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Antibacterial and Smear Layer Removal Efficacy of Moringa (Moringa oleifera): In vitro Study

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Abstract:	Objective: The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion: These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics
Response to Reviewers:	

Response to Reviewers

Dear Editor,

We appreciate you and the reviewers for your precious time in reviewing our paper and providing valuable comments. It was your valuable and insightful comments that led to possible improvements in the current version. The authors have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your high standards. The authors welcome further constructive comments if any.

Below we provide the point-by-point responses. All modifications in the manuscript have been listed.

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Response to Reviewer 1

The authors have written an intriguing study on moringa's antibacterial and smear layer removal efficacy.

Response : Thank you very much

It was exciting to read a simple but clear introduction, objective, and methods of the study. However, some areas require improvement and clarification, which I have listed below:

Abstract:

Line 5: Omit the plant name "moringa": as you have already put the scientific name

Response : We revised the moringa with scientific name of *Moringa oleifera* in the first appear and follow by *M. oleifera*

Line 7: Please write NaOCL and EDTA in full as this is the first time they appear in the text.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 1, line 5-6]

"The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) as well as antimicrobial activities".

Methodology

How many reviewers evaluate the smear layer score, and how do you ensure the raters' reliability?

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 5, line 23-24]

"The smear layer was independently graded by two operators".

To ensure the raters reliability, all the operators are trained assistants

Line 32, page 5: write "Tab" in full i.e table. Make sure to do the necessary changes throughout the entire manuscript.

Response : Thanks for your kind reminders. We revised the all the sentence "tab" to Table and also Fig to Figure

Line 27, page 6: Please include where you obtained the bacteria E. faecalis and S.mutans - from either ATCC or wild type.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 4, line 25]

"The antimicrobial activity of *M. oleifera* leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175".

Line 41, page 6: "single-rooted teeth" - please add some explanation of the teeth - human or animal and what type of teeth are used?

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 5, line 6-7]

"In this investigation, thirty removed human single-rooted teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal".

Line 5, page 7: smear layer removal - should it be smear layer removal ability?

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 5, line 16]

"Smear layer removal ability".

Results:

Do provide numerical data whenever possible, so that it can be easily read without having to refer back to the table.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 6, line 17-22; pg 6, line 25 to pg 7 line 1-2]

"At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) or nearly similar on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm)".

"Regarding the smear layer score, it was observed that *M. oleifera* 2.5% and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both *M. oleifera* 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0%".

Lines 54-58, page 7: Please avoid concluding the results section.

Response : Thanks for your kind reminders. We deleted the sentence.

Lines 34-41, page 9: There is no need to repeat the of doing a Shapiro Wilk test here as you have already described this non-parametric test in your methodology.

Response : Thanks for your kind reminders. We deleted the sentence.

Line 1-3, page 9: Please reserve your comments on the results for the discussion section.

Response : Thanks for your kind reminders. We moved the sentence to discussion section as follows [pg 8, line 18-19]

"This outcome suggests that *M. oleifera* leaves decoction promise an alternative irrigant".

Discussion

* It's really difficult to follow the discussion in the second paragraph. Please reword the entire text. Although the first statement was about a smear layer, the next one was about flavonoids in an acid solution. This is followed by a discussion of saponin. If you're referring to the saponins found in moringa leaves, please be more specific.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 8, line 1-7]

"The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[23] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[24] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[25,26]"

* Please be sure to provide references to back the statements that you have written in this discussion section.

Response : Thanks for your kind reminders. We added references to all statement.

Conclusion

Please make recommendations for future research as well as the study's limitations.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 9, line 4-9]

"Within the limitations of this study, alternating the use of *M. oleifera* leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of *M. oleifera* leaves decoction as an alternative to irrigant solution. Nevertheless, further long-term clinical studies are necessary to confirm these results and evaluate their relevance to treatment outcome".

Tables and figures

The micrographs in Figure 2 are noticeably blurry. Do take into account including more precise and clear images. The labels (a) dentinal tubules without smear layer and (b) smear layer on the surface of dentinal tubules were specified by the authors, however, neither can be seen in the image.

Response : Thanks for your kind reminders. We changed with a good new picture resolution. [pg 16]

Response to Reviewer 2

Reviewer #2: abstract: minor correction in Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 1, line 12-16]

"The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens".

page 5 line 51: how long this solution was stored and used. usually water extracts have limited shelf life as compared to alcoholic extracts.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 4, line 9-10]

"The decoction was prepared immediately before experiment".

Page 8 line 2-20: antimicrobial activity results need to be re-written as they are not clear.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 6, line 15-22]

"Based on the mean value zone of inhibition, the *M. oleifera* leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Figure 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

".

Page 8 & 9 : results are not very clear. language used does not convey the results properly.

Response : Thanks for your kind reminders. We revised the sentence.

Page 9 line 10 : phytochemical compounds were applied or were present in the decoction.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 7, line 10-11]

"Phytochemical screening compounds like tannins, flavonoids, and saponins were present on *M. oleifera* leaves decoction".

Page 10 line 10-17: authors are saying NaOCL does not have surfactant activity that means absence of saponins. immediately in next sentence authors aer saying NaOCL shows saponization reaction. these findings are contradictory.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 8, line 8-10]

"In contrast to *M. oleifera*, NaOCl does not contain surfactant directly. However, dissolution of organic tissue can be verified in the saponification reaction when sodium hypochlorite degrades fatty acids and lipids resulting in soap and glycerol".

Page 10 line 27-31: mechanism of NaOCL is explained and at the end it is claimed moringa is better. but how it is better it is not mentioned. mechanism of action of both agents are not very clear.

Response : Thanks for your kind reminders. We claimed *M. oleifera* is better than NaOCL to remove the smear layer dentinal tubules. It can be seen from the photos of dentin cleanliness but the mechanism of *M. oleifera* not investigated. We only list the general mechanism of saponins and flavonoids as smear layer removal.

"*M. oleifera* leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%" [pg 9, line 5-6].

"Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[24] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds". [pg 7, line 23 to pg 8, line 1-7]

What are the limitations of the study?

conclusion should start as "Within the limitations of the current study....."

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 9, line 4-9]

"Within the limitations of this study, alternating the use of *M. oleifera* leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of *M. oleifera* leaves decoction as an alternative to irrigant solution. Nevertheless, further long-term clinical studies are necessary to confirm these results and evaluate their relevance to treatment outcome.".

Response to Reviewer 3

Reviewer #3: incomplete background and discussion
Response : Thanks for your kind reminders. We revised the background and discussion.

Response to Reviewer 4

Reviewer #4: TITLE OF MANUSCRIPT:
Title is not clear and complete, please modify the title, and also mention type of study.
Response : Thanks for your kind reminders. We revised the sentence as follows
"Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study"

INTRODUCTION:
Please add refrences where required. Most of the time, you wrote about ethanolic and aqueous extract of moringa, but you should also describe about the moringa decoction, Also add some refrences about it.
Response : Thanks for your kind reminders. We added references to all sentences. A decoction is a water-based extraction where the plant is placed in boiling water and simmered for a decent amount of time. In this case decoction resulted water extract.

MATERIALS AND METHODS:
Please mention study setting, study duration and sample size formula, which is not mentioned in manuscript. you didn't mention the source of plant material and its processing before making decoction. I also recommed to re-write the procedure of making moringa decoction. however, there are number of grammatical errors; many of them have been mentioned in the main file. You are advised to correct all those mistakes and have a good proof reading.
Response : Thanks for your kind reminders. We revised the study design as follows [pg 3, line 22-24]
Study design
"This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020".

This study used human teeth that had been extracted at the hospital and then selected based on inclusion and exclusion criteria, so the sample calculation formula was not used.

Source of plant material and its processing before making decoction revised the study design as follows [pg 4, line 3-5]

"The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The leaves were harvested by hand, washed under running tap water and drained".

To improve the readability, proofreading has been carried out on papertrue.com

STATISTICS:

Although the statistical significance evaluation of data is obtained and discussed, there is need to rephrase and explain it a more descriptive way. The statistical tests applied are not so much clear.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 6, line 1-7]

"The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Comparing the smear layer removal efficacy between the five different groups was done by Kruskal–Wallis analysis followed by Mann–Whitney U test for individual comparisons. Value of $p < 0.05$ was considered statistically significant".

RESULTS:

The description in table 2 is not clear, please rephrase table 2. It contains some grammatical and syntax errors. please elaborate Table 3 in more in categorical way. Please use correct terminologies, some of them have been mentioned in the main file.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 17]

DISCUSSION:

Discussion is reasonable. Mostly you discussed and compare the beneficial effects of ethanolic extract of moringa with moringa decoction. Please mention, how both preparations have same effects. By incorporating studies of moringa decoction will improve the discussion.

Response : Thanks for your kind reminders. We revised the sentence and compared to the same procedure of extraction method using water.

REFERENCES:

please try to use references from latest articles, not more than 10 years.

Response : Thanks for your kind reminders. We have made revisions accordingly.

GENERAL COMMENTS:

The study is well planned. Required some correction as suggested and resubmission.

Response : Thanks for your kind reminders.

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

TITTLE PAGE

Type of article: Original
Title of the article: Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

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Thanking you,
Yours' sincerely,

Signature

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January 19, 2023

Professor Abdulmohsen Hamdan Al-Zalabani, MD, ABCM, MHPE
Editor in Chief
Journal of Taibah University Medical Sciences

Dear Professor
Here with we enclosed the submission to the Journal of Taibah University Medical Sciences for publishing. The manuscript entitled "Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study" by Nurhayaty Natsir, Yonathan, Juni Jekti Nugroho, Aries Chandra Trilaksana, Christine Anastasia Rovan, Maria Tanumihardja, and Lukman M.

Endodontic treatment aims at eliminating microorganisms from the infected root canal system by mechanical and chemical methods. Mechanical preparation of the canals leads to the formation of a smear layer. The objective of this study was to compare antibacterial and smear layer removal efficacy of moringa. The leaves of moringa were extracted using hot water extraction. The result suggested that moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. In addition, 5.0% of decoction shows higher antimicrobial activity against both *Enterococcus faecalis* and *Streptococcus mutans*.

Journal of Taibah University Medical Sciences would be an outstanding forum for this paper due to its intention of featuring interdisciplinary research publications; we believe that this paper will be of interest to botanist, dentistry, and pharmacist due to the first report of smear layer removal efficacy of moringa.

All authors have read and approved the final manuscript. We hereby transfer, assign, or otherwise convey all copyright ownership, including any and all rights incidental thereto, exclusively to the journal, in the event that such work is published by the journal.

On behalf of all the contributors I will act and guarantor and will correspond with the journal from this point onward.

Corresponding Author:
Nurhayaty Natsir

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) as well as antimicrobial activities. **Methods**: The *M. oleifera* leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that *M. oleifera* leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. A change in the dentine 'substrate's properties, and hence the interaction of dentine with root filling materials, is one of the effects of root canal irrigation.[2]

The most regularly used irrigating agents are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine.[3] Although NaOCl is the most effective irrigating solution due to its ability to dissolve organic content, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue.[5] The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[6] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial. [4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the eradication of microbes from the root canal system as well as to remove the smear layer.[7]

Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common *M. oleifera* species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[8] *M. oleifera* leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[9]

Methanolic extracts for *M. oleifera* had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity to MDCK epithelial cell.[10] Aqueous extracts of *M. oleifera* leaves showed antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Salmonella typhi.*[11] Ethanol extract of *M. oleifera* leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection.[12] According to Nugroho *et al.* (2021), ethanol extract of *M. oleifera* 5.0% promises an alternative to root canal irrigant.[13]

The presence of isothiocyanates with their glucosinolate precursors is thought to have an antimicrobial effect. Isothiocyanate activity is mainly linked to the reactivity with sulfhydryl groups, and the antimicrobial effect is dose-dependent.[14] The main advantage of *M. oleifera* is a broad safety margin for human and animal consumption.[15]

In the present study, we evaluated the antimicrobial activity of *M. oleifera* leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020.

Materials

The irrigation solutions used in this study can be seen in Table 1.

Preparation of plant decoction

The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The leaves were harvested by hand, washed under running tap water and drained. Decoction of *M. oleifera* 2.5% was made by weighing about 2.5 g of *M. oleifera* dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for *M. oleifera* 5.0%. The decoction was prepared in triplicate. The decoction was prepared immediately before experiment.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[16]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[16]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[16]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[16]

Antimicrobial activity

The antimicrobial activity of *M. oleifera* leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175. Pathogenic bacteria

included *E. faecalis* and *S. mutans*. The fresh bacterial suspension was dispersed on the surface of Muller Hinton agar plates. One hundred microliters of each decoction were incorporated into the wells, and the plate was incubated at 37 °C for 24 h. The NaOCl 2.5% was used as the positive control. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, thirty removed human single-rooted teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal. Root caries, fractures, curve canals, endodontic therapy, internal resorption, and calcification were all ruled out. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The teeth were standardised at 16 mm in length. A safe-sided diamond disk fitted in a low-speed handpiece with a water coolant was used to decorate the teeth. By subtracting 1 mm from the measurement recorded, the working length was determined. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal ability

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1, distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, *M. oleifera* 2.5%; and Group 5, *M. oleifera* 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Chhabra *et al.* (2016) (Table 2),[17] and the results were tabulated. The smear layer was independently graded by two operators.

STATISTICAL ANALYSIS

The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Comparing the smear layer removal efficacy between the five different groups was done by Kruskal–Wallis analysis followed by Mann–Whitney U test for individual comparisons. Value of $p < 0.05$ was considered statistically significant.

RESULT

Phytochemical screening

The phytochemical analysis conducted on *M. oleifera* leaves decoction revealed the presence of tannins as well as flavonoids and saponins (Table 3). These phytochemical components support bioactive activities in medicinal plants and are responsible for the antimicrobial activity of the plant extract studied.

Antimicrobial activity

Based on the mean value zone of inhibition, the *M. oleifera* leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Figure 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Figure 2). Regarding the smear layer score, it was observed that *M. oleifera* 2.5%

and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both *M. oleifera* 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tabel 4).

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of *M. oleifera* decoction between different groups at each level of the smear layer (Table 5). In *M. oleifera* 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both *M. oleifera* (2.5% and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%.

DISCUSSION

Phytochemical screening compounds like tannins, flavonoids, and saponins were present on *M. oleifera* leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[18,19] Chhikara *et al.* (2020), Enerijiofi *et al.* (2021), and Trigo *et al*. (2021) have summarised the several bioactive compounds isolated and identified from *M. oleifera* leaves. However, they also reported tannin, saponin, and flavonoid.[20-22] Specifically, 2-octenoic acid and 1, 2-epoxyhexadecane identified from the leaves water extract showed antimicrobial activities.[20] Thus, *M. oleifera* leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the *M. oleifera* leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[23] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[24] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic

and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[25,26]

In contrast to *M. oleifera*, NaOCl does not contain surfactant directly. However, dissolution of organic tissue can be verified in the saponification reaction when sodium hypochlorite degrades fatty acids and lipids resulting in soap and glycerol.[27] In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[28,29] The findings in this study corroborate with earlier reports from Khallaf *et al.* (202), where they reported leaves extracts of *M. oleifera* showed the least amount of smear layer on canal wall.[30] The result corroborates the use of the plant extracts traditionally as a smear layer removal agent.[31] This outcome suggests that *M. oleifera* leaves decoction promise an alternative irrigant.

Both *M. oleifera* leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[32,33] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal

walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[34,35]

CONCLUSION

Within the limitations of this study, alternating the use of *M. oleifera* leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of *M. oleifera* leaves decoction as an alternative to irrigant solution. Nevertheless, further long-term clinical studies are necessary to confirm these results and evaluate their relevance to treatment outcome.

Source of funding

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM carried out the research and collected the data. NN and MM designed and supervised the study, visualized and validated the data, and reviewed draft material. The data were organized, analyzed, and interpreted by CAR, who also reviewed the article. NN and LM organized, analyzed, and interpreted the data and revised the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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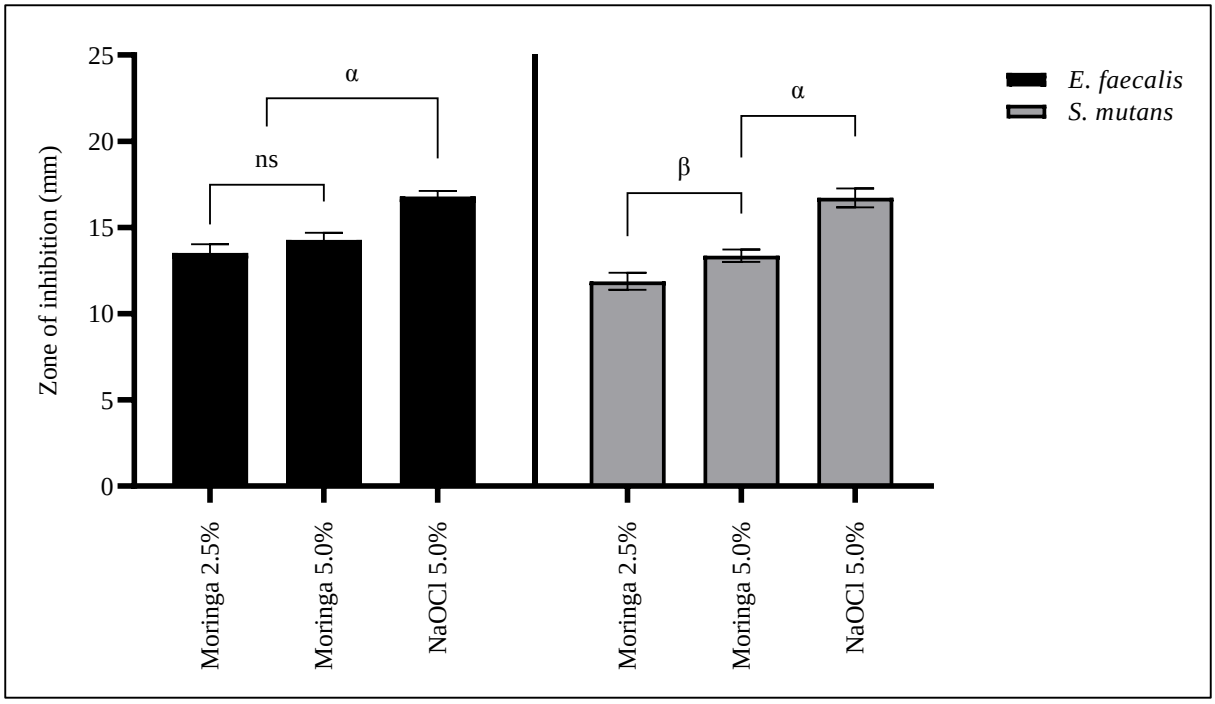

Figure 1 Antimicrobial activity of *M. oleifera* leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to *M. oleifera* 2.5%; $^{(ns)}$ non-significant.

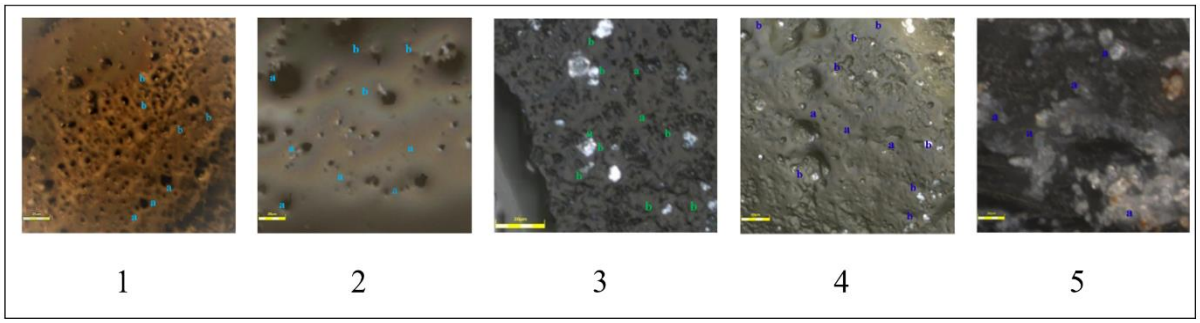

Figure 2 Representative CLSM micrographs (x) in each group: (1) *M. oleifera* 2.5%; (2) *M. oleifera* 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1 Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2 Smear layer evaluation criteria.[17]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer were open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	Complete root canal wall covered by a homogeneous smear layer and no open dentinal tubules
5	Heavy homogeneous smear layer covering the complete root canal wall

Table 3 Phytochemical analysis for *M. oleifera* based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	++

Note: Absent= —, Trace = +, highly present = ++

Table 4 Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
M. oleifera 2.5%	6	1.83	0.41	0.000*	0.001*
M. oleifera 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: ∗ Statistically significant result ($p < 0.05$)

Table 5 Mann-Whitney test value to evaluate the difference between groups.

Group	*M. oleifera* 2.5%	*M. oleifera* 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
M. oleifera 2.5%					
M. oleifera 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: ∗ Statistically significant result ($p < 0.05$)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal of Taibah University Medical Sciences

Antibacterial and Smear Layer Removal Efficacy of Moringa (Moringa oleifera): In vitro Study

--Manuscript Draft--

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Abstract:	Objective: The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion: These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics
Response to Reviewers:	

Response to Reviewers

Dear Editor,

Thank you for giving us the opportunity to submit a revised draft. We appreciate you and the reviewers for your precious time in reviewing our paper and providing valuable comments. It was your valuable and insightful comments that led to possible improvements in the current version. The authors have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your high standards. The authors welcome further constructive comments if any.

Below we provide the point-by-point responses. All modifications in the manuscript have been listed.

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Response to Reviewer 1

We are very grateful for the reviews provided.

Response to Reviewer 3

We are very grateful for the reviews provided.

Response to Reviewer 4

For this study you used 30 extracted teeth, but you didn't mention the sample size formula. how you calculate your sample size? (highlighted in the main file)

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 5, line 11-12]

The sample size was calculated using Federer formula (DOI: 10.1088/1755-1315/131/1/012049).

$t (r - 1) > 15$ t= number of treatments and r =number of replications

$5 (r – 1) > 15$

$5r – 5 > 15$

$5r > 20$

$r > 4$

Federer calculation found more than 4 sample of each treatment, therefore this research using 6 teeth.

In preparation of plant Decoction, please rephrase it in a scientific way, add a sentence about the verification from a botanist about the authenticity of moringa leaves you are using dry leaves of moringa for making decoction, but you didn't mention the method procedure to dry the leaves (Mentioned in mail file)

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 4, line 4-9]

The plants identified by Prof. Gemini Alam. Voucher specimens were deposited in Biological Laboratories, Sekolah Tinggi Ilmu Farmasi Makassar (2534B11). The leaves were harvested by hand, washed under running tap water and drained. The samples were handled with thermal drying using an oven at temperatures of 40°C (Memmert, Germany) for 48 h, then ground with a food grinder (Philips, Indonesia) to produce a fine powder.

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

TITTLE PAGE

Type of article: Original
Title of the article: Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

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January 19, 2023

Professor Abdulmohsen Hamdan Al-Zalabani, MD, ABCM, MHPE
Editor in Chief
Journal of Taibah University Medical Sciences

Dear Professor
Here with we enclosed the submission to the Journal of Taibah University Medical Sciences for publishing. The manuscript entitled "Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study" by Nurhayaty Natsir, Yonathan, Juni Jekti Nugroho, Aries Chandra Trilaksana, Christine Anastasia Rovan, Maria Tanumihardja, and Lukman M.

Endodontic treatment aims at eliminating microorganisms from the infected root canal system by mechanical and chemical methods. Mechanical preparation of the canals leads to the formation of a smear layer. The objective of this study was to compare antibacterial and smear layer removal efficacy of moringa. The leaves of moringa were extracted using hot water extraction. The result suggested that moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. In addition, 5.0% of decoction shows higher antimicrobial activity against both *Enterococcus faecalis* and *Streptococcus mutans*.

Journal of Taibah University Medical Sciences would be an outstanding forum for this paper due to its intention of featuring interdisciplinary research publications; we believe that this paper will be of interest to botanist, dentistry, and pharmacist due to the first report of smear layer removal efficacy of moringa.

All authors have read and approved the final manuscript. We hereby transfer, assign, or otherwise convey all copyright ownership, including any and all rights incidental thereto, exclusively to the journal, in the event that such work is published by the journal.

On behalf of all the contributors I will act and guarantor and will correspond with the journal from this point onward.

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Nurhayaty Natsir

Antibacterial and Smear Layer Removal Efficacy of Moringa: A Preliminary Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. A change in the dentine 'substrate's properties, and hence the interaction of dentine with root filling materials, is one of the effects of root canal irrigation.[2]

The most regularly used irrigating agents are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine.[3] Although NaOCl is the most effective irrigating solution due to its ability to dissolve organic content, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue.[5] The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[6] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial.[4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the eradication of microbes from the root canal system as well as to remove the smear layer.[7]

Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common moringa species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[8] Moringa leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[9]

Methanolic extracts for moringa had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity, using low concentration.[10] Aqueous extracts of moringa leaves showed antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi.*[11] Ethanol extract of moringa leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection.[12] According to Nugroho *et al.* (2021), ethanol extract of moringa 5.0% promises an alternative to root canal irrigant.[13]

The presence of isothiocyanates with their glucosinolate precursors is thought to have an antimicrobial effect. Isothiocyanate activity is mainly linked to the reactivity with sulfhydryl groups, and the antimicrobial effect is dose-dependent.[14] The main advantage of moringa is a broad safety margin for human and animal consumption.[15]

In the present study, we evaluated the antimicrobial activity of moringa leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020.

Materials

The irrigation solutions used in this study can be seen in Tab 1.

TABLE 1

Preparation of plant decoction

The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The plants identified by Prof. Gemini Alam. Voucher specimens were deposited in Biological Laboratories, Sekolah Tinggi Ilmu Farmasi Makassar (2534B11). The leaves were harvested by hand, washed under running tap water and drained. The samples were handled with thermal drying using an oven at temperatures of 40°C (Memmert, Germany) for 48 h, then ground with a food grinder (Philips, Indonesia) to produce a fine powder. Decoction of *M. oleifera* 2.5% was made by weighing about 2.5 g of *M. oleifera* dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for *M. oleifera* 5.0%. The decoction was prepared in triplicate. The decoction was prepared immediately before experiment.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[16]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[16]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[16]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[16]

Antimicrobial activity

The antimicrobial activity of moringa leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175. Pathogenic bacteria included *E. faecalis* and *S. mutans*. The fresh bacterial suspension was dispersed on the surface of Muller Hinton agar plates. One hundred microliters of each decoction were incorporated into the wells, and the plate was incubated at 37 °C for 24 h. The NaOCl 2.5% was used as the positive control. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, the number of samples were calculated using Federer's formula:[17] [t (r – 1) > 15], where: t= number of treatments; r= number of replications. Thirty removed single-rooted human premolars teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal. Root caries, fractures, curve canals, endodontic therapy, internal resorption, and calcification were all ruled out. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The teeth were standardised at 16 mm in length. A safe-sided diamond disk fitted in a low-speed handpiece with a water coolant was used to decorate the teeth. By subtracting 1 mm from the measurement recorded, the working length was determined. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1,

distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, moringa 2.5%; and Group 5, moringa 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Chhabra *et al.* (2016) (Tab 2),[18] and the results were tabulated. The smear layer was independently graded by two operators.

TABLE 2

STATISTICAL ANALYSIS

The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Comparing the smear layer removal efficacy between the five different groups was done by Kruskal–Wallis analysis followed by Mann–Whitney U test for individual comparisons. Value of $p < 0.05$ was considered statistically significant.

RESULT

Phytochemical screening

The phytochemical analysis conducted on moringa leaves' decoction revealed the presence of tannins as well as flavonoids and saponins (Tab 3). These phytochemical components support bioactive activities in medicinal plants and are responsible for the antioxidant activity of the plant extract studied.

TABLE 3

Antimicrobial activity

Based on the mean value zone of inhibition, the moringa leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Fig 1). At

the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) or nearly similar on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

FIGURE 1

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Fig 2). Regarding the smear layer score, it was observed that moringa 2.5% and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both moringa 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tab 4).

FIGURE 2

TABLE 4

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of moringa decoction between different groups at each level of the smear layer (Tab 5). In moringa 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both moringa (2.5% and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%. This outcome suggests that moringa leaves decoction promise an alternative irrigant.

TABLE 5

DISCUSSION

Phytochemical screening compounds like tannins, flavonoids, and saponins were present on moringa leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[19,20] Chhikara *et al.* (2020) and Trigo *et al.*

(2021) have summarised the several bioactive compounds isolated and identified from moringa leaves. However, this report agreed with the findings of Chhikara *et al.* (2020), Enerijiofi *et al.* (2021) and Trigo *et al.* (2021) where they also reported tannin, saponin, and flavonoid.[21-23] Specifically, 2-octenoic acid and 1, 2-epoxyhexadecane identified from the leaves water extract showed antimicrobial activities.[21] Thus, moringa leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the moringa leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[24] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[25] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[26,27]

In contrast to moringa, NaOCl does not contain surfactant directly. However, dissolution of organic tissue can be verified in the saponification reaction when sodium hypochlorite degrades fatty acids and lipids resulting in soap and glycerol.[28] In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions

and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[29,30] The findings in this study corroborate with earlier reports from Khallaf *et al.* (202), where they reported leaves extracts of moringa showed the least amount of smear layer on canal wall.[31] The result corroborates the use of the plant extracts traditionally as a smear layer removal agent.[32]

Both moringa leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[33,34] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[35,36]

CONCLUSION

Within the limitations of this study, alternating the use of moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. Nevertheless, further long-term clinical studies are necessary to confirm these results and evaluate their relevance to treatment outcome.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM carried out the research and collected the data. NN and MM designed and supervised the study, visualized and validated the data, and reviewed draft material. The data were organized, analyzed, and interpreted by CAR, who also reviewed the article. NN and LM organized, analyzed, and interpreted the data and revised the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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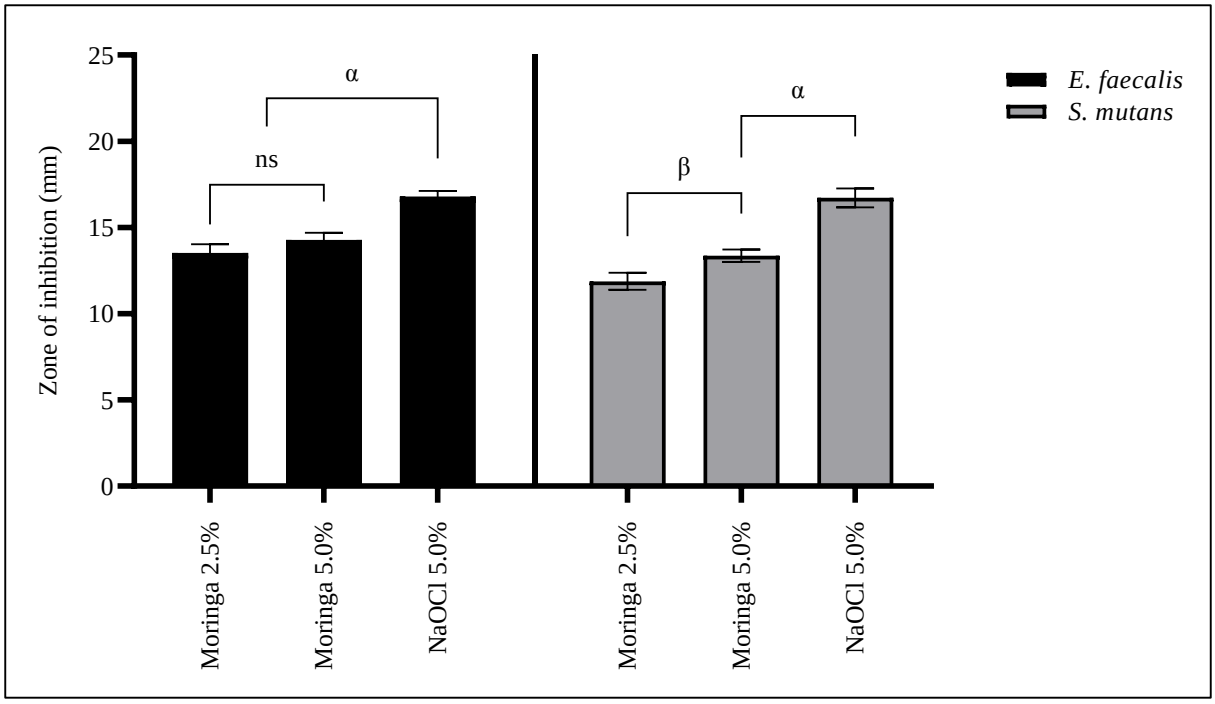

Figure 1 Antimicrobial activity of moringa leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to moringa 2.5%; $^{(ns)}$ non-significant.

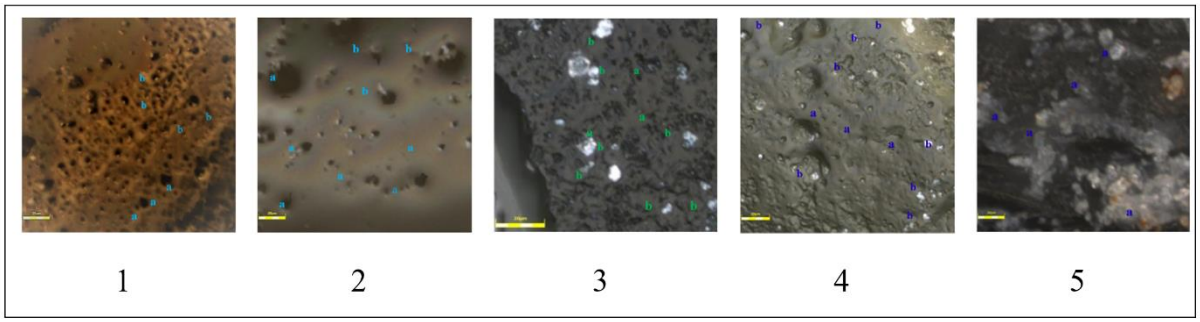

Figure 2 Representative CLSM micrographs (x) in each group: (1) moringa 2.5%; (2) moringa 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1 Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2 Smear layer evaluation criteria.[18]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer were open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	Complete root canal wall covered by a homogeneous smear layer and no open dentinal tubules
5	Heavy homogeneous smear layer covering the complete root canal wall

Table 3 Phytochemical analysis for moringa based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	++

Note: Absent= —, Trace = +, highly present = ++

Table 4 Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
Moringa 2.5%	6	1.83	0.41	0.000*	0.001*
Moringa 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: ∗ Statistically significant result ($p < 0.05$)

Table 5 Mann-Whitney test value to evaluate the difference between groups.

Group	Moringa 2.5%	Moringa 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
Moringa 2.5%					
Moringa 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: ∗ Statistically significant result ($p < 0.05$)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Journal of Taibah University Medical Sciences

Antibacterial and Smear Layer Removal Efficacy of Moringa (Moringa oleifera): In vitro Study

--Manuscript Draft--

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Abstract:	Objective: The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion: These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics
Response to Reviewers:	

Response to Reviewers

Dear Editor,

Thank you for giving us the opportunity to submit a revised draft. We appreciate you and the reviewers for your precious time in reviewing our paper and providing valuable comments. It was your valuable and insightful comments that led to possible improvements in the current version. The authors have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your high standards. The authors welcome further constructive comments if any.

Below we provide the point-by-point responses. All modifications in the manuscript have been listed.

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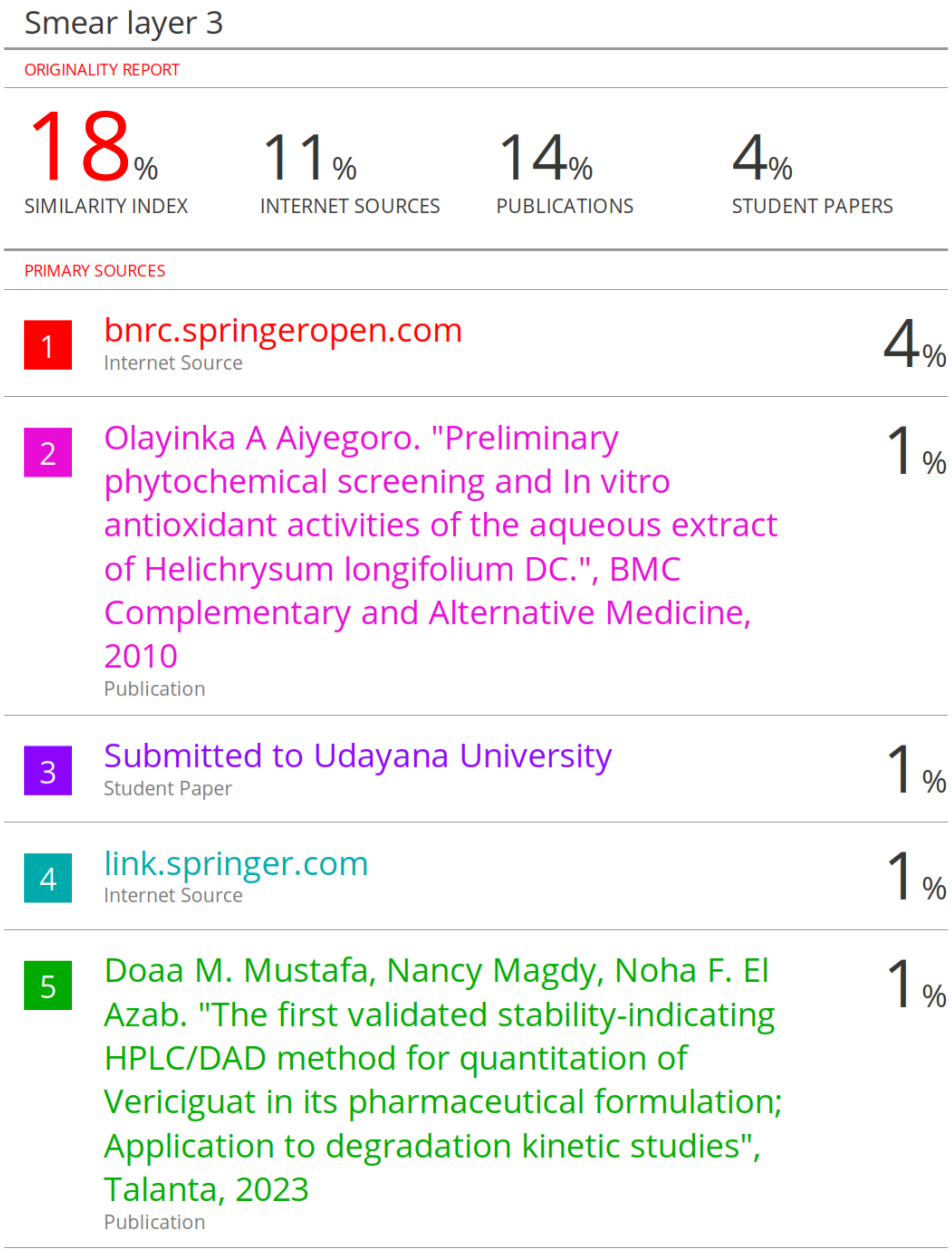
Response to Reviewer 4

We are very grateful for the reviews provided.

Response to Editorial

We have detected a high similarity index (28%) in the revised version of the manuscript. To proceed further, can you please reduce similarity index (must be below 20 %) and resubmit.

Response : Thanks for your kind reminders. We revised the sentence to 18% of similarity index.



Smear layer 3

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Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

TITTLE PAGE

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Title of the article: Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

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Thanking you,
Yours' sincerely,

Signature

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January 19, 2023

Professor Abdulmohsen Hamdan Al-Zalabani, MD, ABCM, MHPE
Editor in Chief
Journal of Taibah University Medical Sciences

Dear Professor
Here with we enclosed the submission to the Journal of Taibah University Medical Sciences for publishing. The manuscript entitled "Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study" by Nurhayaty Natsir, Yonathan, Juni Jekti Nugroho, Aries Chandra Trilaksana, Christine Anastasia Rovan, Maria Tanumihardja, and Lukman M.

Endodontic treatment aims at eliminating microorganisms from the infected root canal system by mechanical and chemical methods. Mechanical preparation of the canals leads to the formation of a smear layer. The objective of this study was to compare antibacterial and smear layer removal efficacy of moringa. The leaves of moringa were extracted using hot water extraction. The result suggested that moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. In addition, 5.0% of decoction shows higher antimicrobial activity against both *Enterococcus faecalis* and *Streptococcus mutans*.

Journal of Taibah University Medical Sciences would be an outstanding forum for this paper due to its intention of featuring interdisciplinary research publications; we believe that this paper will be of interest to botanist, dentistry, and pharmacist due to the first report of smear layer removal efficacy of moringa.

All authors have read and approved the final manuscript. We hereby transfer, assign, or otherwise convey all copyright ownership, including any and all rights incidental thereto, exclusively to the journal, in the event that such work is published by the journal.

On behalf of all the contributors I will act and guarantor and will correspond with the journal from this point onward.

Corresponding Author:
Nurhayaty Natsir

Antibacterial and Smear Layer Removal Efficacy of Moringa: A Preliminary Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. One of the gold of root canal irrigation is a modification of the dentin substrate properties and, therefore, the interaction of dentin with root filling materials.[2]

The three irrigating substances that are most frequently used are chlorhexidine, ethylenediaminetetraacetic acid (EDTA), and sodium hypochlorite (NaOCl).[3] Despite the fact that NaOCl is the best irrigation solution since it can breakdown organic material, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue.[5] The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic agent that chelates Ca^{2+} and Mg^{2+} cations to permeate the outer membrane of Gram-negative bacteria. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[6] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial.[4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the removal of bacteria from the root canal system as well as to remove the smear layer.[7]

Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common moringa species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[8] Moringa leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[9]

Methanolic extracts for moringa had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity, using low concentration.[10] Aqueous extracts of moringa leaves illustrated an antimicrobial activity against *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, and Staphylococcus *aureus***.**[11] Ethanol extract of moringa leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection.[12] According to Nugroho *et al.* (2021), ethanol extract of moringa 5.0% promises an alternative to root canal irrigant.[13]

The presence of isothiocyanates with their glucosinolate precursors is thought to have an antimicrobial effect. The antibacterial effect of isothiocyanates is dose dependent and mostly related to their reactivity with sulfhydryl groups. The antibacterial effect of isothiocyanates is dose dependent and mostly related to their reactivity with sulfhydryl groups.[14] The main advantage of moringa is a broad safety margin for human and animal consumption.[15]

In the present study, we evaluated the antimicrobial activity of moringa leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020.

Materials

The irrigation solutions used in this study can be seen in Tab 1.

TABLE 1

Preparation of plant decoction

The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The plants identified by Prof. Gemini Alam. Voucher specimens were deposited in Biological Laboratories, Sekolah Tinggi Ilmu Farmasi Makassar (2534B11). The leaves were harvested by hand, washed under running tap water and drained. The samples were handled with thermal drying using an oven at temperatures of 40°C (Memmert, Germany) for 48 h, then ground with a food grinder (Philips, Indonesia) to produce a fine powder. Decoction of *M. oleifera* 2.5% was made by weighing about 2.5 g of *M. oleifera* dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for *M. oleifera* 5.0%. The decoction was prepared in triplicate. The decoction was prepared immediately before the experiment.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[16]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[16]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[16]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[16]

Antimicrobial activity

The antimicrobial activity of moringa leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175. Pathogenic bacteria included *E. faecalis* and *S. mutans*. On Muller Hinton agar plates, the recently dissolved bacterial solution was spread out. One hundred microliters of each decoction were inserted into the wells, and the plate was incubated at 37 °C for 24 h. The positive control utilized was NaOCl 2.5%. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, the number of samples were calculated using Federer's formula:[17] [t (r – 1) > 15], where: t= number of treatments; r= number of replications. Thirty removed single-rooted human premolars teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal. Internal resorption, fractures, root caries, curve canals, endodontic therapy, and calcification were all excluded. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The length of the teeth was standardized at 16 mm. The teeth were embellished using a safe-sided diamond disk attached to a low-speed handpiece with a water coolant. The working length was calculated by taking 1 mm away from the measurement that was taken. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1, distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, moringa 2.5%; and Group 5, moringa 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Chhabra *et al.* (2016) (Tab 2),[18] and the results were tabulated. The smear layer was independently graded by two operators.

TABLE 2

STATISTICAL ANALYSIS

Each decoction's zone of inhibition's diameter was measured in triplicate. Mean and standard deviation (SD) were computed. Shapiro Wilk was used to evaluate the normality of data. One-way analysis of variance (ANOVA) was used to compare the mean zone of inhibition between groups, and Tukey's post hoc test was used to confirm the results. Kruskal-Wallis analysis was used to compare the smear layer removal efficacy between the five different groups followed by Mann-Whitney U test was used for individual comparisons. Value of $p < 0.05$ was considered statistically significant.

RESULT

Phytochemical screening

The phytochemical examination conducted on moringa leaves' decoction revealed the presence of flavonoids, and saponins as well as tannins (Tab 3). These phytochemical elements promote the bioactive activities in medicinal plants and are responsible for the antioxidant activity of the plant extract studied.

TABLE 3

Antimicrobial activity

Based on the mean value zone of inhibition, the moringa leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Fig 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) or nearly similar on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

FIGURE 1

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Fig 2). Regarding the smear layer score, it was observed that moringa 2.5% and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both moringa 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tab 4).

FIGURE 2

TABLE 4

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of moringa decoction between different groups at each level of the smear layer (Tab 5). In moringa 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both moringa (2.5% and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%. This outcome suggests that moringa leaves decoction promise an alternative irrigant.

TABLE 5

DISCUSSION

Phytochemical screening compounds like tannins, flavonoids, and saponins were present on moringa leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[19,20] Chhikara *et al.* (2020) and Trigo *et al.* (2021) have summarised the several bioactive compounds isolated and identified from moringa leaves. However, this report agreed with the findings of Chhikara *et al.* (2020), Enerijiofi *et al.* (2021) and Trigo *et al.* (2021) where they also reported tannin, saponin, and flavonoid.[21-23] Specifically, 2-octenoic acid and 1, 2-epoxyhexadecane identified from the leaves water extract showed antimicrobial activities.[21] Thus, moringa leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the moringa leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[24] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[25] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[26,27]

In contrast to moringa, NaOCl does not contain surfactant directly. However, the saponification reaction, in which sodium hypochlorite breaks down fatty acids and lipids to produce soap and glycerol, can demonstrate the dissolution of organic tissue.[28] In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[29,30] The findings in this study corroborate with earlier reports from Khallaf *et al.* (202), where they reported leaves extracts of moringa showed the least amount of smear layer on canal wall.[31] The result supports the traditional usage of the plant extracts as a smear layer removal agent.[32]

Both moringa leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[33,34] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[35,36]

CONCLUSION

Within the limitations of this study, alternating the use of moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. However, additional long-term clinical

investigations are required to verify these findings and assess their applicability to treatment outcomes.

Source of funding

No specific grant for this research was provided by funding organizations in the public, private, or nonprofit sectors.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM conducted the research and collected the data. The study was designed and supervised by NN and MM, who also validated the data and evaluated the drafts. CAR, who also reviewed the article, organized, examined, and analysed the data. NN and LM collected, collated, and reviewed the article. The content and similarity index of the paper are the responsibility of all authors, who also gave the final text a critical assessment and approval.

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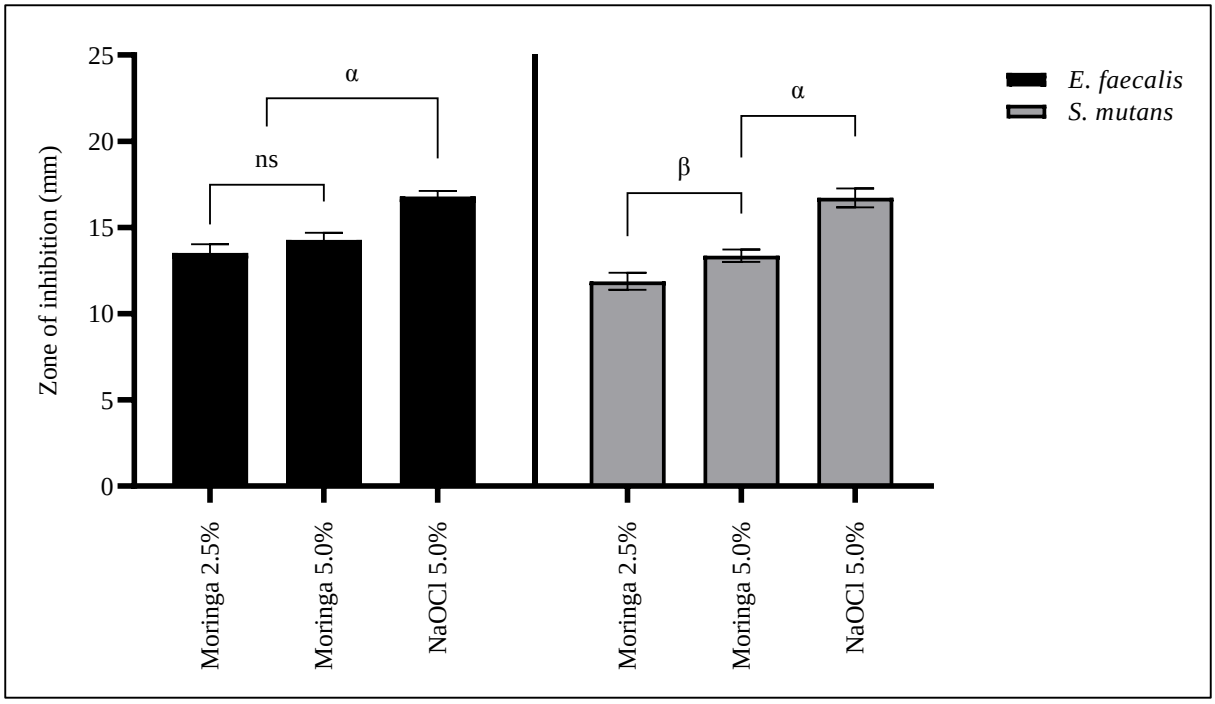

Figure 1 Antimicrobial activity of moringa leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to moringa 2.5%; $^{(ns)}$ non-significant.

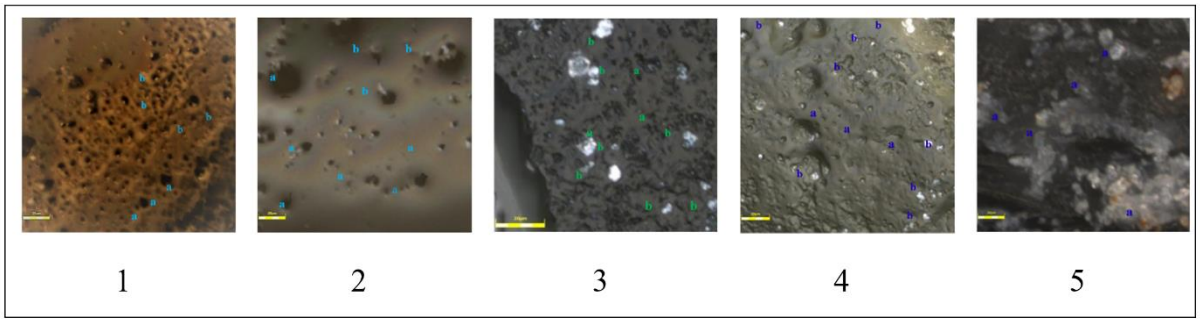

Figure 2 Representative CLSM micrographs (x) in each group: (1) moringa 2.5%; (2) moringa 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1 Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2 Smear layer evaluation criteria.[18]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer were open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	Complete root canal wall covered by a homogeneous smear layer and no open dentinal tubules
5	Heavy homogeneous smear layer covering the complete root canal wall

Table 3 Phytochemical analysis for moringa based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	++

Note: Absent= —, Trace = +, highly present = ++

Table 4 Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
Moringa 2.5%	6	1.83	0.41	0.000*	0.001*
Moringa 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: ∗ Statistically significant result ($p < 0.05$)

Table 5 Mann-Whitney test value to evaluate the difference between groups.

Group	Moringa 2.5%	Moringa 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
Moringa 2.5%					
Moringa 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: ∗ Statistically significant result ($p < 0.05$)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Antibacterial and Smear Layer Removal Efficacy of Moringa (Moringa oleifera): In vitro Study

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Abstract:	Objective: The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion: These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics
Response to Reviewers:	

Response to Reviewers

Dear Editor,

We appreciate you and the reviewers for your precious time in reviewing our paper and providing valuable comments. It was your valuable and insightful comments that led to possible improvements in the current version. The authors have carefully considered the comments and tried our best to address every one of them. We hope the manuscript after careful revisions meet your high standards. The authors welcome further constructive comments if any.

Below we provide the point-by-point responses. All modifications in the manuscript have been listed.

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Response to Editorial

Please provide 5 to 7 keywords. Please include the same in all stages of the manuscript.

Response : Thanks for your kind reminders. We added 6 keywords as follows.
"Antibacterial, Decoction, Irrigants, *Moringa oleifera*, Root canal, Smear Layer"

Please include below statement in authors contribution.

All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 10]
"YY, JJN, ACT, and LM conducted the research and collected the data. The study was designed and supervised by NN and MM, who also validated the data and evaluated the drafts. CAR, who also reviewed the article, organized, examined, and analysed the data. NN and LM collected, collated, and reviewed the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript".

Please provide an editable source file format of the title page.

Response : Thanks for your kind reminders. An editable source of title page was attached in MS Office Word.

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Decoction, Irrigants, *Moringa oleifera*, Root canal, Smear Layer.

TITTLE PAGE

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Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Decoction, Irrigants, *Moringa oleifera*, Root canal, Smear Layer.

Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. One of the gold of root canal irrigation is a modification of the dentin substrate properties and, therefore, the interaction of dentin with root filling materials.[2]

The three irrigating substances that are most frequently used are chlorhexidine, ethylenediaminetetraacetic acid (EDTA), and sodium hypochlorite (NaOCl).[3] Despite the fact that NaOCl is the best irrigation solution since it can breakdown organic material, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue.[5] The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic agent that chelates Ca^{2+} and Mg^{2+} cations to permeate the outer membrane of Gram-negative bacteria. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[6] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial.[4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the removal of bacteria from the root canal system as well as to remove the smear layer.[7]

Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common moringa species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[8] Moringa leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[9]

Methanolic extracts for moringa had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity, using low concentration.[10] Aqueous extracts of moringa leaves illustrated an antimicrobial activity against *Bacillus cereus*, *Escherichia coli*, *Salmonella typhi*, and Staphylococcus *aureus***.**[11] Ethanol extract of moringa leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection.[12] According to Nugroho *et al.* (2021), ethanol extract of moringa 5.0% promises an alternative to root canal irrigant.[13]

The presence of isothiocyanates with their glucosinolate precursors is thought to have an antimicrobial effect. The antibacterial effect of isothiocyanates is dose dependent and mostly related to their reactivity with sulfhydryl groups. The antibacterial effect of isothiocyanates is dose dependent and mostly related to their reactivity with sulfhydryl groups.[14] The main advantage of moringa is a broad safety margin for human and animal consumption.[15]

In the present study, we evaluated the antimicrobial activity of moringa leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020.

Materials

The irrigation solutions used in this study can be seen in Tab 1.

TABLE 1

Preparation of plant decoction

The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The plants identified by Prof. Gemini Alam. Voucher specimens were deposited in Biological Laboratories, Sekolah Tinggi Ilmu Farmasi Makassar (2534B11). The leaves were harvested by hand, washed under running tap water and drained. The samples were handled with thermal drying using an oven at temperatures of 40°C (Memmert, Germany) for 48 h, then ground with a food grinder (Philips, Indonesia) to produce a fine powder. Decoction of *M. oleifera* 2.5% was made by weighing about 2.5 g of *M. oleifera* dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for *M. oleifera* 5.0%. The decoction was prepared in triplicate. The decoction was prepared immediately before the experiment.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[16]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[16]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[16]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[16]

Antimicrobial activity

The antimicrobial activity of moringa leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175. Pathogenic bacteria included *E. faecalis* and *S. mutans*. On Muller Hinton agar plates, the recently dissolved bacterial solution was spread out. One hundred microliters of each decoction were inserted into the wells, and the plate was incubated at 37 °C for 24 h. The positive control utilized was NaOCl 2.5%. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, the number of samples were calculated using Federer's formula:[17] [t (r – 1) > 15], where: t= number of treatments; r= number of replications. Thirty removed single-rooted human premolars teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal. Internal resorption, fractures, root caries, curve canals, endodontic therapy, and calcification were all excluded. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The length of the teeth was standardized at 16 mm. The teeth were embellished using a safe-sided diamond disk attached to a low-speed handpiece with a water coolant. The working length was calculated by taking 1 mm away from the measurement that was taken. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1, distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, moringa 2.5%; and Group 5, moringa 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Tosco *et al.* (2023) (Tab 2),[18] and the results were tabulated. The smear layer was independently graded by two operators.

TABLE 2

STATISTICAL ANALYSIS

Each decoction's zone of inhibition's diameter was measured in triplicate. Mean and standard deviation (SD) were computed. Shapiro Wilk was used to evaluate the normality of data. One-way analysis of variance (ANOVA) was used to compare the mean zone of inhibition between groups, and Tukey's post hoc test was used to confirm the results. Kruskal-Wallis analysis was used to compare the smear layer removal efficacy between the five different groups followed by Mann-Whitney U test was used for individual comparisons. Value of $p < 0.05$ was considered statistically significant.

RESULT

Phytochemical screening

The phytochemical examination conducted on moringa leaves' decoction revealed the presence of flavonoids, and saponins as well as tannins (Tab 3). These phytochemical elements promote the bioactive activities in medicinal plants and are responsible for the antioxidant activity of the plant extract studied.

TABLE 3

Antimicrobial activity

Based on the mean value zone of inhibition, the moringa leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Fig 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) or nearly similar on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

FIGURE 1

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Fig 2). Regarding the smear layer score, it was observed that moringa 2.5% and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both moringa 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tab 4).

FIGURE 2

TABLE 4

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of moringa decoction between different groups at each level of the smear layer (Tab 5). In moringa 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both moringa (2.5% and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%. This outcome suggests that moringa leaves decoction promise an alternative irrigant.

TABLE 5

DISCUSSION

Phytochemical screening compounds like tannins, flavonoids, and saponins were present on moringa leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[19,20] Chhikara *et al.* (2020) and Trigo *et al.* (2021) have summarised the several bioactive compounds isolated and identified from moringa leaves. However, this report agreed with the findings of Chhikara *et al.* (2020), Enerijiofi *et al.* (2021) and Trigo *et al.* (2021) where they also reported tannin, saponin, and flavonoid.[21-23] Specifically, 2-octenoic acid and 1, 2-epoxyhexadecane identified from the leaves water extract showed antimicrobial activities.[21] Thus, moringa leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the moringa leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[24] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[25] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[26,27]

In contrast to moringa, NaOCl does not contain surfactant directly. However, the saponification reaction, in which sodium hypochlorite breaks down fatty acids and lipids to produce soap and glycerol, can demonstrate the dissolution of organic tissue.[28] In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[29,30] The findings in this study corroborate with earlier reports from Khallaf *et al.* (202), where they reported leaves extracts of moringa showed the least amount of smear layer on canal wall.[31] The result supports the traditional usage of the plant extracts as a smear layer removal agent.[32]

Both moringa leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[33,34] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[35,36]

CONCLUSION

Within the limitations of this study, alternating the use of moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution. However, additional long-term clinical

investigations are required to verify these findings and assess their applicability to treatment outcomes.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM conducted the research and collected the data. The study was designed and supervised by NN and MM, who also validated the data and evaluated the drafts. CAR, who also reviewed the article, organized, examined, and analysed the data. NN and LM collected, collated, and reviewed the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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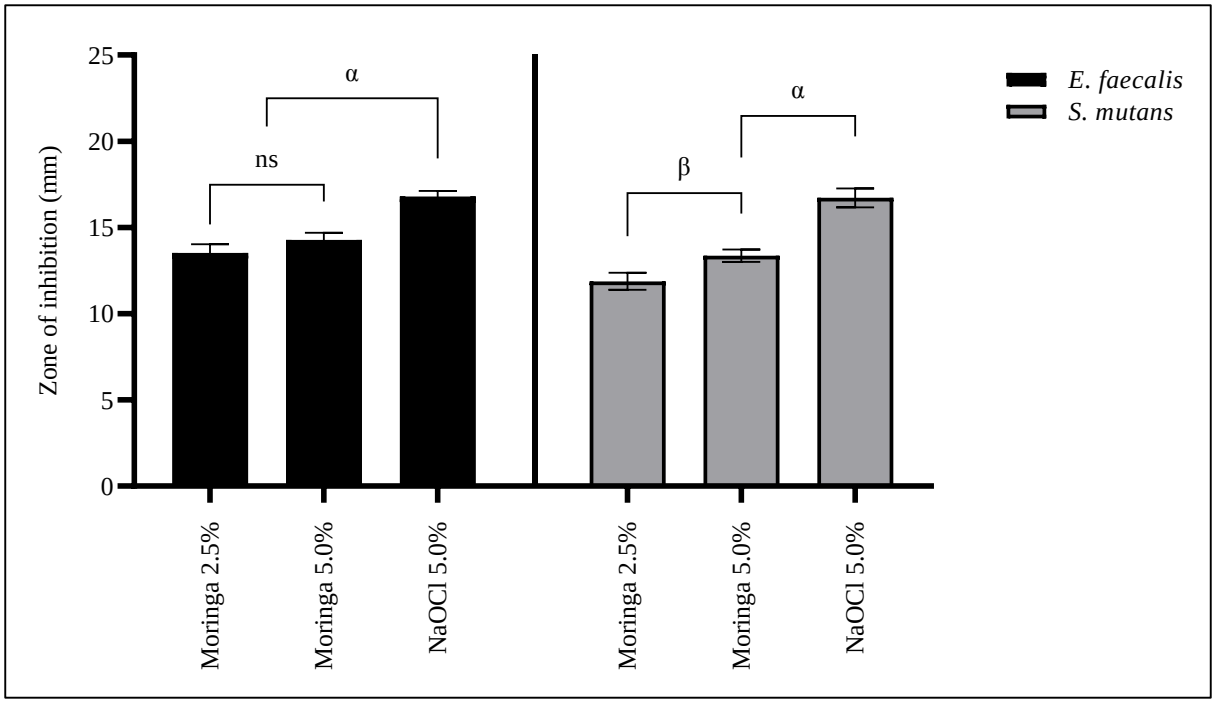

Figure 1 Antimicrobial activity of moringa leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to moringa 2.5%; $^{(ns)}$ non-significant.

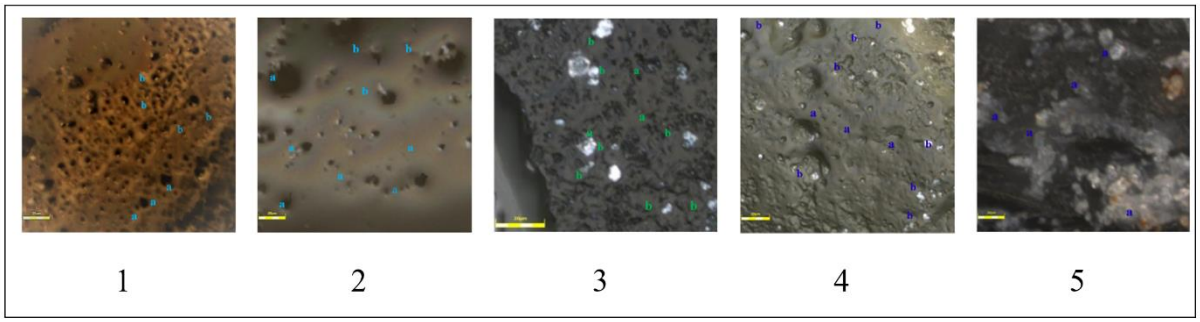

Figure 2 Representative CLSM micrographs (x) in each group: (1) moringa 2.5%; (2) moringa 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1 Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2 Smear layer evaluation criteria.[18]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer were open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	Complete root canal wall covered by a homogeneous smear layer and no open dentinal tubules
5	Heavy homogeneous smear layer covering the complete root canal wall

Table 3 Phytochemical analysis for moringa based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	++

Note: Absent= —, Trace = +, highly present = ++

Table 4 Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
Moringa 2.5%	6	1.83	0.41	0.000*	0.001*
Moringa 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: ∗ Statistically significant result ($p < 0.05$)

Table 5 Mann-Whitney test value to evaluate the difference between groups.

Group	Moringa 2.5%	Moringa 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
Moringa 2.5%					
Moringa 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: ∗ Statistically significant result ($p < 0.05$)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Antibacterial and Smear Layer Removal Efficacy of Moringa: A Preliminary Study

--Manuscript Draft--

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Article Type:	Original Article
Section/Category:	Dentistry
Keywords:	Antibacterial; Irrigants; Moringa oleifera; Smear Layer
Abstract:	Objective The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl to remove the smear layer, however, no significant ($p > 0.05$) compared to EDTA. The in vitro antimicrobial assay results prove that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Antibacterial and Smear Layer Removal Efficacy of Moringa: A Preliminary Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl to remove the smear layer, however, no significant ($p > 0.05$) compared to EDTA. The in vitro antimicrobial assay results prove that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

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Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. A change in the dentine 'substrate's properties, and hence the interaction of dentine with root filling materials, is one of the effects of root canal irrigation.[2]

The most regularly used irrigating agents are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine.[3] Although NaOCl is the most effective irrigating solution due to its ability to dissolve organic content, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue. The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[5] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial. [4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the eradication of microbes from the root canal system as well as to remove the smear layer.[6] Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common moringa species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[7] Moringa leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[8]

Methanolic extracts for moringa had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity, using low concentration.[9] Aqueous extract

of moringa leaves showed antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus, Escherichia coli*, and *Salmonella typhi.*[10] Ethanol extract of moringa leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection. [11] According to Nugroho *et al.* (2021), ethanol extract of moringa 5.0% promises an alternative to root canal irrigant.[12] The presence of chemical compound 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)-benzylisothiocyanate is thought to have an antimicrobial effect. Inhibition of key cellular membrane enzymes is the mechanism of action.[13] The main advantage of moringa is a broad safety margin for human and animal consumption.[14]

In the present study, we evaluated the antimicrobial activity of moringa leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and Methods

Materials

The irrigation solutions used in this study can be seen in Tab 1.

TABLE 1

Preparation of plant decoction

Decoction of moringa 2.5% was made by weighing about 2.5 g of moringa dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for moringa 5.0%. The decoction was prepared in triplicate. The resulting decoctions were stored at 4 °C for future use.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[15]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[15]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[15]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[15]

Antimicrobial activity

The antimicrobial activity of moringa leaves decoction against pathogenic bacteria was investigated. Pathogenic bacteria included *E. faecalis* and *S. mutans*. The fresh bacterial suspension was dispersed on the surface of Muller Hinton agar plates. One hundred microliters of each decoction were incorporated into the wells, and the plate was incubated at 37 °C for 24 h. The NaOCl 2.5% was used as the positive control. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, thirty removed single-rooted teeth were employed. A radiograph was taken of each tooth to establish the presence of a single canal. Root caries, fractures, bent canals, endodontic therapy, internal resorption, and calcification were all ruled out. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The teeth were standardised at 16 mm in length. A safe-sided diamond disk fitted in a low-speed handpiece with a water coolant was used to decorate the teeth. By subtracting 1 mm

from the measurement recorded, the working length was determined. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1, distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, moringa 2.5%; and Group 5, moringa 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Chhabra *et al.* (2016) (Tab 2)[16], and the results were tabulated.

TABLE 2

Statistical analysis

The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Whereas Kruskal Wallis method was used to determine the differences between the means and variables between or among groups of smear layer remover were compared using the Mann-Whitney U test. Value of $p < 0.05$ was considered statistically significant.

Results

Phytochemical screening

The phytochemical analysis conducted on moringa leaves' decoction revealed the presence of tannins as well as flavonoids and saponins (Tab 3). These phytochemical components support bioactive activities in medicinal plants and are responsible for the antioxidant activity of the plant extract studied.

TABLE 3

Antimicrobial activity

Based on the mean value zone of inhibition, the moringa leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Fig 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) or nearly similar on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% were significantly different from those of 5.0% ($p < 0.05$). However, NaOCl 5% was more effective against both *E. faecalis* and *S. mutans*.

FIGURE 1

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Fig 2). Regarding the smear layer score, it was observed that moringa 2.5% and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both moringa 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tab 4). The Shapiro Wilk test examination showed that the data was not normally distributed in the population, so the differences between the means were evaluated by Kruskal Wallis, followed by the Mann-Whitney U test to determine between or among groups smear layer score.

FIGURE 2

TABLE 4

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of moringa decoction between different groups at each level of the smear layer (Tab 5). In moringa 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both moringa (2.5%

and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%. This outcome suggests that moringa leaves decoction promise an alternative irrigant.

TABLE 5

Discussion

Phytochemical screening compounds like tannins, flavonoids, and saponins were applied on moringa leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[17,18] Chhikara *et al.* (2020) and Trigo *et al.* (2021) have summarised the several bioactive compounds isolated and identified from moringa leaves. The moringa contains polyphenols, flavonoids, alkaloids, glucosinolates, glycosides, terpenoids, and carotenoids.[19,20] Specifically, phenolic glycoside, 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)-benzylisothiocyanate isolated from the leaves ethanol extract showed cytotoxic, hypotensive, antimicrobial, and anti-inflammatory activities.[21,22] Thus, moringa leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the moringa leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva. In an acid solution, flavonoids as polyphenols are weak acids. They decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[23,24] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have

distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[25,26]

In contrast to moringa, NaOCl does not contain surfactant directly. Its high surface tension causes NaOCl to be less able to penetrate the deeper dentinal tubules of the root canal. Due to the saponisation reaction of NaOCl in breaking organic matter and fats into fatty acids in soap and glycerol, the surface tension of the remaining solution is reduced. In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[27,28] This is the reason why moringa leaves decoction was more effective than NaOCl

Both moringa leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[29,30] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[31,32]

Conclusion

Under the conditions of the current study, alternating the use of moringa leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the

use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of moringa leaves decoction as an alternative to irrigant solution.

Source of funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM conceived and designed the study, conducted research, provided research materials, and collected and organized data. NN, CAR, and MM analyzed and interpreted data. NN and LM wrote initial and final draft of article and provided logistic support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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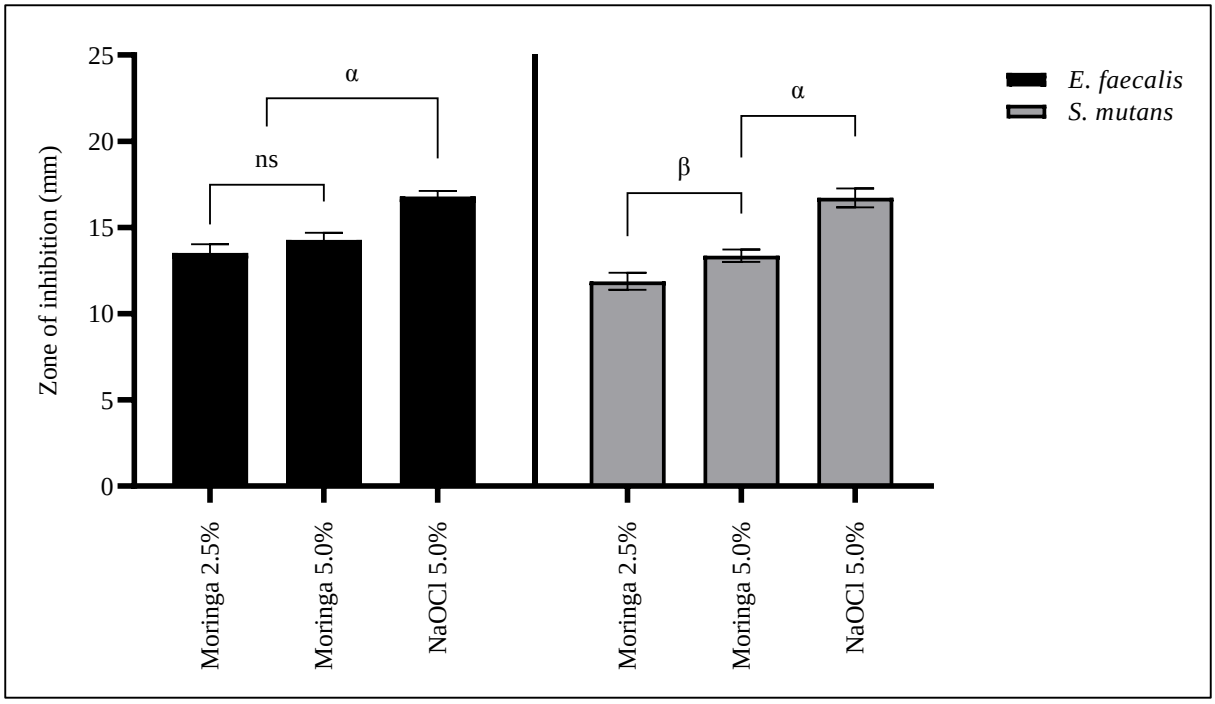

Figure 1: Antimicrobial activity of moringa leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to moringa 2.5%; $^{(ns)}$ non-significant.

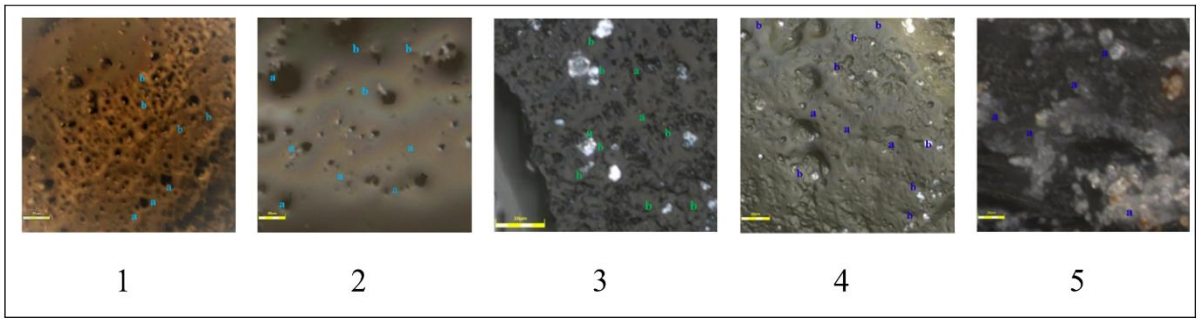

Figure 2: Representative CLSM micrographs (x) in each group: (1) moringa 2.5%; (2) moringa 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1: Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2: Smear layer evaluation criteria.[16]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	There are no exposed dentinal tubules, and a uniform smear layer completely coats the root canal wall
5	A thick, homogenous smear layer coats the whole root canal wall

Table 3: Phytochemical analysis for moringa based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	+

Note: + = presence; − = absent.

Table 4: Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
Moringa 2.5%	6	1.83	0.41	0.000*	0.001*
Moringa 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: (*) Superscript means differ ($p < 0.05$)

Table 5: Mann-Whitney test value to evaluate the difference between groups.

Group	Moringa 2.5%	Moringa 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
Moringa 2.5%					
Moringa 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: (*) Superscript means differ ($p < 0.05$)

Journal of Taibah University Medical Sciences

Antibacterial and Smear Layer Removal Efficacy of Moringa (Moringa oleifera): In vitro Study

--Manuscript Draft--

Manuscript Number:	JTUMED-D-23-00132R1
Article Type:	Original Article
Section/Category:	Dentistry
Keywords:	Antibacterial; Irrigants; Moringa oleifera; Smear Layer
Abstract:	Objective: The study aimed to evaluate the effectiveness of the moringa (Moringa oleifera) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. Methods: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on Enterococcus faecalis and Streptococcus mutans bacteria using the agar diffusion method. Results: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The in vitro antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. Conclusion: These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics

Response to Reviewer 3

Reviewer #3: incomplete background and discussion
Response : Thanks for your kind reminders. We revised the background and discussion.

Response to Reviewer 4

Reviewer #4: TITLE OF MANUSCRIPT:
Title is not clear and complete, please modify the title, and also mention type of study.
Response : Thanks for your kind reminders. We revised the sentence as follows
"Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study"

INTRODUCTION:
Please add refrences where required. Most of the time, you wrote about ethanolic and aqueous extract of moringa, but you should also describe about the moringa decoction, Also add some refrences about it.
Response : Thanks for your kind reminders. We added references to all sentences. A decoction is a water-based extraction where the plant is placed in boiling water and simmered for a decent amount of time. In this case decoction resulted water extract.

MATERIALS AND METHODS:
Please mention study setting, study duration and sample size formula, which is not mentioned in manuscript. you didn't mention the source of plant material and its processing before making decoction. I also recommed to re-write the procedure of making moringa decoction. however, there are number of grammatical errors; many of them have been mentioned in the main file. You are advised to correct all those mistakes and have a good proof reading.
Response : Thanks for your kind reminders. We revised the study design as follows [pg 3, line 22-24]
Study design
"This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020".

This study used human teeth that had been extracted at the hospital and then selected based on inclusion and exclusion criteria, so the sample calculation formula was not used.

Source of plant material and its processing before making decoction revised the study design as follows [pg 4, line 3-5]

"The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The leaves were harvested by hand, washed under running tap water and drained".

To improve the readability, proofreading has been carried out on papertrue.com

STATISTICS:

Although the statistical significance evaluation of data is obtained and discussed, there is need to rephrase and explain it a more descriptive way. The statistical tests applied are not so much clear.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 6, line 1-7]

"The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Comparing the smear layer removal efficacy between the five different groups was done by Kruskal–Wallis analysis followed by Mann–Whitney U test for individual comparisons. Value of $p < 0.05$ was considered statistically significant".

RESULTS:

The description in table 2 is not clear, please rephrase table 2. It contains some grammatical and syntax errors. please elaborate Table 3 in more in categorical way. Please use correct terminologies, some of them have been mentioned in the main file.

Response : Thanks for your kind reminders. We revised the sentence as follows [pg 17]

DISCUSSION:

Discussion is reasonable. Mostly you discussed and compare the beneficial effects of ethanolic extract of moringa with moringa decoction. Please mention, how both preparations have same effects. By incorporating studies of moringa decoction will improve the discussion.

Response : Thanks for your kind reminders. We revised the sentence and compared to the same procedure of extraction method using water.

REFERENCES:

please try to use references from latest articles, not more than 10 years.

Response : Thanks for your kind reminders. We have made revisions accordingly.

GENERAL COMMENTS:

The study is well planned. Required some correction as suggested and resubmission.

Response : Thanks for your kind reminders.

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to NaOCl and EDTA as well as antimicrobial activities. **Methods**: The moringa leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that moringa leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

Antibacterial and Smear Layer Removal Efficacy of Moringa (*Moringa oleifera*): *In vitro* Study

Objective: The study aimed to evaluate the effectiveness of the moringa (*Moringa oleifera*) leaves decoction for removal of smear layer compared to sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA) as well as antimicrobial activities. **Methods**: The *M. oleifera* leaves were extracted using hot water decoction at two different concentrations (2.5% and 5.0% b/v). A total of 30 extracted human single-rooted teeth were prepared to assess the smear layer removal efficacy. The presence of a smear layer at middle third of the root canal was calculated by Confocal Laser Scanning Microscope (CLSM). Then, antibacterial activity was performed on *Enterococcus faecalis* and *Streptococcus mutans* bacteria using the agar diffusion method. **Results**: The 2.5% and 5.0% of decoction were significantly ($p < 0.05$) more effective than 0.25% NaOCl in removing the smear layer, however, no significant ($p > 0.05$) difference observed when compared to EDTA. The *in vitro* antimicrobial assay results have shown that 5.0% of decoction shows higher antimicrobial activity against both the test pathogens. **Conclusion:** These findings suggest that *M. oleifera* leaves decoction can be considered an effective irrigant in endodontics.

Keywords: Antibacterial, Irrigants, *Moringa oleifera*, Smear Layer.

Introduction

A variety of microorganisms in the root canal produce pulpal and peri-radicular infections. Root canal therapy aims to remove germs from the root canal and provide an environment conducive to tissue recovery. Endodontic treatment's success is determined by proper biomechanical preparation, irrigation, and root canal obturation.[1] Irrigation is crucial during root canal therapy for teeth with complex interior structures. A change in the dentine 'substrate's properties, and hence the interaction of dentine with root filling materials, is one of the effects of root canal irrigation.[2]

The most regularly used irrigating agents are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine.[3] Although NaOCl is the most effective irrigating solution due to its ability to dissolve organic content, it also has various drawbacks, including being poisonous and potentially irritating to periapical tissues and having a disagreeable odor and taste.[3,4]

One of the most important requirements of an ideal endodontic irrigant is to possess smear layer remover, antibacterial effect, and minimum toxic effect on the periapical tissue.[5] The smear layer may prevent intracanal medicaments and sealants from reaching the dentinal tubules. EDTA 17.0% is effective for smear layer removal and a bacteriostatic compound that permeabilizes the outer membrane of Gram-negative bacteria by chelating Ca^{2+} and Mg^{2+} cations. Therefore, EDTA can reduce the dentin microhardness and react with calcium ions in dentine, causing calcium chelation and promoting dentine decalcification.[6] While NaOCl 2.5% removed the smear layer in the third apical area incompletely, it has a strong antibacterial. [4] An alternative irrigant was needed to overcome this problem. It needed to have antimicrobial activity and smear layer removing capacity without damaging the dentin. In the past decade, considerable efforts have been made to develop new irrigants from medicinal herbs to facilitate the eradication of microbes from the root canal system as well as to remove the smear layer.[7]

Moringa species are common plant herbs listed in ancient records because of their extraordinary nutritional and medicinal properties. *Moringa oleifera* is the most common *M. oleifera* species. It contains a variety of phytochemical substances, such as alkaloids, tannins, flavonoids, saponins, triterpenoids, and antimicrobial properties.[8] *M. oleifera* leaves extract at 8% b/v can inhibit the growth of *Staphylococcus epidermidis* with an inhibition zone around 14 mm.[9]

Methanolic extracts for *M. oleifera* had an antibacterial effect against *Enterococcus faecalis* after incubation for 24 and 48 h without any toxicity to MDCK epithelial cell.[10] Aqueous extracts of *M. oleifera* leaves showed antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Salmonella typhi.*[11] Ethanol extract of *M. oleifera* leaves exhibited cariogenic biofilm formation due to *Streptococcus mutans* infection.[12] According to Nugroho *et al.* (2021), ethanol extract of *M. oleifera* 5.0% promises an alternative to root canal irrigant.[13]

The presence of isothiocyanates with their glucosinolate precursors is thought to have an antimicrobial effect. Isothiocyanate activity is mainly linked to the reactivity with sulfhydryl groups, and the antimicrobial effect is dose-dependent.[14] The main advantage of *M. oleifera* is a broad safety margin for human and animal consumption.[15]

In the present study, we evaluated the antimicrobial activity of *M. oleifera* leaves decoction against *E. faecalis* and *S. mutans*, and its effect on the smear layer using a Confocal Laser Scanning Microscope (CLSM).

Materials and methods

Study design

This study was a laboratory experimental study that used a post-test only control group design in between January and March 2020.

Materials

The irrigation solutions used in this study can be seen in Table 1.

Preparation of plant decoction

The *M. oleifera* leaves used in this study was obtained from Toraja, South Sulawesi, Indonesia in January 2020. The leaves were harvested by hand, washed under running tap water and drained. Decoction of *M. oleifera* 2.5% was made by weighing about 2.5 g of *M. oleifera* dried leaves and put with distilled water (till 100 ml), while the water temperature was maintained at 90 °C (within ± 2 °C) for 30 min. The mixture was filtered under hot conditions over a Buchner funnel, and hot water was directly poured on sample to reach 100 ml. The same procedure was conducted for *M. oleifera* 5.0%. The decoction was prepared in triplicate. The decoction was prepared immediately before experiment.

Phytochemical qualitative screening

The presence of phytochemical qualitative analysis was determined using the following conventional procedures on decoction.[16]

Test for tannin

The 2 ml of decoction received approximately 10 ml of bromine water. The discolouration of bromine revealed the presence of tannins.[16]

Test for saponin

A 5.0 ml of decoction was taken in a test tube, and a few drops of olive oil was mixed in it. After homogenising vigorously, the appearance of foam showed the presence of saponins.[16]

Tests for flavonoid

A few magnesium ribbons and concentrated HCl were combined with decoction and allowed to stand for a few minutes. The pink tint indicated the existence of flavonoids.[16]

Antimicrobial activity

The antimicrobial activity of *M. oleifera* leaves decoction against pathogenic bacteria was investigated: *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175. Pathogenic bacteria

included *E. faecalis* and *S. mutans*. The fresh bacterial suspension was dispersed on the surface of Muller Hinton agar plates. One hundred microliters of each decoction were incorporated into the wells, and the plate was incubated at 37 °C for 24 h. The NaOCl 2.5% was used as the positive control. The zone of inhibition was recorded on each plate.

Specimen selection

In this investigation, thirty removed human single-rooted teeth were used. A radiograph was taken of each tooth to establish the presence of a single canal. Root caries, fractures, curve canals, endodontic therapy, internal resorption, and calcification were all ruled out. After removing calculus and soft-tissue debris, the teeth were disinfected with 70% ethanol for 1 h before being preserved in the saline solution until instrumentation.

Specimen preparation

The teeth were standardised at 16 mm in length. A safe-sided diamond disk fitted in a low-speed handpiece with a water coolant was used to decorate the teeth. By subtracting 1 mm from the measurement recorded, the working length was determined. A protaper universal nickel-titanium rotary system was used to prepare the root canals.

Smear layer removal ability

Each tooth was divided into equal sections of the middle third with a diamond disk. Teeth were divided into five groups (n = 6) according to the irrigant used, as follows: Group 1, distilled water; Group 2, NaOCl 2.5%; Group 3, EDTA 17%; Group 4, *M. oleifera* 2.5%; and Group 5, *M. oleifera* 5.0%. After each file size, 5.0 ml of irrigant solution was used to irrigate each group. Five millilitres of distilled water were used as a final rinse. Images of each third of the canal were taken using CLSM. Cleanliness was evaluated using criteria described by Chhabra *et al.* (2016) (Table 2),[17] and the results were tabulated. The smear layer was independently graded by two operators.

STATISTICAL ANALYSIS

The diameter of the zone of inhibition of each decoction was obtained at triplicate values. The mean and standard deviation (SD) were calculated. The normality of the data was assessed using Shapiro Wilk. The statistical difference of the mean zone of inhibition between groups was carried out by one-way analysis of variance (ANOVA) followed by Tukey's post hoc. Comparing the smear layer removal efficacy between the five different groups was done by Kruskal–Wallis analysis followed by Mann–Whitney U test for individual comparisons. Value of $p < 0.05$ was considered statistically significant.

RESULT

Phytochemical screening

The phytochemical analysis conducted on *M. oleifera* leaves decoction revealed the presence of tannins as well as flavonoids and saponins (Table 3). These phytochemical components support bioactive activities in medicinal plants and are responsible for the antimicrobial activity of the plant extract studied.

Antimicrobial activity

Based on the mean value zone of inhibition, the *M. oleifera* leaves decoction's antibacterial activity ability depended on the concentrations of the decoction dan bacterial used (Figure 1). At the concentration of 2.5% and 5.0% of decoction, *E. faecalis* was non-significant ($p > 0.05$) on the mean zone of inhibition (12.70 ± 0.50 mm and 13.82 ± 0.42 mm, respectively). For *S. mutans*, the zones of inhibition of the decoction at 5% (9.76 ± 0.49 mm) were significantly different ($p < 0.05$) from those of 5.0% (10.87 ± 0.36 mm). However, NaOCl 5% was more effective against both *E. faecalis* (16.76 ± 0.32 mm) and *S. mutans* (13.45 ± 0.55 mm).

Smear layer remover efficiency

A comparison of smear layer covering in the middle third at tooth between groups was performed (Figure 2). Regarding the smear layer score, it was observed that *M. oleifera* 2.5%

and 5.0% had similar effectiveness (score of 1.83 ± 0.41). Both *M. oleifera* 2.5% and 5.0% were more effective than NaOCl 2.5% and EDTA 17.0% (Tabel 4).

Generally, the Mann-Whitney U test showed a significant difference in the cleanliness of *M. oleifera* decoction between different groups at each level of the smear layer (Table 5). In *M. oleifera* 2.5% and 5.0%, there was no significant difference concerning the cleanliness of dentin ($p = 1$). Our results revealed a significant difference between the smear layers, both *M. oleifera* (2.5% and 5.0%) and standard irrigant (NaOCl 2.5%), but more effective than NaOCl 2.5%.

DISCUSSION

Phytochemical screening compounds like tannins, flavonoids, and saponins were present on *M. oleifera* leaves decoction. These compounds are known to be helpful in the treatment of infection in both pre-clinical and clinical studies.[18,19] Chhikara *et al.* (2020), Enerijiofi *et al.* (2021), and Trigo *et al*. (2021) have summarised the several bioactive compounds isolated and identified from *M. oleifera* leaves. However, they also reported tannin, saponin, and flavonoid.[20-22] Specifically, 2-octenoic acid and 1, 2-epoxyhexadecane identified from the leaves water extract showed antimicrobial activities.[20] Thus, *M. oleifera* leaves decoction containing this compound may be a potential source of bioactive compounds against pathogen bacteria. Besides killing the microbes, one of the properties of the irrigant solutions is eliminating the smear layer on the dentin.[2] For this reason, we also evaluated the effectiveness of the *M. oleifera* leaves for the removal of the smear layer compared to NaOCl and EDTA.

The smear layer consists of inorganic and organic components. The inorganic components are apatite particles, while the organic components include microorganisms and saliva.[23] Generally, flavonoids decompose hydroxyapatite, releasing calcium ions (Ca^{2+}) and hydrogen phosphate (HPO_4^{2-}), soluble in water. As a result, demineralisation occurs.[24] Saponin acts as emulsifiers to reduce the surface tension of the solution. Saponin consists of hydrophilic

and hydrophobic groups. The hydrophilic group will bind to polar compounds from the organic smear layer, and hydrophobic groups will bind to non-polar compounds from the inorganic smear layer. Saponins also have distinctive physicochemical properties, namely foaming when soaked in the water. The chemical structure of saponins – consisting of glycosides (polar compounds) and triterpenes (non-polar compounds) – indicates that it belongs to a class of surfactants with detergent-like properties. This class of surfactants can dissolve polar and non-polar compounds.[25,26]

In contrast to *M. oleifera*, NaOCl does not contain surfactant directly. However, dissolution of organic tissue can be verified in the saponification reaction when sodium hypochlorite degrades fatty acids and lipids resulting in soap and glycerol.[27] In addition, saponisation reactions occur between NaOCl and root canal organic matter through neutralisation reactions and chlorination reactions. Amino acid neutralisation reactions occur when NaOCl neutralises amino acids into brine by removing hydroxyl ions, thereby lowering the pH. Chlorination is a reaction between hypochlorous acid contained in NaOCl solution in contact with organic matter, ending in a hydrolysis process.[28,29] The findings in this study corroborate with earlier reports from Khallaf *et al.* (202), where they reported leaves extracts of *M. oleifera* showed the least amount of smear layer on canal wall.[30] The result corroborates the use of the plant extracts traditionally as a smear layer removal agent.[31] This outcome suggests that *M. oleifera* leaves decoction promise an alternative irrigant.

Both *M. oleifera* leaves decoction (2.5% and 5.0%) and EDTA showed similar ability to remove the smear layer. EDTA 17.0% had a chelating effect. The chelating effect on EDTA occurred because at high pH (alkaline), excess hydroxyl ions will prolong the decomposition of hydroxyapatite and limit the number of calcium ions available. Thus, a negatively charged chelating agent will bind positively charged calcium ions from enamel or dentin.[32,33] Several researchers have reported that the chelating effect of EDTA use causes erosion of root canal

walls due to hyper decalcification. Therefore, the EDTA solution can be applied for a shorter time and in smaller volumes to minimize erosion.[34,35]

CONCLUSION

Within the limitations of this study, alternating the use of *M. oleifera* leaves decoction showed significantly better ability to remove the smear layer dentinal tubules compared to the use of NaOCl 2.5% and EDTA 17.0%. Therefore, we recommend a possible use of *M. oleifera* leaves decoction as an alternative to irrigant solution. Nevertheless, further long-term clinical studies are necessary to confirm these results and evaluate their relevance to treatment outcome.

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Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

The research protocol was approved by the Human Ethics Review Committee of the Faculty of Dentistry, Hasanuddin University (No 0032/PL.09/KEPK FKG-RSGM UNHAS/2018).

Authors contributions

YY, JJN, ACT, and LM carried out the research and collected the data. NN and MM designed and supervised the study, visualized and validated the data, and reviewed draft material. The data were organized, analyzed, and interpreted by CAR, who also reviewed the article. NN and LM organized, analyzed, and interpreted the data and revised the article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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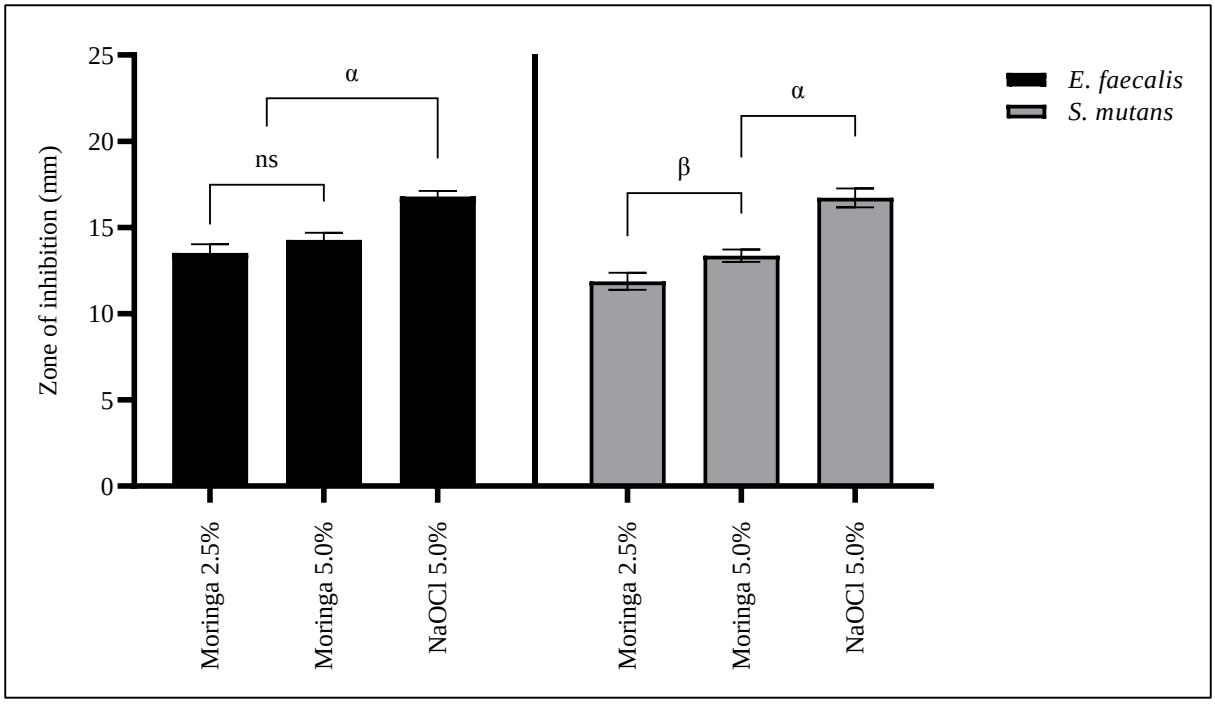

Figure 1 Antimicrobial activity of *M. oleifera* leaves decoction represented as the zone of inhibition mean (mm) for tested bacteria. Values are expressed as Mean ± SD (n = 3); analysis was performed with One-Way ANOVA followed by Tukey test with Post Hoc multiple comparisons; $^{(\alpha)}$ compared to NaOCl 5.0%; $^{(\beta)}$ compared to *M. oleifera* 2.5%; $^{(ns)}$ non-significant.

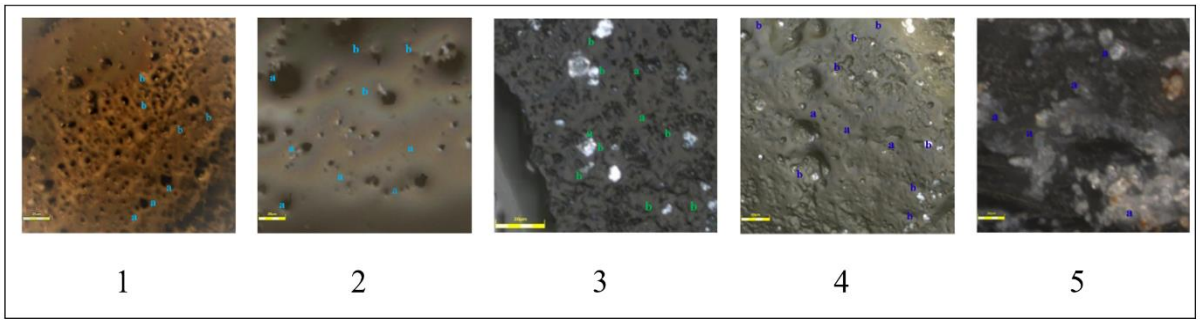

Figure 2 Representative CLSM micrographs (x) in each group: (1) *M. oleifera* 2.5%; (2) *M. oleifera* 5.0%; (3) NaOCl 2.5%; (4) EDTA 17.0%; (5) distilled water. (a) Dentinal tubules without smear layer, (b) Smear layer on the surface of dentinal tubules.

Table 1 Specifications of irrigants used.

Irrigant	Brand	Concentration (%)	Manufacture Country
EDTA	Onemed	17.0	PT Jayamas Medica Industri, Indonesia
NaOCl	Onemed	2.5	PT Jayamas Medica Industri, Indonesia

Table 2 Smear layer evaluation criteria.[17]

Score	Description
1	There is no smear layer, and all of the dentinal tubules are exposed
2	Some dentinal tubules and a little bit of smear layer were open
3	Only a few dentinal tubules are exposed due to a homogeneous smear film covering the root canal wall
4	Complete root canal wall covered by a homogeneous smear layer and no open dentinal tubules
5	Heavy homogeneous smear layer covering the complete root canal wall

Table 3 Phytochemical analysis for *M. oleifera* based on the preliminary decoction leaves' screening.

Phytochemical compounds	Presence
Tannin	+
Saponin	+
Flavonoid	++

Note: Absent= —, Trace = +, highly present = ++

Table 4 Means ± SD score of smear layer in the middle third of different groups, and the results of the Shapiro Wilk and Kruskal-Wallis tests.

Group	N	Mean	SD	Shapiro Wilk (P)	Kruskal-Wallis (p)
M. oleifera 2.5%	6	1.83	0.41	0.000*	0.001*
M. oleifera 5.0%	6	1.83	0.41	0.000*	
NaOCl 2.5%	6	2.33	0.52	0.000*	
EDTA 17.0%	6	2.83	0.41	0.001*	
Distilled water	6	4.83	041	0.000*	

Note: ∗ Statistically significant result ($p < 0.05$)

Table 5 Mann-Whitney test value to evaluate the difference between groups.

Group	*M. oleifera* 2.5%	*M. oleifera* 5.0%	NaOCl 2.5%	EDTA 17.0%	Distilled water
M. oleifera 2.5%					
M. oleifera 5.0%	1.000				
NaOCl 2.5%	0.001*	0.001*			
EDTA 17.0%	0.092	0.092	0.019*		
Distilled water	0.002*	0.002*	0.001*	0.001*	

Note: ∗ Statistically significant result ($p < 0.05$)