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Antimicrobial Efficacy of Sonically Activated Salvadora persica and Glycyrrhiza glabra Extracts against *Enterococcus Faecalis* An in-vitro study

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Abstract— **The aim**: is to evaluate antibacterial efficacy of sonically activated (Salvadora persica / Glycyrrhiza glabra Extracts) compared to sonically activated (sodium hypochlorite / EDTA) against Enterococcus Faecalis in an in-vitro model. **Material and methods**: Sixty Human mandibular premolarswere collected and a standardized tooth length of 18mm was established. Then root canals were instrumentedand all samples wereautoclaved. 15 specimens were then subjected to microbial analysis and served as negative control group.The remaining 45 specimens were inoculatedwith*E faecalis*suspension.15 specimens served as Positive control group,15 specimens served as Group (A) where sonically activated (2.5 % NaOCl / 17% EDTA) was applied and15specimensserved as Group B where sonically activated (Glycyrrhiza glabra / Salvadora persica Extracts) was applied . For all specimens were subjected for microbial analysis. **Results**: group A (NaOCl / EDTA) recorded the lowest median bacterial count at 0.6 log CFU while; group B (G. glabra /S. persica) showed median bacterial count of 1.2 log CFU. The results showed a statistically significant difference in comparison with positive control group $p < 0.001$ indicating the strong antibacterial activity of both irrigation protocols of group (A, B). Furthermore group A showed a statistically significant more reduction in bacterial count than did group B at p value < 0.001; indicating the superior antibacterial activity of group A. **Conclusion:** sonically activated G. glabra / S. persicacan be considered as a strong antimicrobial protocol.However;sonically activated NaOCl / EDTAstill is a superior antimicrobial protocol.

Introduction:

Microorganisms play a major role in initiation of pulpal and periapical diseases. Therefore, elimination of the microorganisms present in the complex root canal system is the fundamental objective of endodontic treatment; while this statement seems simple it isn't. Mechanical debridement and shaping of the root canal falls short of totally eradicating all microorganisms from the root canal hence ,different irrigating solutions are used not only to help in removing debris from the root canal but also in dissolving soft tissues and killing bacterial especially from areas inaccessible to instruments. [1, 2, 3, 4, 5]

Enterococcus Faecalis is one of the most commonly identified microorganisms in failed endodontic treatments. [6, 7] This opportunistic facultative anaerobic gram positive cocci is known for its resistance among root canal flora, and has been frequently isolated in both pulpal and periapical lesions. Many studies have linked this to its virulence factors which include lytic enzymes, lipoteichoic acid, pheromones, cytolysinand aggregation substances, its high affinity to bind to dentin collagen, invade the dentinal tubules and its high ability to

suppress lymphatic activity which potentially contributes to endodontic treatment failure.[6, 7, 8, 9]

Sodium hypochlorite has been considered for decades as the gold standard endodontic irrigant with soft tissue dissolving characteristic, wide range antimicrobial activity and lubrication[10, 11, 12]. On the other hand many side effects have been observed over the years as toxicity to the periapical tissue, allergic reactions, reduction of flexure strength of dentin leading to its weakness and susceptibility to deformation and fracture, extruded into the periapical tissue causes serious side effects as sever inflammation, ecchymosis, hematoma, necrosis and Parathesia [13, 14]. These inherent drawbacks of chemical irrigants and constant increase in antibiotic resistance create an ongoing urge to discover and explore new natural alternative medicament

Over the last decades, plant antimicrobial activity has been studied in different regions of the world [15].Glycyrrhiza.glabra, commonly called as Licorice, is one of the important traditional medicinal plants grows in the various part of the world and has been used for medicinal purposes foryears. Root of this plant has several usefulpharmacological properties such as anti-inflammatory, antiviral, antimicrobial, and anticancer activities in addition to immune-modulatory, hepato-protective and cardio-protective effects [16].It is a soothing plant that is beneficial in alimentary tract disorders and mouth ulcers [17]. Although thereare some studies on antimicrobial activity of Licorice on skin, respiratory and urinary system pathogens but there is very little research about endodontic pathogens [18]

Salvadora persica (miswak)belongs to the family Salvadoraceae[19]. *S. persica* (arak) is mainly present in the Arabian Peninsula, India, Iraq, Sri Lanka, Pakistan, and Africa[20]**.** *S. persica* was the firstplant used in oral hygiene as a toothbrush; it is named the toothbrush tree (miswak)[21].In 2000, WHO approved miswak as an effective and low-cost toothbrush, which can beused routinely [22]. A variety of studies found that the leaves of this plant can be used fortheir hypoglycemic, antiplasmodial, analgesic, diuretic, antiseptic, antifungal, antibacterial,and anticaries properties[23, 24]. The stem of *S. persica* has profound antifungal, antimicrobial, and antiplaque actions [24]. Miswak contains multiple bioactive ingredients. The most important andeffective ingredient is benzyl isothiocyanate, a major antimicrobial volatile oil [25]. This plant also contains *N*-benzyl-2-phenylacetamide, whichhas shown efficacy against *Escherichia coli* [26]. The endodontics literature elucidates the variety of uses of *S. persica* for root canal irrigation[27], smear layer removal [28],and intracanalmedication[29].

In terms ofroot canal irrigation, various devices have been developedto enhance the efficiency of the root canal irrigants.Activated irrigation may be defined as using a method to agitate and improve the flow of irrigants to the intricacies of root canal system by mechanical or other energy forms.There are many mechanical irrigant activation systems available in the market. One of which is an Innovative sonic-powered irrigation (EDDY) uses flexible polyamide tips to preventcutting dentin and changing root canal morphology duringsonic activation at high frequency, which is useful in removing debris and organic tissues from canal walls [30 , 31]

The null hypothesis is that there is no difference in antimicrobial efficacy between using sonically activated (Salvadora persica and Glycyrrhiza glabra Extracts)and sonically activated (sodium hypochlorite and ethylene diaminetetraacetic acid)against Enterococcus Faecalis in an in-vitro model.

Aim of the study:

The aim of this study is to evaluate antibacterial efficacy of sonically activated (Salvadora persica and Glycyrrhiza glabra Extracts) compared to sonically activated (sodium hypochlorite and EDTA)against Enterococcus Faecalis in an in-vitro model.

Materials and Methods

[1] Study design:

Comparative- control in vitro study.

[2] Sample size calculation

Based on a previous study by Saha et al, [32] sample size for antibacterial action as primary outcome was calculated using power of G. it was found that the appropriate sample size for the study was a total of 60teeth. GroupA, B (15 teeth each) and15 teeth as negative control and 15 teeth as positive control. The power is 80% and α error probability =0.05.

[3] Ethical regulation

- **a.** Before commencing the study; itwas approved by the ethical committee of Faculty of Dentistry, Minia University. Under no. (98/793)
- **b.** Patients or Parents of the patients signed a written informed consent for using their teeth in the study.
- **c.** Following completion of the study teeth were collected in hazardous waste container and disposed by incineration.

[4] Selection and preparation of teeth

- **a.** Sixty Human mandibular premolars extracted for periodontal or orthodontic reasons were collected from department of oral and maxillofacial surgery, Faculty of Dentistry, Minia University.With fully formed apices; only intact teeth with single root canal were included in this study.

- **b.** Teeth were thoroughly cleaned of external surface debris, soft tissue remnants and calculus; then stored in saline till use.

- **c.** All samples were flattened to establish a standardized tooth length of 18mm using a diamond stone (Diatech, Coltene, Switzerland) to establish uniform specimens. Then root canals were instrumented using Protaper system to size F4 (Dentsply, Maillefer, Switzerland); in between instruments 5.25% NaOCl

was used as irrigation, and at the end of the preparation a final flush with 5ml of distilled water.

d. Root canals were dried with paper points and the apices were sealed with composite resin, two layers of nail varnish were applied to cover the external root surface to avoid bacterial leakage.

e. Each specimen was fixed with silicon impression material in an Eppendorf vial (this was to facilitate handling and identification); then placed into a carrier box placed which were then inserted in an autoclave sachet and autoclaved for 20 min at 121^{o}C.

f. 15 specimens were then subjected to microbial analysis and served as negative control group.

[5] Contamination of the specimens

a. *E. Faecalis* preparation:

A pure E Faecalis culture (ATCC 29212) was grown over night in brain heart infusion (BHI) broth at 37^{o}C. The turbidity was adjusted to 0.5 Mac Farlandy standard and the obtained cell density was 1.5× 10 ^{8} cell\ml.

b. Specimen contamination:

The remaining 45 specimens were filled with *E faecalis* suspension using sterile micropipettes and a sterile K- file # 15 was used to ensure bacterial suspension penetration in to the working length. All contaminated specimens were incubated at 37^{o}C for 4 weeks and the root canal contents were refreshed every 3 days.

[6] Preparation of the irrigation solutions:

a. Sodium hypochlorite; NaOCl (Egyptian company for household bleach-Egypt); the concentration was adjusted at Faculty of Pharmacy, Minia University (2.5 % w\v NaOCl solution)
b. Ethylene diaminetetraacetic acid ; EDTA 17%(Dentsply, Maillefer, Switzerland)
c. Glycyrrhiza glabra Extractwas prepared and concentration adjusted; Roots of *G. glabra*Roots of the plant (500gr) were dried and then powdered using a mechanical grinder. The extraction was carried out using ethanol (80%, v/v) for a period of 72 hours without any heating procedure. The final volume of the filtrate was removed using a rotary vacuum evaporator at 40˚c to give the concentrated extract. The irrigation concentration was set at25mg/ ml[33]

d. Salvadora persica Extract was prepared and concentration adjusted;the fresh ground roots were extracted with 10% water in ethanol, which was then evaporated to dryness. The S. persica extract was dissolved in dimethyl sulfoxide to prepare a 400 mg/ml stock solution; working concentrations 5 mg/ml were prepared [34]

[7] Classification of the groups: specimens were randomly allocated to the groups

1. Negative control group: (n=15) specimens were autoclaved and no further treatment was done (This group ensured that no contamination of the autoclaved specimens occurred).
2. Positive control group: (n=15) specimens were infected after autoclaving and no further treatment was carried out. (This group ensured that proper infection took place for all the infected specimens)
3. Group A: (n= 15) specimens wereautoclaved and infected; then alternating(2.5 % NaOCl and 17% EDTA) irrigationwas delivered into the root canal and sonically activated.
4. Group B: (n= 15) specimens were autoclaved and infected; then alternating (Glycyrrhiza glabra Extract and Salvadora persica Extract)was delivered into the root canal and sonically activated.

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[8] Irrigation protocol:

a. All irrigation procedures were performed in laminar flow hood under a septic condition, with sterile gloves and sterile syringe for each specimen.
b. For irrigation protocol; the same standardized procedure was carried out for all specimens.
c. A 30-gauge side-vented closed end irrigation needle (Shanghai Fanta Dental Materials, Shanghai) was used to deliver the irrigation solution; and the needle tip was placed in the root canal 1mm from the working length (at 17mm).
d. A total of 3 ml irrigation solution was used for each specimen according to the group:
    I. Group A: 3 ml of 2.5 % NaOCl activated for 2 cycles using eddy tip(VDW, Munich, Germany) sonic activation each cycle 30 seconds followed by 3ml of 17% EDTA activated by 2 more cycles using eddy tip sonic activation each cycle 30 seconds
    II. Group B: 3 ml of Glycyrrhiza glabra Extract activated for 2 cycles using eddy tip sonic activation each cycle 30 seconds followed by 3ml of Salvadora persica Extract activated by 2 more cycles using eddy tip sonic activation each cycle 30 seconds
e. One investigator assigned to perform all the irrigation protocols.
f. At the end of the tested irrigation protocols all specimens were irrigated with 5ml sterile saline.

[9] Microbial analysis:

Following irrigation of the specimens 3 sterile paper points F4 (Dentsply, Maillefer, Switzerland) were introduced into the root canals to the full working length for 60 seconds each; then transferred to a labeled Eppendorf vials containing 1 ml of sterile PSB (Phosphate-Saline Buffer). Vialswere vortexed for 1 minute. Tenfold standard sequential dilution of each vial was performed and the bacterial count in colony forming units for each ml was calculated.

[10] Statistical analysis:

The log transformation of each CFU\ ml count was performed, the collected data were coded, tabulated, and statistically analyzed using SPSS program (Statistical Package for Social Sciences) software version 25. Distribution of the data was done by Shapiro Wilk test. Descriptive statistics were done for non-parametric quantitative data by median and interquartile range (IQR). Analyses were done for non-parametric quantitative data between the five groups using Kruskal Wallis test followed by Mann Whitney test between each two groups. The level of significance was set at (P value < 0.05).

Results:

The median log and IQR of bacterial count in (log CFU \ml $^{-1}$) for all study groups and statistical analysis (Table 1, Figure 1).All specimens of the negative control group showed zero bacterial count while the specimens of positive control group recorded the highest bacterial count.

Regarding the tested irrigation protocols; group A(NaOCl / EDTA) recorded the lowest median bacterial count at 0.6log CFU while group B (S. persica / G. glabra) showed median bacterial count of 1.2 log CFU. The results showed a statistically significant difference in comparison with positive control group p < 0.001 indicating the strong antibacterial activity of both irrigation protocols of group (A, B). Furthermore group A showed a statistically significant more reduction in bacterial count than did group B at p value < 0.001; indicating the superior antibacterial activity of group A (NaOCl / EDTA).

		+Ve Control	Group A	Group B	P value
		N=15	N=15	N=15	
Bacterial count (Log CFU /ml)	*Median* *IQR*	6.1[a] (6-6.3)	0.6[c] (0.4-1.2)	1.2[b] (0.8-1.6)	***<0.001****

- ***Kruskal Wallis Test for non-parametric quantitative data between the three groups followed by Mann Whitney test between each two groups.***
- ***Superscripts with different small letters refer to the significant difference between each two groups.***
- ****: Significant difference at P value < 0.05***

As regards the previous table, there was a significant decrease in the bacterial count in both groups A and B compared with the +Ve control group, also group A showed a significant decrease in bacterial count compared than group B.

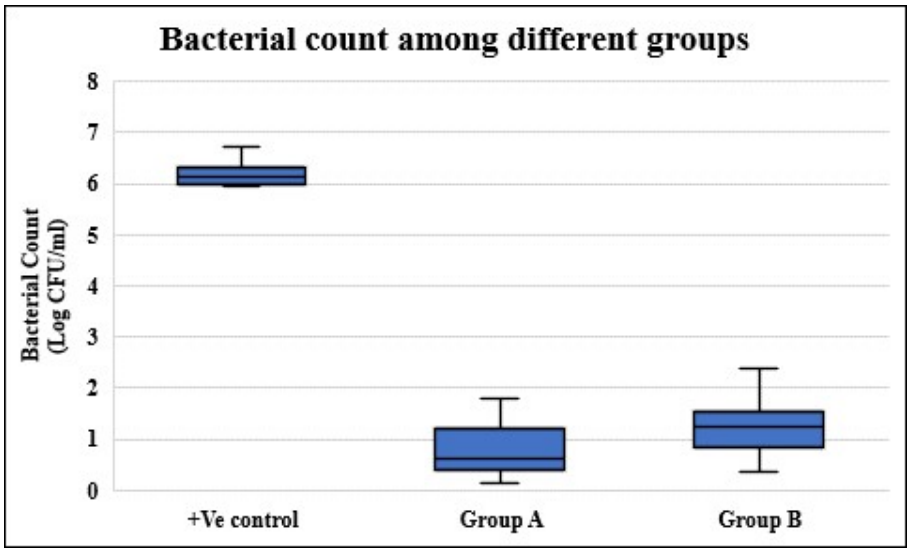

Fig. (1) Boxplot of Median and interquartile range (IQR) ofbacterial count in (log CFU\ml $^{-1}$) for all groups

Discussion

The goal of endodontic treatment is complete debridement and disinfection of the root canal system; this is established not only by instrumentation but utilizing variable irrigation protocols. This justifies the ongoing search for new irrigation solutions and techniques. In this study, a single-species biofilm model E. faecalis was used to establish an intracanal infection modelas the microorganism usually isolated from failed endodontic treatmentdue to its ability to survive harsh root canal environmental stresses; use of different irrigation solution, intracanalmedicaments, moreover its ability to form protective biofilms all of which is well documented. Furthermore ;*E. faecalis* possesses many virulence factors including lipoteichoic acid, cytolysin, pheromones, lytic enzymes and aggregation substances, its ability to adhere to tooth substance and its high ability to suppress lymphatic activity all of which potentially contributes to endodontic treatment failure.[35,36,37]

Intact Human single rooted teeth were prepared and contaminated with *E Faecalis* and incubated for 4 weeks to ensure formation of a biofilm, this is to create a model that was clinically more relevant and replicate the usual endodontic significance. Many studies have confirmed that microorganisms grow in biofilms and that they reach 1000 times more resistance to antimicrobial agents than the planktonic bacteria.All samples included in the study were standardized to the same length and preparation procedures. The tested irrigation protocols were also standardized to have the same solution (volume, temperature, and irrigation duration and sonic activation cycle duration). [38]

Regarding the present study 60 samples were included all samples were autoclaved, 15samples servedas negative control and were autoclaved only andyield no bacterial count ensuring that specimenswere completely sterile and no contamination, in addition to 15 more

specimens served as positive control which werecontaminated and did not undergo further treatment and showed the highest bacterial count ensuring theuniform contamination and microbial loading levelsof all specimens.

In this present study group (A); NaOCl and EDTA were chosen as irrigation solution as they are the most commonly used endodontic combination in clinical practice[10, 11, 12]
While for group B (S. persica / G. glabra) was chosen to verify and compare its antibacterial activity of this proposed combination; as G.glabra, commonly called as Licorice, is one of the important traditional medicinal plants has several useful pharmacological properties such as anti-inflammatory, antiviral, antimicrobial, and anticancer activities in addition to immune-modulatory[16]moreover its saponine content which has been proved to possess strong dissolution effects on human pulp tissues[39]

On the other hand*S. persica* (miswak)has antiseptic, antifungal, antibacterial,and anticaries properties [23, 24]. The stem of *S. persica* has profound antifungal, antimicrobial, and antiplaque actions [24].

To further enhance the antibacterial efficacy of tested irrigations An Innovative sonic-powered tip (EDDY) was used in this study. This flexible tippreserves root canal morphology, whilestripping off the biofilm from dentin wall[30, 31]

In the present study group A (which was treated with sonically activated NaOCl / EDTA) showed lowest bacterial count with a median of 0.6log CFU\ml which is significantly lower than that of the positive control indicating its strong antibacterial efficacy. The results came in accordance with many previous studies.[40]**Gianrdino et al** [41]showed that NaOCl was able to eradicate *E. faecalis* biofilm in 30 seconds, in addition to **Dunavantetal**[42]whom demonstrated that NaOCl killed all bacterial colonies within an organized biofilm. This is mainly attributed to its ability to dissolve organic debris due to its proteolytic effect along with the chlorine release which affects a broad range microbe together with release of oxygen that eradicates anaerobic bacteria.[42]

While group B (which was treated with sonically activated G. glabra / S. persica) showed median bacterial count of 1.2log CFU\ml. The results showed a statistically significant difference in comparison with positive control group with $p < 0.001$ indicating the strong antibacterial activity

This comes in accordance with findings by **Chakotiyaet al** [43] whom found that The main ingredient of licorice roots glycyrrhizin or glycyrrhizic acid (GA); atriterpinoidsaponin; Whichwas found to have strong antibacterial action and they attributed this effectiveness to saponine ability to target the physiological parameters of the bacteria by increased cell membrane permeability , efflux activityeventually leading to adecreased biofilm formation.
Abuletal[44]evaluated the antimicrobial effect of G. glabra extract against E. faecalis and related it to the saponins. Further illustrating that the mode of action of antibacterial effects of saponins seems to involve membranolytic propertiesin which it interact with membrane lipids and disrupt the cell membrane integrity causing the cells to leave the intracellular organelles,

rather than simply altering the surface tension of the extracellular medium, thus decreasing microbial population density.
Moreover the flavonoid content of licorice extract is also a strong inhibitor of oxygen consumption in bacterial cell[45, 46]

Regarding *S. persica*ourresults come in agreement with previousstudies by **Khalil et al,Al-Sabawietal , Baltoetal, Al-Sabawiet al** [25, 26, 27, 28, 29]; whom all concur with the significant antibacterial activity of *S. persica*. This may be due to its most important andeffective ingredientwhichis benzyl isothiocyanate, a major antimicrobial volatile oil [25]. This plant also contains *N*-benzyl-2-phenylacetamide, whichhas shown efficacy against *Escherichia coli*[26].

Moreover**Sofataetal**[25]in a transmission electron microscope study; which evaluated the effect of S. persica on bacteria found that the extract composed of 98% benzyl isothiocyanate which is the component responsible for dramatic effect on the cell membrane of bacteria and eventually loss of the bacterial cell membrane integrity

Furthermore group A showed a statistically significant more reduction in bacterial count than did group B at p value < 0.001; indicating the superior antibacterial activity of groups A (NaOCl / EDTA).

This comes in agreement with many studies that have demonstrated the superiority of sodium hypochlorite against microorganism especially E faecalis [10, 11, 12]
This is due to three main reactions;the saponification, amino acid neutralization and chloramination reactions that occur inthe presence of microorganisms and organic tissue leadto the antimicrobial and tissue dissolution process. Theantimicrobial activity is related to bacterial essentialenzymatic sites promoting irreversible inactivation byhydroxyl ions and the chloramination reaction.[48]

Sodium hypochlorite neutralizes amino acidsforming water and salt. With the exit of hydroxyl ions, there is a reduction of PH.Hypochlorous acid, a substance present in sodium hypochlorite solution, when in contact with organic tissue acts as a solvent, releases chlorine that,combined with the protein amino group, forms chloramines. Hypochlorous acid and hypochlorite ions lead toamino acid degradation and hydrolysis.[49]Moreover the high pH of sodium hypochlorite interferesin the cytoplasmic membrane integrity causing irreversible enzymatic inhibition, biosynthetic alterations incellular metabolism and as end result phospholipid degradation.[50]

Although the present study was conducted with standardprotocols and guidelines but even then it has certain limitations.Major limitation of the present study wasthat when secondary root canal infection occurs, it's nevermono microbial rather poly microbial in nature. Hencethe results might change in the presence of other microbes due to various microbial interactions.

Finally; though sonically activatedG. glabra / S. persica significantly reduces the bacterial load of E faecalis; sonically activated NaOCl / EDTA excides it significantly thereby the null hypothesis was rejected.

Conclusion:

Under the limitation of the present study; sonically activated G. glabra / S. persica significantly reduced microbial colonies of E faecalis with in the root canal system and can be regarded as an alternative for NaOCl / EDTA if patient shows sensitivity to NaOCl. However sonically activated NaOCl / EDTA still significantly reduced more colony forming units of E faecalis compared to G. glabra / S. persica.

Further antibacterial evaluation ofsonically activatedG. glabra / S. persica is needed to compare different concentrations and to evaluate their efficacy in dissolving organic tissues and smear layer removal.

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