control and negative control. Interpretation of the bacteriostatic and bactericidal activity of the test extract by comparing the decrease in bacterial colonies in multiples of 1000 exposed to anti-bacterial compounds from the test extract relative to the number of initial bacterial colonies. In MIC, it will show 90% inhibition, and on MBC, it will show the death of 99.9% of Enterococcus faecalis bacterial colonies. Four different observers repeated the calculation three times, taking the average.

The data analysis tests carried out in this study were the Kolmogorov-Smirnov test for normality and the Levene's test for homogeneity. The parametric test used One-way ANOVA to determine the difference in the mean number of bacteria between the treatment groups and continued with the significance test using the LSD Post-Hoc test. All of the analyses used SPSS version 21.

RESULTS

Based on the observation of blood agar media, there were differences in bacterial growth between the groups. The mean number of colonies growing in the negative control group was more than 100,000 CFU. While in the positive control group containing BHIB media, 0.05 ml of standardized bacterial suspension with 0.5 *McFarland* (1.5 x 108 CFU/ml) and 2% CHX, the number of bacteria that grew was 0 CFU/ml. This means that 2% CHX is still strong enough to kill 100% of bacterial growth relative to the number of bacteria. CHX 2% is bactericidal that has strong and broad anti-bacterial activity and a permanent increase in anti-bacterial activity and can inhibit the intracellular proteolytic activity of bacteria.[6,9] This condition is in accordance with previous research that 2% CHX can still be recommended for use as anti-bacterial. However, its effectiveness against mycobacteria is also limited.

Based on the MIC and MBC between the groups, we found the first group with 3.125% green meniran extract; the mean average bacterial growth was 10.00±0.82 CFU/ml or 94%. We can conclude the MIC green meniran extract against Enterococcus faecalis was 3.125%. While in the 100%, %0%, 25%, 12.5% and 6.25% meniran leaf, we didn't observe any bacterial growth; thus, the MBC for the first group was 6.25%. That minimum concentration of meniran extract can kill 99.9% of the bacteria. We suggest that the higher the green meniran extract

concentration, the higher the anti-bacterial power against Enterococcus faecalis.

In the second treatment group with a 0.78% green meniran extract and 2% CHX, we found the average bacterial growth was 10.50 CFU/ml compared to the negative control of 158.00±3.37 CFU/ml. We didn't find any bacteria concentration in the 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56% of green meniran leaf; we didn't find any bacteria concentration; thus, the MBC for the second group was 1.56% that can kill 99.9% bacteria. The 0.78 concentration can be stated as MBC but not as MIC against Enterococcus faecalis due to the absence of a comparison group. The results above prove that a combination of green meniran extract and 2% CHX both have very strong anti-bacterial power with no visible bacterial growth at concentrations of 100% to 1.56%. This combination is expected to overcome the pitfall of 2% CHX as a synthetic sterilizing agent, which is still ineffective in removing the Enterococcus faecalis bacteria's biofilm.[13,14]

We analyzed the average bacteria growth between groups, using the One Way ANOVA test followed by the Least Significant Difference (LSD). We found significant differences in bacteria growth between treatment groups ($p<0.05$). The average bacteria growth in the negative control group was 158.00±3.37 CFU/ml, in the 0.78% green meniran extract group was 57, 00±2.16 CFU/ml, in 1.56% green meniran extract group was 25.75±2.22 CFU/ml, in 3.125% meniran extract group was 10, 00±0.82 CFU/ml, and in 0.78% meniran extract combine with 2% CHX was 10.50±1.29 CFU/ml.

The results of the LSD test showed that the negative control group was significantly different from all treatment groups ($p<0.05$). The negative control group was significantly different from the 3.125% meniran extract group and the 0.78% meniran extract combined with the 2% CHX group ($p<0.05$). The 1.56% meniran extract group was also significantly different from the 3.125% meniran extract group and the 0.78% meniran extract combined with the 2% CHX group ($p<0.05$). The green meniran extract with concentrations of 3.125%, 1.56%, and 0.78%, and the combination of 0.78% green meniran extract with 2% CHX has quite effective anti-bacterial power.

The anti-bacterial power in the 0.78% green meniran extract was 64%, the 1.56% meniran green extract has 84% power, the 3.125% meniran extract group has 94% power, and the 0.78% green meniran extract and 2% CHX has 93% power compared to the control group.

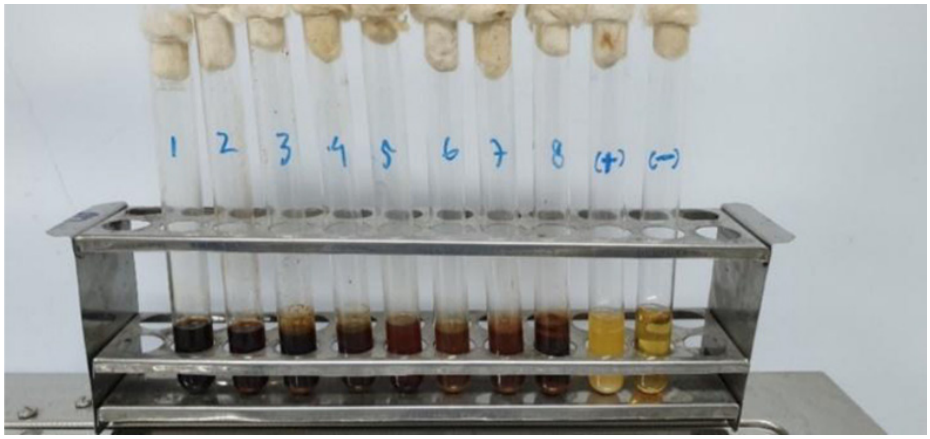

Figure 1. The serial dilutions of Meniran extract (*Phyllanthus niruri Linn*) against Enterococcus faecalis bacteria.

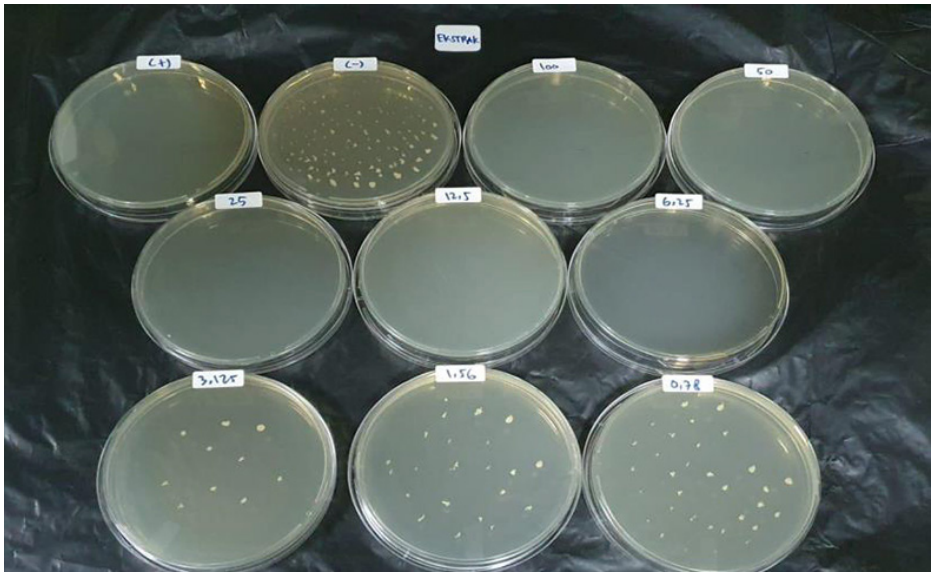

Figure 2. Enterococcus faecalis colony growth results on agar media at 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0.78% meniran extract concentrations, positive control, and negative control.

Table 1. The mean bacteria number between groups after treatment

Variable	Group	n	Mean±SB (CFU/ml)	p
Bacteria	Negative control	4	158.00±3.37	< 0.001
	0.78% meniran extract	4	57.00±2.16	
	1.56% meniran extract	4	25.75±2.22	
	3.125% meniran extract (MIC)	4	10.00±0.82	
	0.78% meniran extract and 2% CHX	4	10.50±1.29	

CHX= chlorhexidine digluconate; MIC=minimum inhibitory concentration

DISCUSSION

There were two treatment groups in this study, the first large group consist of meniran extract with various concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, and 0.78 %. The second large group consisted of meniran extract with various concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56%, 0, 78% and CHX 2%. In the first treatment group, meniran extract with 3.125%, 1.56% and 0.78% concentration still showed Enterococcus faecalis growth. We can conclude that a higher meniran extract concentration will increase the bioactive anti-bacterial ingredients and power. While in the second treatment group, Enterococcus faecalis growth was only seen in the group treated with 0.78% meniran extract combined with 2% CHX. From this result, we can conclude that the anti-bacterial activity of 2% CHX can compensate for the lack of bioactive ingredients in meniran extract compared to the positive control group.

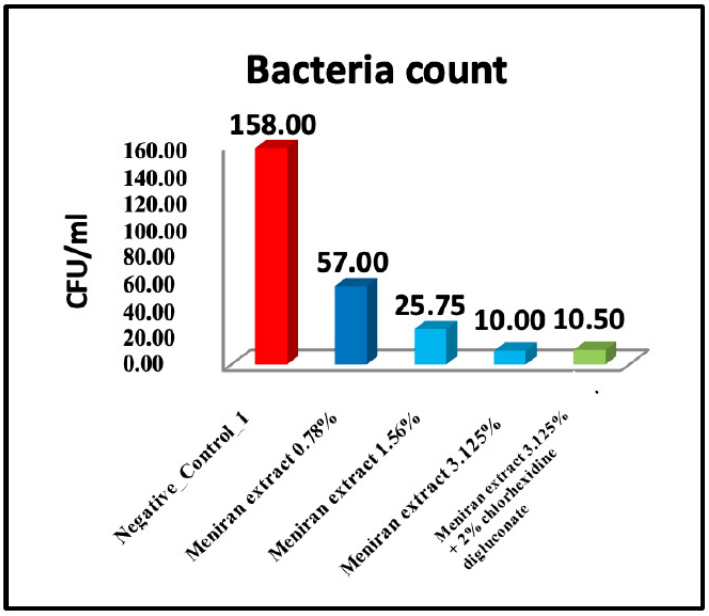

Figure 3. The average number of Enterococcus faecalis colony

Table 2. The descriptive analysis of bacterial count

Variable	Group	n	Mean	SB	Min	Max
Bacteria	Negative control	4	158.00	3.37	154	162
	0.78% meniran extract	4	57.00	2.16	55	60
	1.56% meniran extract	4	25.75	2.22	23	28
	3.125% Meniran extract (MIC)	4	10.00	0.82	9	11
	0.78% meniran and 2% CHX	4	10.50	1.29	9	12

CHX= chlorhexidine digluconate; MIC=minimum inhibitory concentration

Table 3. Post-Hoc Test between Treatment Groups

Group comparison	Mean difference	P
Negative control - 0.78% meniran extract	101.00	<0.001
Negative control - 1.56% meniran extract	132.25	<0.001
Negative control - 3.125% meniran extract (MIC)	148.00	<0.001
Negative control - 0.78% meniran extract and 2% CHX	147.50	<0.001
0.78% meniran extract - 1.56% meniran extract	31.25	<0.001
0.78% meniran extract - 3.125% meniran extract	47.00	<0.001
0.78% meniran extract - 0.78% meniran extract dan CHX 2%	46.50	<0.001
1.56% meniran extract - 3.125% meniran extract (MIC)	15.75	<0.001
1.56% meniran extract - 0.78% meniran extract and 2% CHX	15.25	<0.001
3.125% meniran extract (MIC) - 0.78% meniran extract and 2% CHX	-0.50	0.772

CHX= chlorhexidine digluconate; MIC=minimum inhibitory concentration

Several studies showed that meniran leaf has several bioactive compounds such as alkaloids, flavonoids, terpenoids, tannins and saponins, which have anti-bacterial activity.[12,15,16] Alkaloids have anti-bacterial activity by inhibiting esterase, DNA, RNA polymerase, and cellular respiration and play a role in DNA intercalation. Alkaloids work as anti-bacterial by destroying the peptidoglycan component in bacterial cells so that the wall layer is not formed completely and causes bacterial cell death. Meanwhile, as antifungal, biologically alkaloids cause damage to cell membranes. Alkaloids will bind strongly to ergosterol to form holes or channels, causing the cell membrane to leak and lose some intracellular materials such as electrolytes (especially potassium) and small molecules. This results in permanent damage to cells and cell death.[16-18]

A study from Tjandrawinata et al. stated that the flavonoid component in meniran leaf acts as an anti-bacterial.[14] The anti-bacterial mechanism of flavonoids occurred through their interaction with bacterial DNA and the formation of extracellular flavonoid-protein complexes through hydrogen bonds, increasing the permeability of cell membranes and inhibiting bacterial cell division. Flavonoids work by damaging the cell walls and cytoplasmic membranes of bacteria. Through this reaction, the protein content in the cell wall and cytoplasmic membrane of bacteria becomes unstable, thus inhibiting its biological activities, causing bacterial cell lysis and, finally, bacterial cell death.[13-15]

The saponin compound act as anti-bacterial by forming a foam that will disrupt the cell wall surface tension. This condition is because the content of saponins can interact with the bacterial cell wall so that the cell wall will cause lysis.[5] The other active component is tannin which also acts as anti-bacterial. Tannins will inactivate the activity of adhesins, enzymes, protein coagulants,

and glucosyltransferase activity which will damage bacterial cell membranes and precipitate bacterial proteins through the copolymers bound with the bacteria.[15,16] Tannins also work by shrinking the cell wall or cell membrane, thereby disrupting the permeability of the cell itself. Due to the disruption of permeability, cells cannot carry out living activities so that their growth is inhibited or even dies. Due to the mechanism of action of these tannins, it will result in the inactivation of enzymes and bacterial genetic material to cause bacterial cell death.[17,18]

Those four active compounds in meniran extract work synergistically in inhibiting and killing Enterococcus faecalis bacteria. It is necessary to consider the anti-bacterial power of green meniran extract (*Phyllanthus niruri Linn*) as an herbal ingredient that has potential as alternative medicine in root canals treatment.[14,17,19] Moreover, current root canal infection treatment is based on inadequate synthetic materials to eliminate biofilms from Enterococcus faecalis bacteria and still have many side effects.[20,21] Finally, this study stated that green meniran extract (*Phyllanthus niruri Linn*) at a certain MIC and MBC has a strong anti-bacterial effect against Enterococcus faecalis bacteria.

The limitation of this study is we haven't determined the secondary metabolite compounds of green meniran extract (*Phyllanthus niruri Linn*), which can inhibit and kill the biofilm of Enterococcus faecalis bacteria via in vitro and also the toxicity test and biocompatibility test of green meniran extract (*Phyllanthus niruri Linn*); thus, further research is needed.

CONCLUSION

From this study, we can conclude there were significant differences in bacterial growth, the MIC and MIB between green meniran leaf extract with 2% chlorhexidine digluconate and meniran extract only against the growth of Enterococcus faecalis**.** The combination of green meniran extract (*Phyllanthus niruri Linn*) and 2% CHX has better anti-bacterial power than the single-use of green meniran extract with a minimum bactericidal concentration of 1.56%.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this study.

ETHICS CONSIDERATION

This research was conducted based on the ethical conduct of research from the Ethics Committee of the Medical Faculty, Udayana University/Sanglah Hospital Denpasar No. 1531/UN14.2.2.VII.14/LT/2020.

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AUTHOR CONTRIBUTION

All authors contributed equally to this study.

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