Antimicrobial Efficacy of a Novel Herbal Endodontic Irrigant Against Enterococcus Faecalis in Root Canals of Permanent Teeth: An in Vitro Study

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ABSTRACT
Background: A successful endodontic treatment is aimed at the sterilization of the entire pulp space. The use of extracts from *Rhamnus prinoides* as a novel irrigating material for root canal has not been studied . Hence, the antimicrobial efficacy of the alcoholic extract of *Rhamnus prinoides* as an irrigation material against *E. faecalis* was evaluated in comparison with the 2.5% sodium hypochlorite (NaOCL) solution used for root canals of permanent teeth. **Methods:** A total of 30 single-rooted human permanent teeth were thoroughly cleaned, shaped, and disinfected. Then, each tooth was subjected to a two-week infection with *Enterococcus faecalis* at 37 °C . Afterward, the samples were divided into three groups (10 teeth per group): 0.9% normal saline, 2.5% sodium hypochlorite, and 250 µg/ml *Rhamnus prinoides*. Paper points were used before and after irrigation to collect samples. Bacterial growth was evaluated after 24 h. Bacterial colonies were counted. The data were examined statistically via one-way analysis of variance, Dunnett's T3 post hoc test, and Shapiro–Wilk test. The level of significance was set at $p<0.05$. **Results:** The 2.5% sodium hypochlorite and 250 µg/ml *Rhamnus prinoides* demonstrated higher effectiveness against biofilm of *Enterococcus faecalis* than normal saline ($p<0.05$). The percentage of antibacterial effectiveness were 94.094 ±3.342, 93.685 ±5.280, and 25.603 ±3.912. **Conclusions:** *Rhamnus prinoides*, when used as root canal irrigant solution, was effective the sodium hypochlorite against the *E. faecalis* biofilm.

1. Introduction

Failure of root canal treatment can be attributed to various reasons, such as pathogen persistence, improper disinfection, failed coronal seals, and untreated missing canals. Thus, chemomechanical debridement in endodontic treatment mainly aims to eradicate or greatly decrease the amount of germs, which are of different types although obligate anaerobic bacteria are dominant, detected in infected root canals.[1]

Enterococcus faecalis (*E. faecalis*) is the main cause of periradicular infections through root canal therapy (RCT). This bacterium is an anaerobic, facultative coccus and can tolerate malnutrition because of its physicochemical features, including biofilm growth, antibacterial resistance, and the potency to enter dentinal tubules to a depth close to the cementum .[2] Therefore, the neutralization of bacteria and inactivation of their toxins depend on the effectiveness and proper biomechanical preparation and the application of a potent endodontic irrigation, which is very important in root canal cleaning, especially in areas difficult to be cleaned by mechanical devices, and aid in reducing the number of bacteria. [3]

Sodium hypochlorite (NaOCl) is one of the most popular irrigating solutions used for root canals. This mixture is the most commonly used solution due to its capability to break down organic tissues during chemomechanical root canal debridement.[4] However, the solution suffers from tissue-disintegrating activity that causes cytotoxicity in vital tissues;[5] in addition, if extruded accidentally beyond the apical foramen, it may cause heavy bleeding, severe postoperative pain, and swelling.[6]

Most existing (or currently used) endodontic irrigants have limits. Accordingly, an irrigant with a wide antibacterial efficacy, a short disinfecting time, and biocompatibility must be developed.[7] Herbs are increasingly gaining popularity in dental and medical treatment because of their biocompatibility.[8] Herbal therapy has experienced a rapid growth due to its beneficial characteristics, availability, and minimal level of side effects.[9] The increased resistance of biofilm bacteria to antibiotics, in addition to the adverse effects of such medications, prompted the investigation of medicinal plants as a possible antibacterial and anti-inflammatory compound. One of these

therapeutic plants is *Rhamnus prinoides* (Rhamnaceae). The Rhamnus fruit is edible and has been used for various medicinal treatments, including those for infectious disorders. This plant is well-known for its ethnomedicinal usage, such as nose, ear, and throat infections.[10] Its leaves can be prepared as liniment, which can reduce the pain caused by joint sprains, and the extract may be used to treat chest pains, common colds, stomach problems, fevers, malaria, diarrhea, and ringworm infections.[11]Depending on the qualitative phytochemical screening results, its methanol fraction may contain alkaloids, flavonoids, terpenoids, saponins, polyphenols, and tannins. These metabolites render the plant with antioxidant, anti-inflammatory, and antibacterial activities.[10]

Irrigation in root canal treatment using natural plant extracts as a substitute for NaOCl has been studied previously.[12-15] However, studies on *Rhamnus prinoides* as an alternative root canal irrigant are limited. Therefore, this study was conducted to evaluate the antimicrobial efficacy of *Rhamnus prinoides* alcoholic extract against *E. faecalis* compared with NaOCl.

2. Methods

Teeth Selection and Preparation

A total of 30 single-rooted human permanent teeth were used in this work. The exclusion criteria comprised teeth with fractures, internal or external resorption, or calcification. The root surfaces were cleaned carefully using periodontal curettes to remove any remanent of soft tissues, calculus if present, and bone. The teeth were disinfected through immersion in 5.25% sodium hypochlorite for 60 min prior to storage in a 0.2% thymol crystal solution.[16] Crown dissection was performed at the Cemento Enamel Junction . Tooth root length was reduced to 14 mm. The working length of each tooth was determined through the subtraction of 1 mm portion from the distance measured using the K-file and 1 mm from the apical foramen.[17] For canal preparation, Pro-Taper rotary files were used to prepare the canals up to F3 (the master apical file). Between applications of the rotary files, canal irrigation was performed with 5.25% NaOCl using a 5 ml syringe with an endodontic needle. After preparation, to remove the smear layer, we used a 30-gauge double-side vented endodontic needle to irrigate each canal for 3 min with 1 ml ethylenediaminetetraacetic acid (17%), 5 ml normal saline solution (0.9%), and 1 ml NaOCl. Finally, each canal was sprayed with 5 mL saline solution. Following biomechanical instrumentation, \ the apical foramen with composite fillings was sealed. The root surfaces then were painted with two coats of fingernail polish. [18] Next, the teeth were placed in screw cup glasses and autoclaved for 30 min at 21°C with 15 IB. [19]

Plant Material Collection and Extraction

Rhamnus prinoides leaves were collected after being confirmed to be free of insecticides. Plant extraction occurred in the Biology Science Department, College of Science, University of Diyala, Iraq. Extraction was carried out by obtaining 50 g clean and dry *Rhamnus prinoides* leaves before grinding them to powder using an electrical grinder converter. The powder was placed in a thimble (cylindrical container) and then placed in the location assigned to the Soxhlet apparatus. Using 90% methanol as the solvent, extraction was performed for 6 h at a temperature of 60 °C. Then, the extract was incubated with 70% methanol for two days. A filter paper (Whattman 1) was used for extract filtration. The alcoholic extract was treated with HCl (1%) and filtered again using Whattman 1. After the addition of diethyl ether in the funnel, the mixture was left for 24 h to allow separation of the two layers of the mixture. The superior layer (diethyl ether layer) was removed, and the inferior one was collected and adjusted to pH 8. [20] A lyophilizer (Operan, Korea Vacuum Limited, Korea) was used to dry the extract from alcohol and obtain the extracted powder, which was placed in a container where it was mixed with deionized water and vibrated in a water bath for 15 min to acquire the extract irrigation solution.

Bacterial Isolation

Swabs were used to isolate *E. faecalis* from infected root canals of healthy children aged 7– 14 years during their first visit for RCT. The children received no antibiotic therapy in the last three months,

had no systemic diseases, and were not taking any medication.[2] Prior to specimen collection, all parents of the participants signed a consent form.[21] Several samples were collected from roots that were suspected of harboring *E. faecalis*, and bacteria were identified after 24 h of culture via bile esculin test (selective test for the isolation of *E. faecalis* when a black deposit appeared on the agar plate). [22] A Vitek 2 system was used for the more accurate detection of *E. faecalis*. [23]

Antibacterial Activity

An agar well diffusion test was performed to evaluate the antibacterial property of the produced samples (*Rhamnus prinoides* leaf extract) against the microorganisms.[24] Aseptically, approximately 25 mL Mueller– Hinton (MH) agar (MHA) was placed in sterile Petri dishes. A sterile wire loop was used to gather microbial species from their stock cultures.[25] After growing the microorganisms, a sterile tip was used to create 6 mm-diameter wells on the agar plates. Various sample concentrations were introduced into the wells. At 37 °C, overnight incubation of the cultured plates (containing the samples and tested microorganisms) was performed before measurement, and the average diameter of the zone of inhibition [26] obtained during result analysis was recorded using GraphPad Prism program (Figure 1).

Determination of the Suitable Concentration of the *Rhamnus prinoides* Alcoholic

Extract Minimum Inhibition Concentration (MIC)

MH broth was used for microbroth dilution assays to determine the plant extract MIC against *E. faecalis*. The extract's concentrations were 15.62, 31.25, 62.5, 125, 250, and 500 µg/ml. After 24 h of aerobic incubation at 37 °C, the lowest plant extract concentration that caused neither visible microbial growth nor turbidity in the microbroth dilution assay was considered the MIC.[27] (Figure 2).

Minimum Bactericidal Concentration (MBC)

From the microbroth assay, 100 µL of the culture from each well was subcultured at 37 °C for 24 h in MHA plates to obtain the MBC of the plant extracts (the lowest extract concentration that reduced the viability of an initial microbial inoculum by 99% (no microbial growth); Figure 3).

Steps of Methodology [27]

1. A pure culture of (*Enterococcus faecalis*) was cultivated in nutritional broth.
2. A serial dilution of the antibacterial agent (*Rhamnus prinoides* alcoholic extract powder) was obtained through its dilution in deionized water at various concentrations (15.62, 31.25, 62.5, 125, 250, and 500 µg/ml).
3. In each tube, 8 ml nutrient broth, 20 µml culture, and the antimicrobial agent were added. Then, incubation at the appropriate temperature was conducted for 24 h.
4. After incubation, the series of dilution vessels was inspected for microbiological development at the bottom of vessels, and The MIC (Figure 2) and MBC (Figure 3) were determined.

Sample Inoculation

E. faecalis colonies were grown in a 5 mL Brain/Heart Infusion Broth (BHI) at 37 °C for 4 h. McFarland standard was used to adjust the turbidity of *E. faecalis* suspension to 0.5. [28] A sterile micropipette was used to deliver bacterial suspension to mechanically enlarged root canals. Then, the teeth were placed and kept for 2 weeks in BHI broth at 37 °C to allow the formation of a large bacterial colony.[29] To prevent nutrient depletion and the accumulation of hazardous end products, the culture medium was changed every other day. [30]

Antibacterial Efficacy of Irrigants

After incubation, all the teeth samples were carefully removed from the tubes, and a pre-irrigating sample (S1) was collected through insertion of a sterilized paper point (# 25) into the root canals for

1 min and then withdrawn. Afterward, the teeth samples were randomly divided into three experimental groups (for each group n=10). [31]

Irrigation Procedure

Group I (control): A 10-teeth sample irrigated with 5 ml 0.9% normal saline.

Group II: A 10-teeth sample irrigated with 5 ml 2.5% sodium hypochlorite.

Group III: A 10-teeth sample treated with 5 ml irrigation of 250 µg/ml *Rhamnus prinoides* leaf extract.

The irrigation for each sample lasted 5 min. Each solution was administered into the lumen of the canal using sterile plastic syringes (5 mL) with endodontic needles. At room temperature, all irrigation techniques were carried out by the same operator in a sterile environment.[6] Postirrigation sampling was then performed using sterile paper points (S2). The pre- and postsamples were vortexed for 30 s in Eppendorf tubes containing 1 ml normal saline.[31] Dilution was repeated . Each dilution was then pipetted and cultured for 24 h at 37 °C on blood agar plates. The number of colony-forming units (CFU) was counted, and the CFU/ml value was calculated.

Data Analysis

The data in this study were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Shapiro–Wilk test was used to test the normality distribution of the collected numerical data. Paired t-test was performed to compare the mean in pre- and postirrigation for each group, and one-way analysis of variance and Dunnett's T3 post hoc test were used to compare the mean reduction between groups. The significance level was set at $p<0.05$.

3. Results and Discussion

The maximum growth inhibition zone of *Rhamnus prinoides* leaf extract against *E. faecalis* in the agar diffusion test was detected at 500 µg/ml (27 mm), and the minimum inhibition zone was recorded at 62 µg/ml (23 mm) (Figure 1). The MIC of *Rhamnus prinoides* leaf extract against *E. faecalis* was 125 µg/ml (Figure 2), and the MBC was 250 µg/ml.

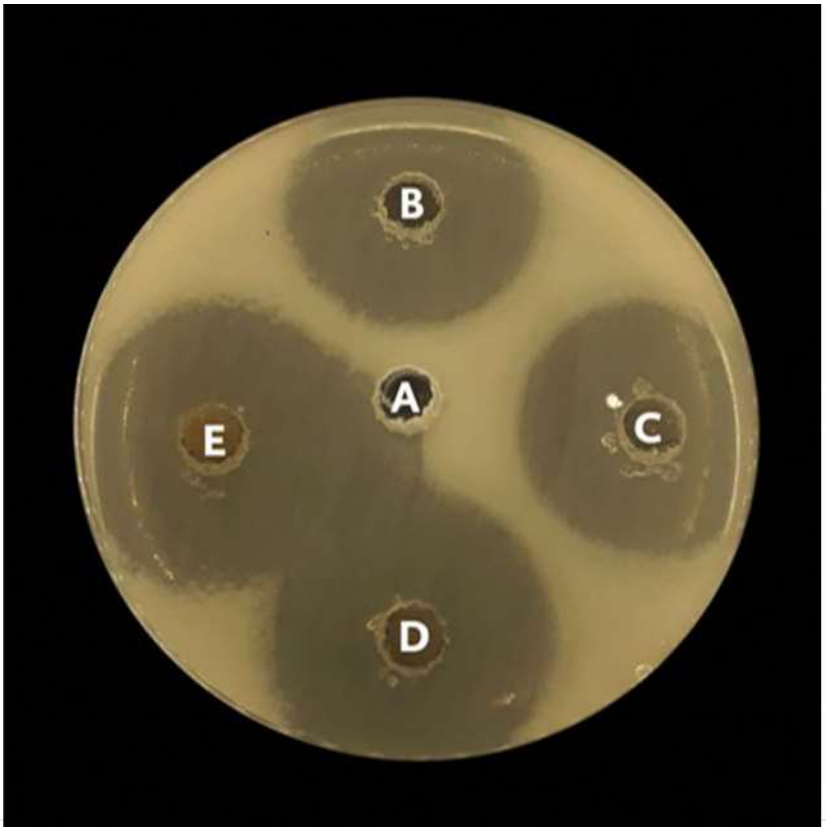

Figure 1. *Rhamnus prinoides* leaves extract antibacterial activity against *E. faecalis*

Control (6mm), B. 62.5 microgram/ml (23 mm), C, 125 microgram/ml (24mm), D, 250 microgram/ml (26mm), E. 500 microgram/ml (27mm).

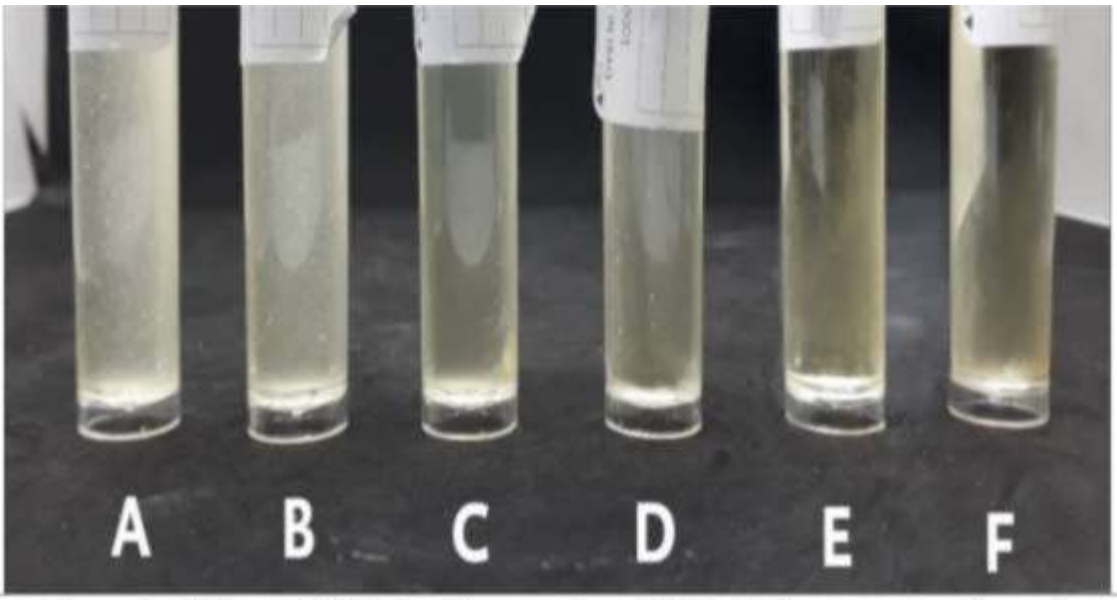

Figure 2. Minimum Inhibition Concentration (MIC) of *Rhamnus prinoides* against *Enterococcus faecalis.*

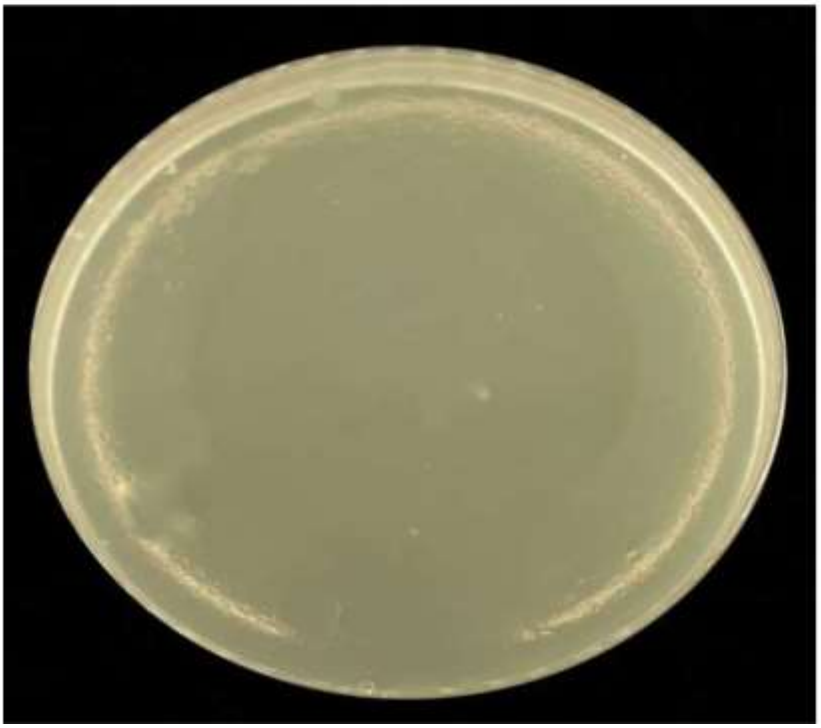

Figure 3. (MBC) For (Enterococcus faecalis) (250 µg/ml)

In this study, all three irrigation solutions used substantially decreased the mean of *E. faecalis* (CFU) (Table 1). The NaOCL group had the greatest effect size, followed closely by the *Rhamnus prinoides*

leaf extract group, whereas the normal saline group exhibited the lowest effect size. A significant difference was found between the normal saline group and the NaOCL and *Rhamnus prinoides* leaf-extract groups. However, no significant difference was found between the later groups (Table 2).

Table 1. Descriptive and statistical test of *E. Faecalis* (CFU×10^4) among groups:

Groups		**Pre**	**Post**	**P Value**	**Efficiency (Mean ±SD)**
Normal Saline	Mean	2881.700	2087.400	0.006*	25.603±3.912
	±SD	2352.254	1678.956		
2.5% NaOHCL	Mean	2883.400	83.100	.00407*	94.094 ±3.342
	±SD	2352.163	42.094		
Rhamnus Prinoides	Mean	2886.000	111.900	.00431*	93.685 ±5.280
	±SD	2353.079	76.580		
F		0.00	14.011		
P value		1.00 NS	0.000 **		

*Significantly different resulting from paired t- test

** Significantly different resulting from one-way ANOVA test and post hoc Tukey's tests Table

2. Multiple Comparisons of *Enterococcus faecalis* Post Irrigation Using Dunnett's T3

Groups		**Mean difference**	**P value**
Normal Saline	2.5% NaOHCL	2004.300	0.012*
	Rhamnus Prinoides	1975.500	0.014*
2.5% NaOHCL	*Rhamnus Prinoides*	-28.800	0.662

* Significant difference $p<0.05$

In dentistry, studies on the possible application of natural herb substances have remarkably increased.[32] To the best of the researcher's knowledge, this work is the first in vitro study to investigate the potential use of *Rhamnus prinoides* as an endodontic irrigant. Irrigation is essential for effective RCT. This process involves several important functions, some of which depend on the irrigant being used, including decreasing the friction between the instruments used and internal root surface, promoting the files and cutting efficiency, decreasing the generated heat of the tooth and file, and attaining chemical debridement and antibiofilm/antimicrobial effects.[33] In addition, the use of irrigating solution is the only means to reach areas in the root canal system that cannot be reached by mechanical instruments.[3]

The phrase "phytotherapy, ethnopharmacology, or phytomedicine" refers to the use of herbs to treat various illnesses. The use of herbal medicine has remarkably expanded in recent years. The use of herbal alternatives offer benefits, including the simplicity of access, long shelf life, affordability, minimum toxicities, and absence of resistant microbes, due to their excellent antimicrobial and anti-inflammatory properties, biocompatibility, and antioxidant effects; herbal alternatives to standard

RCT are also gaining popularity. [34-35]

The level of *E. faecalis* is low (4%–40%) in initial endodontic infections and greater (24%–77%) in chronic infections.[36-37] Given its unique properties, *E. faecalis* can prevent chemomechanical instruments from functioning properly during root endodontic therapy. Such attributes include the ability to colonize and produce biofilms in peripheral and inaccessible regions. Apical deltas, auxiliary canals, and isthmuses are examples of areas located distantly from primary canals. They can be protected by dentinal and remaining tissues, dead cells, and human serum, which lower the efficiency of antibacterial drugs. In addition, *E. faecalis* uses several strategies to survive in harsh environments. These strategies include the activation of certain survival genes, inhabiting a nutrient-rich environment via various metabolic pathways, and synergizing and aggregating germs. [38]

Given its antibacterial and tissue-disintegrating characteristics, NaOCl is the most extensively utilized endodontic irrigant. However, NaOCl suffers from disadvantages, such as an undesirable odor and taste, intense corrosion to metals, and extrusion of NaOCl outside the tooth apex, which causes toxicity.[39] Herbal alternatives in RCT are becoming increasingly popular. One of these herbals is *Rhamnus prinoides* leaf extract, which has an antimicrobial effect that may be related to the synergistic or additive effect of the plant's secondary metabolites.[40] In addition, *Rhamnus prinoides* possess triterpenoids, which exert anti-inflammatory and antimicrobial effects, and tannins, which have antioxidant and antimicrobial activity.[41] The initial findings of the present study on showed that *Rhamnus prinoides* had a MIC of 125 µg/ml and MBC of 250 µg/ml against *E. faecalis* . In regard to the cytocompatibility of *Rhamnus prinoides* leaf extract, the MBC used in this study deviates from the result utilizing median lethal concentration, which was used to determine a cytotoxic concentration of 5000 µg/ml in a previous study.[20]

The current study's findings revealed the high efficiency of using extracts (herbal product) as an irrigant to kill *E. faecalis* in the root canal. Accordingly, the extract can be used as an alternative to replace the traditional sodium hypochlorite. These results can be related to the anti-inflammatory effect of *Rhamnus prinoides* leaf extract. This outcome is consistent with that of a prior study which proved that glycosides and flavonoids not only constitute this plant's essential constituents but are also responsible for its powerful anti-inflammatory and antioxidant effects. [42]

4. Conclusion

Overall, the findings of this current study highlight the potential of *Rhamnus prinoides* extract as an irrigating solution for the improved efficacy of endodontic bacterial elimination. We have proven that this novel endodontic irrigation solution attained the same level of efficiency as the conventional NaOCl irrigant in reducing *E. faecalis* CFU counts. Successful outcomes are expected from endodontic treatments, and this novel irrigating solution has the potential to reduce failures. Within the limitations of this study, at 2.5% NaOCl, *Rhamnus prinoides* extract served as an effective irrigant against *E. faecalis*. This substance may serve as an alternative to conventional NaOCl because of its affordability and antimicrobial properties and can be a reliable substitute for other commercially available irrigants.

Recommendations

The outcomes of the present research may vary in practical settings. Further clinical studies are needed to demonstrate and prove the clinical applicability and efficiency of various dosages of *Rhamnus prinoides* extract as an irrigating solution against *E. faecalis* and other types of microorganisms related to endodontic diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

The Research Ethics Committee at the University of Baghdad, College of Dentistry, approved the conduction of this study (letter ref: 727 dated the 28th of December 2022).

Authors contributions

H R R: conceptualization, data curation, investigation, methodology, writing of original draft; writing of review and editing. A H A: supervision, validation, visualization, writing review and editing. 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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