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Evaluation of the Antibacterial and Cytotoxic Effect of MyrtusCommunis Essential Oil Irrigate as Root Canal Irrigant Using Confocal Laser Scanning Microscopy (An in-vitro study)

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Ethical approval:

The protocol of this trial was reviewed and approved by the Research Ethics Committee (REC) – Faculty of Dentistry, October 6 University with approval number (RECO6U/28-2023)

- The study was conducted in accordance with the Declaration of Helsinki

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ABSTRACT

Objective: Evaluation of the antimicrobial and cytotoxic effect of Myrtus communis (M. communis) essential oil as well as chlorohexidine 2%, compared to that of sodium hypochlorite (NaOCl) in mature single-canaled maxillary central incisor teeth. **Materials and Methods:** 47 mature maxillary central incisor teeth were used in the study. All teeth were autoclaved at a temperature of 121◦C for 30 minutes, two cycles, then Enterococcus faecalis was introduced into the teeth. Samples were kept under humidity at 37 ◦C for three weeks at the incubator. After 3 weeks teeth were divided into 4 groups; Group 1 (control group) 2 teeth. Group 2 (Myrtus communis) 15 teeth. Group 3 (2% CHX) 15 teeth. Group 4 (5.25% NaOCL) 15 teeth. The antibacterial effect was assessed using confocal laser microscopy. The cell viability of all teeth was evaluated using the MTT colorimetric assay. **Results:** The 5.25% NaOCl group showed the highest percentage of dead cells. The cell viability percentage was highest for Myrtus communis essential oil, while 5.25 % NaOCL had lower viability than the 2% CHX at different concentrations. **Conclusions:** M.communis essential oil has a more positive impact on cell viability. However, 5.25% NaOCL exhibits the highest antibacterial effect.

Keywords: Antibacterial effect, cytotoxicity effect, Myrtus communis essential oil, sodium hypochlorite, confocal laser microscopy,

Introduction

Microorganisms play a crucial role in causing inflammation and necrosis of the pulpal tissues, leading to the formation of periapical lesions. So, Root canal treatment is mandatory. It is important to eliminate these microorganisms, but it is difficult due to the complex and irregular root canal configuration and the inability of instruments to touch all the dentinal canal walls. Therefore, mechanical enlarging and chemical disinfecting are essential for proper disinfection (1). Chemical and mechanical root canal debridement are the primary methods to remove all dead tissue, bacteria, and microbial byproducts from the canal. NaOCL is a great option for disinfecting surfaces, but if it goes beyond the apex, it can have harmful effects such as a burning sensation, and destroying vital cells and organs. The disinfection protocol during root canal treatments uses chlorhexidine 2% as the gold standard of irrigation solutions. The utilization of herbal products as root canal disinfectants has been extensively researched in endodontics due to their proven effectiveness, safety, and availability. Natural herbal irrigants like tea tree oil and Aloe vera are natural antibacterial agents that can be effectively used as root canal disinfectants and can be used to inhibit bacterial biofilm (2). Essential oils and extracts have demonstrated effective antibacterial and antifungal properties. One of the essential oils subjected to investigation in dentistry is Myrtus communis, which has strong antibacterial effects without being harmful to the tissue due to its active natural ingredients. Cytotoxicity is the ability of a substance to affect cellular viability and can be assessed through various physiological endpoints, including reduced cell growth and proliferation, necrosis, apoptosis, or a combination of these factors. Assessing the cytotoxic activity of intracanal medicaments is crucial as it influences the biological and physiological behavior of cells. The study aims to evaluate the antimicrobial and cytotoxic effect of Myrtus communis (M. communis) essential oil as well as chlorohexidine 2%, compared to that of sodium hypochlorite (NaOCl) in mature single-canaled maxillary central incisor teeth.

Materials and Methods

A. Sample selection:

47 Mature human maxillary central incisor teeth were collected in this study from the oral surgery department at the Faculty of Dentistry, October 6 University. Teeth were inspected to exclude any obvious fractures, caries, craze lines, curvature, and external resorption. Teeth were examined radiographically to exclude any sample with calcification or internal resorption.

B. Sample preparation and Sterilization:

The chosen samples were planned with an ultrasonic scaler (Suprasson P5 Booster, Satelec, France) to weed out any calculus, hard deposits, and soft tissue remnants on the root or remaining bone. Decoronation was performed by fine tapered diamond stone mounted on a high-speed hand-piece (PANA AIR, NSK, Japan) at the level of cementoenamel junction under copious flow of coolant. The length of selected samples was adjusted to be fixed at 15 mm. The root apices of each tooth were sealed by a small amount of composite resin, to prevent seepage of the bacterial suspension during the incubation period in the autoclave, as well as to aid in the retention of the irrigating solution within the root canal to ensure their presence among the contact period to get the maximum and standardized effect to simulate in vivo apical counter pressure. Then sterilized by autoclaving at a temperature of 121◦C for 20 minutes (3).

C. Root canal preparation:

All selected samples were prepared as the following:

1. Pulp tissue extirpation from all root canals using # 15 -20 K-St St file (MANI, Inc, Japan)
2. Root canal patency checked using # 15 K-St St to create a pathway and glide path for rotary files.
3. Mechanical preparation through crown down technique using Pro-Taper Next (PTN, Dentsply Maillefer, Switzerland) rotary system.

4. The Pro-Taper Next files were mounted on a contra-angle handpiece that was attached to a torque-limiting motor E-connect Pro endo motor rotary device (Changzhou Eighteenth Medical Technology Co., Ltd, China) at a speed of 300 RPM and torque
(0.8 N) as preferable by the manufacturer with a sequence X1, X2, X3 to a final size X4 (40/0.06).
5. Irrigation was performed between each rotary file with normal saline.

D. Preparation of irrigating solution:

Myrtus communis essential oil 70 % methanol extract:

A 100g of Myrtus communis powder was mixed with 500 ml of 70% methanol in a sterile jar. It was left at room temperature for 2 days then filtration with No.1 filter paper 150 µm diameter was applied. The filtered solution was dried in a humidity field at 70°C for 1 week. The dried powdered extract was kept in a firmly sealed vial at 4°C until needed. Concentrations ranging from 10 to 500 mg/mL were produced from a stock solution in distilled water .100 grams of Myrtus communis were immersed in 1,000 milliliters of
sterilized boiling water.After 4 hours, the solution was filtered using No. 1 filter paper (Whatman Co, Germany) and dried in a water bath at 70°C. The stock solution was divided into 10 to 500 mg/ml concentrations (4).

Microorganism Biofilm preparation:

Proper preparation of growth media for brain heart infusion (BHI)**,** followed by a pure bacterial culture of gram+ *cocci* (#29212, ATCC Manassas, VI), *Enterococcus faecalis* obtained from American-type culture**.** *Enterococcus faecalis* was introduced in 7 ml of (BHI)**,** and incubated at 37°C for 24 hours. Then suspension was prepared on the BHI plates under the same incubation conditions. Bacterial cells were resuspended in saline and adjusted to 3x10^8 cells/ml using sterile loops, following #1 McFarland turbidity standard. *Enterococcus faecalis* was inoculated into 5 ml of sterilized (BHI) blended with 5 ml of bacterial inoculum using micropettes to fill each root canal, and the samples were allowed to incubate for two weeks to allow bacterial biofilm formation. *,* However, every 72-hour interval, this trial was repeated using pure cultured samples adjusted to the #1 McFarland standard.

Samples were kept under appropriate environment and humidity at 37 ◦C for three weeks at the incubator to ensure complete biofilm formation. The samples should be centrifuged twice during the incubation period at a specific time interval.

Biofilm confirmatory group:

Before irrigating the root canal, 2 samples were used as a negative control group to ensure complete bacterial biofilm formation. The root canal was then scanned using confocal laser microscopy.

Sample classification and irrigation:

45 Samples were classified according to the final irrigation solution into three experimental groups.

Group (A): 15 samples were irrigated with 5 ml Myrtus communis essential oil 5 mg/ml by plastic syringe with a 23-gauge needle for 2 minutes (5).

Group (B): 15 samples were irrigated with 5 ml CHX 2% by plastic syringe with a 23-gauge needle for 2 minutes (5).

Group (C): 15 samples were irrigated with 5 ml NaOCl 5.25% by plastic syringe with a 23-gauge needle for 2 minutes (5).

All canals were dehydrated by paper points after 10 minutes and then irrigated with Sodium thiosulfate to offset the effect of sodium hypochlorite. Samples were kept at a low temperature of -5 ◦C to prevent the growth of microorganisms.

Method of evaluation:

1. Antibacterial effect using confocal laser scanning microscopy:

- **Sample cutting:**

The extracted human teeth were divided using a 0.3 mm Isomet saw under steady cooling with sterile distilled water to enable imaging. From each root, two 1-millimeter sections were taken, which represented the apical and middle third of the root respectively. The sample size was 5*5*1 millimeters. All the needed samples were kept in an ice box at a temperature of -4 °C for fixation of microorganism growth.

Sample preparation for imaging by confocal laser microscopy:

Washing the dentin segments carefully with 100 µl sterilized distilled water for one minute and then dried samples smoothly. Put the washed dentin segments at the bottom of the Eppendorf tube. 10 µL of propidium iodide (PI) red fluorescence and 100 µL of each 0.01 % acidrine orange (AO) green fluorescence were added to the selected specimen to be stained in a dark room, remaining for 15 minutes after centrifuging for 10 seconds. Removing the specimen from the tube and then washing it twice with 100 µl sterile distilled water to eliminate surplus dye. After that specimen was transferred to glass-covered slips, covered with immersion oil before performing any image acquisition

Stain and laser specification:

Confocal radiance was performed by application an argon laser microscope that was fitted to 460 nm emission for (PI) and 500 nm for (AO). Fluorescence from the tinged specimen cells was viewed through using cone focal laser scanning microscopy. Dye material can bind bacterial DNA radiating green fluorescence and bacterial RNA emitting red fluorescence, after staining, the corresponding specimen was immediately analyzed by CLSM technique. PI enters only the dead cells membrane exhibiting red fluorescence, while AO stains the living cells exhibiting green fluorescence.

Image analysis:

Analyzing the fluorescent image with the software of ZEN imaging (zen 2012 Blue edition), viewing the image stacks with the LSM browser. Initial stacks comprising both red and green fluorescence were divided into separate item color channels, saving it as grayscale image and then measured to set voxel size. Bacterial life was demonstrated as the ratio % of green voxels from entire fluorescence. For evaluation of viability, confocal laser images were analyzed into 2 dimensions, and quantitative analysis obtained through using the software Zeiss 2012 that produced information on the total biofilm population.

Results

Statistical analysis:

Statistical analysis was performed with SPSS 20®, Graph Pad Prism®, and Microsoft Excel 2016. (Cairo, Egypt)

I. Bacterial count reduction and Biofilm:

The Mean and standard deviation of eradicated bacteria, remaining bacteria count, and percentages of eradicated bacteria in all groups were presented in Table (1) and Figures (1,2).

Comparison between different groups was performed by using OneWay ANOVA test which exhibited a significant difference between all groups regarding eradicated bacteria and remaining bacteria count and percentage of eradicated bacteria as $p<0.05$, followed by Tukey`s Post Hoc test for multiple comparisons which revealed that:

In the remaining bacterial count: group A (51462.1±9241.66), Group B (50475.1±8434.05), and the negative control group (50633.25±7700.96) were significantly the highest with insignificant differences between them, while group C (42361.6±3359.6) was significantly the lowest.

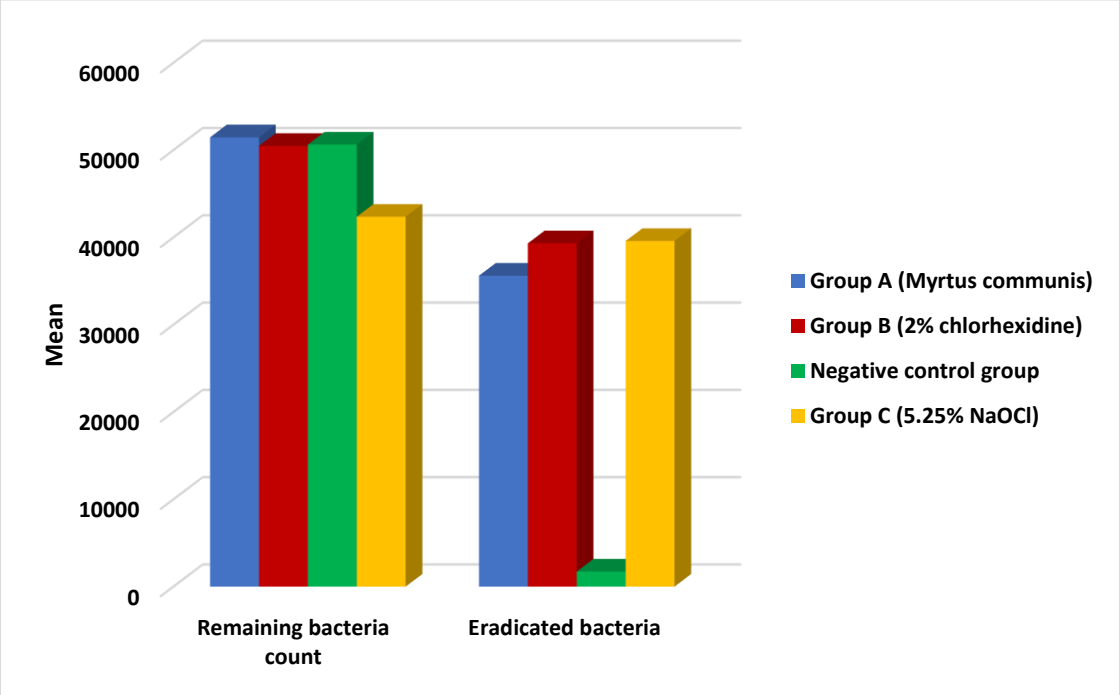
In eradicated bacteria: the negative control group (1719.92±729.88) was significantly the lowest, while group C (39588.65±3879.36)was significantly the highest.

In % of dead bacteria: the negative control group (3.24±1.2)was significantly the lowest, while group C (47.68±4.42) was significantly the highest.

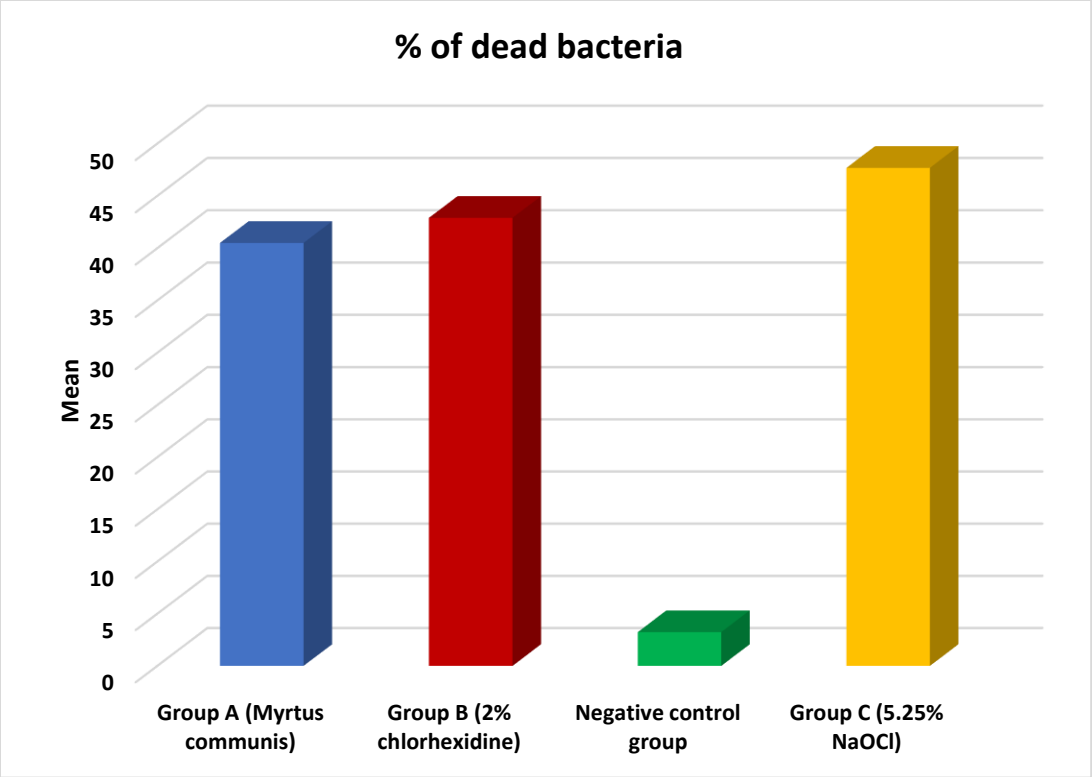
(Table 1): Mean and standard deviation of eradicated bacteria and remaining bacteria count in all groups and comparison between them using One WayANOVA test followed by Tukey`s Post Hoc test:

	Group A (		Group B		Negative control group		Group C		P value
	M	SD	M	SD	M	SD	M	SD	
Remaining bacteria count	51462.1 a	9241.66	50475.1 a	8434.05	50633.25 a	7700.96	42361.6 b	3359.6	0.004*
Eradicated bacteria	35607.4 a	9455.61	39323.3 a	11503.10	1719.92 b	729.88	39588.65 a	3879.36	0.05*
% of dead bacterial	40.5 a	2.58	42.9 a	4.82	3.24 b	1.20	47.68 c	4.42	0.0001 *

**Significant difference as p<0.05.*
Means with the same superscript letters were insignificantly different a p>0.05. Means with different superscript letters were significantly different a p<0.05.



(Fig.1): Bar chart showing eradicated bacteria and remaining bacteria count in all groups.



(Fig.2): Bar chart showing % of dead bacteria in all groups.

Discussion

Long-standing root canal infections tend to harbor a several count of facultative anaerobes, and *E. faecalis* is frequently cultured from failed root canals. The presence of biofilm around bacteria makes them highly resistant to antimicrobial agents, complicating treatment. Therefore, using irrigating solutions with strong antimicrobial activity is crucial for successful root canal therapy.

CHX is a powerful tool in endodontics, acting as an effective irrigating substance. Its broad-spectrum antimicrobial activity, and lubricating properties. It is odor-free and water-soluble. It is effective against Gram-positive and Gram-negative bacteria, facultative and strict anaerobes (7), yeasts, and fungi, particularly Candida albicans (8).

CHX kills bacteria in 30 seconds or less in its liquid form, while in the gel form, it takes from 22 seconds (2% CHX gel) to 2 h (0.2% CHX gel) (9). A drawback compared to NaOCl is its inability to dissolve necrotic and vital tissue.

NaOCL is the most widely employed irrigant due to its potent antimicrobial properties, tissue-dissolving capabilities, and lubricating action. It is used in various concentrations, and while its antimicrobial activity is not concentration-dependent, its ability to dissolve tissue and disrupt biofilm is concentration-dependent.

Despite its high antimicrobial activity, effective tissue dissolution, accessibility, and lower cost, NaOCL has certain disadvantages. These include its cytotoxic effects on surrounding tissues, and its potential to cause bad odor and allergic reactions. Recent findings have also shown that it has a detrimental impact on dentin elasticity and bending resistance.

To avoid NaOCL complications, many authors have demonstrated the effectiveness of herbal agents as an alternative or adjunct to conventional root canal irrigant solutions. One of the natural essential oils in dentistry is Myrtus communis, which has strong antibacterial effects without being harmful to the

tissue due to its active natural ingredients.

The selected samples were stored in an aqueous solution containing disinfectant to avoid dehydration and further bacterial growth (10). *E. faecalis* was chosen for our study because it is a pathogenic microorganism capable of life-saving extreme challenges. *Enterococci* are infrequently found in primary endodontic infections, but they make up a substantial 29-77% of secondary endodontic infections. This divergence in incidence has been linked to post-endodontic coronal leakage., iatrogenic causes, or leaving the root canal exposed to the oral environment (11).

CLSM is critical because it is an effective approach for distinguishing live and dead bacteria within the tubules in situ, to define the viability profile, architecture, and geographic distribution in microbial biofilms (12).

CLSM offers benefits for observing surviving bacteria in root canal walls, discovering labeled bacteria through in situ hybridization, and exploring the structural organization of biofilms in 3D.

The CLSM analysis provides intelligence about the severity of dentine infection and the survival of bacteria within the dentinal tubules at the cellular level. This method shows potential in elucidating the relevance of remaining microorganisms in an uncultivable form (13).

The ongoing study aims to unveil the antibacterial behavior of Myrtus communis essential oil versus sodium NaOCL and CHX. Using results obtained by CLSM we calculated the mean value of the percentage of dead cells. All irrigant solutions showed antibacterial effects against *Enterococcus faecalis* biofilm. The NaOCl group showed the highest percentage of dead cells (48.65%) which was significantly higher than the CHX group (42.9%) and Myrtus communis essential oil (40.5%) as ($P<0.05$), while the negative control group showed the least percentage of dead cells (3.24%).

Chemical composition of Myrtus communis: 1,8-cineole, α-pinene,and linalool are the main components of M. communis essential oil and have been

extensively proven as strong antimicrobial substances (14).

M. communis essential oil is prominent for its antimicrobial activity, which is usually attributed to its chemical composition ample in monoterpene hydrocarbons and oxygenated monoterpenes such as linalool, carvacrol, α-pinene, and 1,8-cineole (15-17).

There are few studies in the literature that explore the Myrtle EO's ability to prevent the creation of biofilms by pathogens (18).

This came inagreement with Mehrsorosh et al (19) who informed its effectiveness in suppressing biofilm formation by various Candida species, including *C. albicans, C. parapsilosis, and C. tropicalis*. The essential oils with high monoterpenes hydrocarbons are very active against microorganisms (20). In another study, Nashaat et al (21) compared the antibacterial activity of sodium hypochlorite, chlorhexidine, and nano silver irrigant solutions against Enterococcus Faecalis biofilm, sodium hypochlorite showed the highest antibacterial activity, with a statistically significant difference followed by chlorhexidine and nano-silver irrigants.

The effectiveness of NaOCl is due to its ability to eliminate microorganisms within the root canal. Hypochlorite acid is a potent oxidizing agent that develops an antimicrobial effect by irreversibly oxidizing the hydrosulfuric groups of bacterial enzymes. This disruption affects the metabolic functions of the bacterial cell, ultimately leading to the death of bacterial cells. In addition, by binding to bacterial cytoplasm components, chlorine creates highly toxic N-chloro compounds, effectively eradicating the microorganisms (22).

The Studies have demonstrated a notable antibacterial efficacy of sodium hypochlorite against prevalent oral pathogens. This efficacy was found to be higher when compared to the essential oil extracted from Myrtus communis leaves (17).

Conclusion:

M. communis essential oil is represented naturally and efficiently as a root canal irrigation for reducing *E.faecalis* biofilm but it is not highly effective.

Clinical Significance:

M. communis essential oil extract is a good natural root canal irrigation because it is the lowest toxic for tissues. Still, it is not better in antibacterial effect on microorganisms than NaOCL and CHX.

List of Abbreviations:

Sodium Hypochlorite	NaOCL
Chlorohexidine	CHX
Mytrus Communis	*M. communis*
Confocal Laser Scanning Microscopy	CLSM
Brain heart infusion	BHI

The highlights:

1-Antibacterial effect of Myrtus communis essintiol oil extract

2- Cytotoxicity effect of Myrtus communis essintiol oil extract

3- confocal laser scanning microscopy identification

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