Antibacterial effect of ethanol extract of *moringa oleifera* seeds against *enterococcus faecalis* atcc 29212

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ABSTRACT

Background: *One of the important stages in endodontic treatment is root canal irrigation. The most widely used irrigant is sodium hypochlorite (NaOCl), but it is toxic to periapical tissue. Irrigant solutions from natural ingredients that have antibacterial potential are needed, one of which is Moringa oleifera seeds. Bacteria that are often found persistent after root canal treatment are Enterococcus faecalis. The purpose of this study was to determine the antibacterial effect of the ethanol extract of M. oleifera seeds at concentrations of 37.5%, 50%, 67.5% and 75% against E. faecalis.*
Method: *Experimental laboratory research with post-test only control group design was carried out with 4 times replication. Antibacterial activity was tested against E. faecalis ATCC 29212 using paper disc diffusion method on Mueller Hinton Agar (MHA) media. The diameter of the inhibition zone formed was measured. Data were analysed by one-way ANOVA followed by LSD.*
Result: *The 2.5% NaOCl group produced the largest inhibition zone of 16.38 ± 0.95 mm, followed by the 75% extract group of 13.51 ± 0.49 mm, and the smallest was the 37.5% extract group of 4.42 ± 0.27 mm. The negative control (DMSO) did not produce an inhibition zone. The increase in the concentration of the ethanolic extract of M. oleifera seeds resulted in significantly better inhibiting the growth of E. faecalis ($p<0.05$).*
Conclusion: *Ethanol extract of M. oleifera seeds at 37.5%, 50%, 62.5% and 75% had antibacterial effects against E. faecalis, with the 75% had the strongest antibacterial effect compared to other extract concentrations.*

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INTRODUCTION

Root canal treatment must be performed following the principles of the endodontic triad, comprises of biomechanical preparation, sterilization which includes irrigation and disinfection, and root canal filling[1]. Endodontic treatment failure is generally due to difficulty of mechanical preparation of complex root canals, resulting in the removal of infected tissue and inadequate elimination of bacteria[2]. Root canal irrigation is vital for cleaning and disinfecting areas inaccessible to instrumentation.[3-4]

Stuart et al. (2006) stated that in 24-77% of failed root canal treatments, *Enterococcus faecalis* was found[5]. *Enterococcus faecalis* is a Gram-positive bacteria that can cause primary and persistent infection[6]. The persistence of *E. faecalis* in root canals is due, among other things, to its ability to survive long enough in conditions of minimal nutrient intake and resistance to medication during root canal treatment.[5]

The endodontic irrigant known to be the most effective for eliminating *E. faecalis* is sodium hypochlorite (NaOCl). Chlorine in NaOCl solution can interfere with bacterial cell metabolism by inhibiting enzymes, impairing DNA synthesis, and hydrolyzing amino acids.[5-7] Concentration of 2.5% NaOCl solution is the most widely used and recommended irrigant, but it is toxic to periapical tissue[(8)]. This solution also has a taste and odor that can cause discomfort in children.

Currently, the use of drugs is starting to lead to natural extracts from plants to reduce the toxic effects of chemicals. Natural extracts from plants are expected to provide optimal antibacterial effects and minimal side effects.[9] *Moringa oleifera* is a plant with antibacterial properties in several parts, including leaves, roots, and seeds.[10] *M. oleifera* leaf extract has been shown to have antibacterial activity against *E. faecalis* at concentrations of 75% and 100%.[11] Another study by Kafi et al. (2014) showed that the ethanolic extract of *M. oleifera* seeds had a better antibacterial effect than the leaf extract against all Gram-positive bacteria tested, including *Staphylococcus aureus, Staphylococcus epidermidis, Diphtheroids, Streptococcus faecalis, Streptococcus pyogenes, Listeria monocytogenes*, and *Streptomyces somaliensis*. The results showed that the ethanol extract of *M. oleifera* seeds had the broadest antibacterial spectrum against the test bacteria.[12] The antibacterial activity of *M. oleifera* seed extract has also been investigated by Wigunarti et al. (2019), which showed that at the highest concentration of 75% was effective in inhibiting the growth of *S. aureus* and *Escherichia coli.*[13]

The antibacterial activity of *M. oleifera* seeds is known to come from the active substance 4-(α-L-rhamnosyloxyl) benzyl isothiocyanate and other important phytochemical compounds such as saponins, tannins, alkaloids, and flavonoids contained in large amount.[14] The active substance 4-(α-L-rhamnosyloxyl) benzyl isothiocyanate inhibits bacterial growth by interfering with the enzyme synthesis mechanism and cell membranes.[10] This study aimed to determine the antibacterial effect of the ethanol extract of *M. oleifera* seeds at concentrations of 37.5%, 50%, 67.5%, and 75% against *E. faecalis*.

RESEARCH METHOD

This research is an experimental laboratory using a post-test-only control group design. The research object used was *Enterococcus faecalis* ATCC 29212. There were 6 treatment groups; ethanol extract of *Moringa oleifera* seeds 37.5%, 50%, 67.5%, 75%, sodium hypochlorite (NaOCl) 2.5% as positive control and dimethyl sulfoxide (DMSO) 10% as negative control. Each treatment

group was replicated four times. This research has obtained ethical clearance No. 00608/KKEP/FKG/UGM/EC/2021 from the Ethics Commission of the Faculty of Dentistry, Universitas Gadjah Mada.

The materials used in this study included ethanol extract of *M. oleifera* seeds 37.5%, 50%, 67.5%, 75%, NaOCl 2.5%, DMSO 10%, *Enterococcus faecalis* ATCC 29212, Muller Hinton Agar (MHA) medium, sterile distilled water, Brain Heart Infusion (BHI) media, and paper disk. The tools used include an analytical balance, autoclave, incubator, test tube, petri dish, sterile cotton swab, micropipette, caliper (millimeter scale), Erlenmeyer flask, vortex, filter paper, laminar flow, and rotary evaporators.

The ethanol extract of *M. oleifera* seeds was made by maceration[13]. Plant identification was carried out at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada, with No. 070/S.Tb/IV/2022. Peeled fresh seeds are dried at a temperature of 25-30^{o}C, then mashed using a blender and sieved to become a smooth simplicia. A total of 250 grams of simplicia was put into an Erlenmeyer, then poured with a liquid filter (96% ethanol) until submerged, then closed and left for 24 hours protected from light while stirring[13]. Maceration was carried out until the filtrate was obtained, then evaporated using a rotary evaporator until a thick extract with a concentration of 100% was obtained. The weight of the thick extract obtained was 30.3 grams. The extract was then suspended in DMSO to obtain concentrations of 37.5%, 50%, 62.5%, and 75%.

E. faecalis bacteria were obtained from the culture in the form of blood agar, taken two oses, and then incubated in liquid BHI media for 2x24 hours at 37°C. *E. faecalis* culture results were taken with a needle and put into 10 ml of sterile saline to obtain a suspension of *E. faecalis*. The density of the suspension of *E. faecalis* bacteria was standardized with the McFarland standard of 0.5 to obtain 10^{8} CFU/ml. The standardized bacterial suspension was applied to the MHA medium using a sterile cotton swab.

Antibacterial activity was tested using the paper disk diffusion method on MHA media. Paper disk was immersed for 15 minutes into the stock solution of ethanol extract of *M. oleifera* seeds with concentrations of 37.5%, 50%, 67.5%, and 75%, 2.5% NaOCl as a positive control, and 10% DMSO as a negative control. The paper disk was then carefully placed on the MHA surface on a laminar airflow. The prepared media was incubated for 48 hours at 37°C, and the inhibition zone formed around the paper disc was measured using a caliper.

RESULT

The antibacterial activity test of the ethanolic extract of *M. oleifera* against *E. faecalis* was carried out at concentrations of 37.5%, 50.%, 62.5%, and 75%. The test material inhibits bacteria growth if a clear area is formed around the paper disk. The mean and standard deviation between the treatment groups are presented in Table 1.

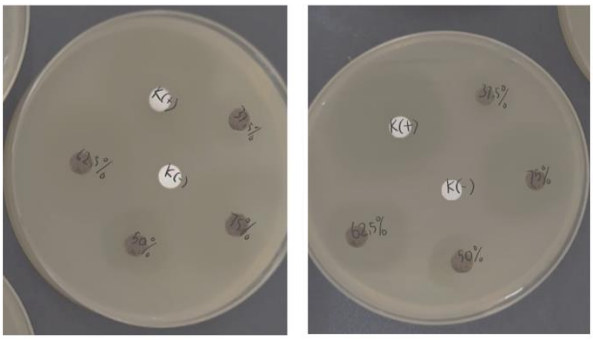

Figure 1. Inhibition zone formed from each treatment groups against *Enterococcus Faecalis.*

Table 1. Mean and standard deviations of inhibition zone diameter between treatment groups

Treatment Groups	Inhibition Zone (mm)	
	n	Mean ± Standard deviations
Negative control (DMSO 10%)	4	0
Ethanol extract of *M. oleifera* seeds 37,5%	4	4,42 ± 0,27
Ethanol extract of *M. oleifera* seeds 50%	4	7,35 ± 0,21
Ethanol extract of *M. oleifera* seeds 62,5%	4	10,44 ± 0,29
Ethanol extract of *M. oleifera* seeds 75%	4	13,51 ± 0,49
Positive Control (NaOCl 2,5%)	4	16,38 ± 0,95

Table 1 shows that the positive control and each concentration of the ethanol extract of *M. oleifera* seeds showed antibacterial activity against *E. faecalis* by forming a clear zone. The four extract concentrations tested in this study showed that the higher the concentration of the ethanol extract of *M. oleifera* seeds, the larger the diameter of the inhibition zone formed. The 75% concentration of *M. oleifera* seeds extract formed an average inhibition zone diameter of 13.51 ± 0.49 mm, followed by concentration of 62.5%, 50%, and the lowest 37.5% was 4.42 ± 0.27 mm. The positive control group had stronger antibacterial activity against *E. faecalis* compared to the ethanol extract of *M. oleifera* seeds. The diameter of the inhibition zone formed in the positive control group was 16.38 ± 0.95 mm. The negative control group did not produce an inhibition zone.

The one-way Anova was tested to compare the inhibition zone between groups with the results shown in Table 2. The test results obtained the calculated F value of 646.59 with $p < 0.001$. The p-value <0.05 indicates a significant difference in the average inhibition zone formed between the six treatment groups. It shows that the different concentrations of the ethanol extract of *M. oleifera* seeds affected inhibiting the growth of *E. faecalis* ATCC 29212. The LSD test was continued to compare the differences between groups (Table 3).

Table 2. One way Anova test of inhibition zone diameter mean differences

Treatment Groups	Mean	F	P
Negative control (DMSO 10%)	0	646.59	.000
Ethanol extract of *M. oleifera* seeds 37,5%	4,42 ± 0,27		
Ethanol extract of *M. oleifera* seeds 50%	7,35 ± 0,21		
Ethanol extract of *M. oleifera* seeds 62,5%	10,44 ± 0,29		
Ethanol extract of *M. oleifera* seeds 75%	13,51 ± 0,49		
Positive Control (NaOCl 2,5%)	16,38 ± 0,95		

Table 3. LSD Test between treatment groups

Treatment Groups	K (-)	P1	P2	P3	P4	K (+)
Negative control (DMSO 10%) **(K-)**	-					
Ethanol extract of *M. oleifera* seeds 37,5% **(P1)**	0.000*	-				
Ethanol extract of *M. oleifera* seeds 50% **(P2)**	0.000*	0.000*	-			
Ethanol extract of *M. oleifera* seeds 62,5% **(P3)**	0.000*	0.000*	0.000*	-		
Ethanol extract of *M. oleifera* seeds 75% **(P4)**	0.000*	0.000*	0.000*	0.000*	-	
Positive Control (NaOCl 2,5%) **(K+)**	0.000*	0.000*	0.000*	0.000*	0.000*	-

(*) = significant (p<0,05)

The results of the LSD test showed that there was a significant difference in the average inhibition zone formed between the negative control group and the other treatment groups ($p<0.05$). It means that the ethanolic extract of *M. oleifera* seeds at concentrations of 37.5%, 50%, 62.5%, 75%, and 2.5% NaOCl had an effect on inhibiting the growth of *E. faecalis* ATCC 29212. Compared to positive control, *M. oleifera* seeds ethanol concentrations of 37.5%, 50%, 62.5%, and 75% showed a significant difference in antibacterial effect against *E. faecalis* ($p<0.05$). It means that 2.5% NaOCl produces stronger antibacterial properties than the ethanol extract of *M. oleifera* seeds. Significant differences were also seen between the extract concentration groups. Ethanol extract of *M. oleifera* seeds with a concentration of 75% produced a stronger antibacterial effect in inhibiting the growth of *E. faecalis* ATCC 29212 compared to concentrations of 37.5%, 50%, and 62.5 ($p<0.05$).

DISCUSSION

Moringa oleifera is known to have extensive medicinal properties from almost all parts of the plant. The seeds of *M. oleifera* are known to contain several phytochemical compounds with antimicrobial properties, such as saponins, flavonoids, tannins, and alkaloids. They contain active antimicrobial substances in the form of polypeptide 4 (α-L-rhamnosyloxy) benzyl isothiocyanate[15]. This study was conducted to determine the antibacterial activity of the ethanolic extract of *M. oleifera* seeds against *Enterococcus faecalis* ATCC 29212 bacteria. *E. faecalis* is a bacterium that can cause primary infection and is resistant to medication during endodontic treatment, thus inhibiting healing of the apical area, and is commonly found in root canals post-endodontic treatment failure[6].

The antibacterial activity test of the ethanolic extract of *M. oleifera* against *E. faecalis* was carried out at several concentrations of 37.5%, 50.%, 62.5%, and 75%. The positive control used in this study was 2.5% NaOCl. Research by Walton (2002) proved that 2.5% and 5.25% NaOCl had the same antibacterial activity against *E. faecalis*, where lower concentrations had lower tissue toxicity[16]. The negative control used was 10% dimethyl sulfoxide (DMSO) to determine whether the solvent affected the growth of *E. faecalis* ATCC 29212. In this study, the negative control group did not form an inhibition zone. It means that 10% DMSO can dissolve the extract well without affecting the growth of the test bacteria.

The results of this study showed that the positive control group had the strongest inhibition among all groups, followed by the ethanolic extract of *M. oleifera* seeds at concentrations of 75%, 62.5%, 50%, and 37.5% against bacteria *E. faecalis* ATCC 29212. NaOCl, a high-level disinfectant, contains a strong oxidant in the form of chlorine which is very stable in liquid form and has a strong antibacterial effect[17]. The antibacterial mechanism of chlorine inhibits bacterial enzymes, damages DNA synthesis, hydrolyzes amino acids, and disrupts cell metabolism[18].

The ethanol extract of *M. oleifera* seeds with a concentration of 75% had a significantly better ability to inhibit the growth of *E. faecalis* ATCC 29212 bacteria compared to concentrations of 62.5%, 50%, and 37.5%. Based on the diameter of the inhibition zone formed, the antimicrobial activity was grouped into four categories according to Davis and Stout[19] (Table 4).

Table 4. Classification of inhibition zone diameter

Clear Zone Diameter	Inhibitory Activity
≤ 5 mm	Weak
5-10 mm	Medium
11-20 mm	Strong
≥ 21 mm	Very Strong

The ethanol extract of *M. oleifera* seeds 37.5% was classified as weak with inhibition zone diameter of 4.42 mm, concentrations of 50% and 62.5% were classified as medium inhibitory with diameter of 7.35 mm and 10.44 mm respectively. Subsequently, the concentration of 75% with inhibition zone of 13.51 mm was classified as strong inhibition but still below 2.5% NaOCl.

The higher the concentration the higher the inhibitory activity produced by the antibacterial agent. It could be due to the greater number of active antibacterial compounds released by the extract, thus facilitating the penetration of these compounds into cells[20]. The results of this study are in line with the research of Wigunarti et al. (2019), which showed that the ethanolic extract of *M. oleifera* seeds most effectively inhibited the growth of the *Staphylococcus aureus* and *Escherichia coli* at a concentration of 75%, followed by concentrations of 50% and 25%[13]. Research by Firdaus et al (2022) and Jangnga et al (2018) also stated that the increase in the inhibition zone occurred along with the increase in concentration of the tested antibacterial agent on the growth of *E. faecalis*[21,22]

The antimicrobial activity of *M. oleifera* seeds refers to its chemical compound 4 (α-L-rhamnosyloxy) benzyl isothiocyanate[23-24]. These polypeptides easily penetrate and interfere with the synthesis of bacterial cell membranes or the synthesis of enzymes that play a role in bacterial growth[25-26]. Wen et al (2022) proved that the active substance 4 (α-L-rhamnosyloxy) benzyl isothiocyanate could inhibit the growth of the test bacteria by inducing cell membrane damage, decreasing cell membrane integrity, increasing cell membrane permeability, interfering with energy metabolism and DNA replication causing the cell to death. Increased concentration of 4 (α-L-rhamnosyloxy) benzyl isothiocyanate resulted in increased bacterial cell membrane damage[27]. The substance of 4 (α-L-rhamnosyloxy) benzyl isothiocyanate in *M. oleifera* seeds has also been known to have antibacterial activity against several other Gram-positive bacteria, including; *Staphylococcus epidermidis, Bacillus subtilis, Streptococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*[26-28].

The solvent used in the extraction process also influences the antimicrobial effect. The ethanol solvent is very effective in obtaining antimicrobial phytochemical content in *M. oleifera* seeds, such as saponins, flavonoids, tannins, and alkaloids, because they have polar similarities[29]. Research by Chhetri et al. (2008) showed that the ethanol extract of *M. oleifera* seeds had a higher inhibitory ability against bacteria when compared to the solvent n-hexane[29].

An acute toxicity test of the ethanolic extract of *M. oleifera* seeds in mice showed that the lethal dose (LD50) was 282.84 mg/kg BW intraperitoneally[30]. Meanwhile, another study showed that the LD50 of *M. oleifera* seed extract given orally was high at 3873 mg/kg BW[31]. The lower LD50 on intraperitoneal administration could be due to the extract not going through a metabolic process as in oral administration. It shows that below that dose, *M. oleifera* seed extract does not cause harmful effects when given orally. It is still safe for consumption and treatment[31].

Further research is needed to obtain an *M. oleifera* seed extract solution with antibacterial potency equivalent to or better than 2.5% NaOCl. In addition, the irrigation solution must meet the

requirements, including having a low surface tension and a high flow rate so that the solution can flow easily and penetrate into the dentinal tubules[32]. Toxicity tests on periapical tissue also need to be carried out to consider its use as an alternative to root canal irrigation.

CONCLUSION

It was concluded that the ethanol extract of *Moringa oleifera* seeds with concentrations of 37.5%, 50%, 62.5%, and 75% had antibacterial activity against *E. faecalis* ATCC 29212. Ethanol extract of *M. oleifera* seeds 75% had better antibacterial effect than 62.5%, 50%, and 37.5% in inhibiting the growth of *E. faecalis* ATCC 29212.

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