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# EVALUATION OF THE ANTIMICROBIAL ACTION AND CLEANING ABILITY OF DIFFERENT NATURALLY BASED VINEGARS USED AS ENDODONTIC IRRIGANTS (IN-VITRO STUDY)

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***KEYWORDS***

*Endodontic irrigants, Phenolic compounds, Smear layer, vinegars.*

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## *ABSTRACT*

**Introduction:** Irrigation of the root canals is an important step during endodontic treatment. Irrigants serve to eradicate microbial infection and to remove the resulting smear layer. NaOCl/EDTA irrigation is the gold standard irrigation protocol. However, it has several side effects. So, it was urgent call to investigate herbal alternatives as potential endodontic irrigants. **Aim:** To evaluate the antimicrobial action and smear layer removal ability of apple, dates and pomegranate vinegars compared to NaOCl/EDTA irrigation. **Materials &Methods:** Sixty-five sterile roots were infected with *E.faecalis*, incubated for three weeks then grouped into five groups; apple vinegar group, dates vinegar group, pomegranate vinegar group, NaOCl/EDTA group and control group, respectively. Three Microbial samples were obtained from the root canals: before irrigation, immediately after irrigation and one week after irrigation. The reduction% of mean Log10 CFU was calculated. Five roots from each group were split bucco-lingually, scanned with SEM and processed using ImageJ software. Statistical analysis was performed by applying the t-test, ANOVA (one and two way) followed by post hoc Tukey's test at 95% significance level. **Results:** Dates vinegar group showed high antimicrobial action and smear layer removal ability comparable to NaOCl/EDTA group and the difference was statistically insignificant. Pomegranate and apple vinegar groups showed less antimicrobial action and smear layer removal ability than dates vinegar and NaOCl/EDTA which was statistically significant. **Conclusion:** Phenolic compounds in dates vinegar are responsible for its antimicrobial action and smear layer removal ability making it potential alternative to NaOCl/EDTA.

## INTRODUCTION

Far away, microbial infection has been recognized as the primary etiological factor in the development of pulp and periapical lesions. Today, there is no doubt that microorganisms, either remaining in the root canal space after treatment or re-colonizing the filled root canal system, are the main cause of endodontic failure[(1)].

Successful root canal treatment depends on thorough debridement of necrotic pulp tissues, dentin debris and infective microorganisms, it is impossible to eradicate intra-radicular infection with mechanical instrumentation alone, therefore, irrigants are required to complete this task[(2)]. An ideal irrigant should have a strong antimicrobial action and simultaneously remove the smear layer but unfortunately no available single irrigant can effectively do both functions.

Today, the most commonly used irrigation protocol comprises main irrigation with sodium hypochlorite (NaOCl) followed by final irrigation with ethylene diamine tetra-acetic-acid (EDTA)[3].

Taking into consideration the limitations and side effects caused by synthetic agents like NaOCl and EDTA which are toxic to vital tissues and negatively affect the mechanical properties of dentin and the increasing resistance among bacterial strains, it is increasingly important to investigate herbal alternatives as potential endodontic irrigants.

Vinegar has been traditionally used as anti-microbial agent due to its low pH and presence of acetic acid as a key element. Recently, fruit vinegars comprising 0.1% acetic acid was found effective against the progression of food-borne pathogens[4]. The antibacterial activities of apple, dates and pomegranate vinegars have been reported in many medical and agricultural studies[5–7]. However, in the endodontic field, data about these vinegars are lacking either as antibacterial irrigants or as chelating agents for smear layer removal. So, the aim of the current study was to evaluate the antimicrobial action and smear layer removal ability of apple, dates and pomegranate vinegars compared to NaOCl followed by EDTA as a final rinse.

The null hypothesis tested in the current study was that there was no significant difference between apple, dates and pomegranate vinegars compared to NaOCl followed by EDTA regarding the antimicrobial action as well as smear layer removal.

## MATERIALS AND METHODS

### 1. Study design

The protocol for this study was subjected to and approved by the Research Ethics Committee (REC) of the faculty of dentistry, Suez Canal university (approval number 190/ 2019).

### 2. Sample size determination

The sample size was calculated according to G*Power software version 3.1.9.3[8]. For the microbiological evaluations, a minimum total sample size of 65 samples was sufficient to detect the effect size of 0.25 with a power (1-β=0.95) of 95% at a significance probability level of $p<0.05$ partial eta squared of 0.06. According to sample size calculation each group of irrigants (I, II, III, IV and V) at the three sampling time points (S1, S2, S3) was represented by a minimum of 13 samples. For SEM imaging evaluation, a minimum total sample size of 25 samples was sufficient to detect the effect size of 0.40 with a power (1-β=0.85) of 85% at a significance probability level of $p<0.05$ partial eta squared of 0.14. According to sample size calculation, each group of irrigants (I, II, III, IV and V) at the three root canal thirds (coronal, middle and apical) was represented by a minimum of 5 samples.

### 3. Teeth selection

Sixty-seven single rooted non-identified premolars freshly extracted for orthodontic reasons were selected to be used in this study. The teeth were collected from the department of oral surgery at the Faculty of Dentistry, Suez Canal University. The teeth were radiographed in mesiolingual and buccolingual views using conventional periapical radiographic technique and selected according to the following inclusion criteria: Single rooted teeth, straight roots, single root canal, root length not less than 14mm, no previous endodontic treatment, completely formed roots, no carious lesions and no internal or external resorption defects.

### 4. Specimen preparation

Teeth were placed in 0.5% NaOCl (Clorox, Cairo, Egypt) for 24 hours for surface disinfection,

scaled by ultrasonic scaler under water spray and stored in distilled water until use. The crowns were resected with isomet saw (Isomet 4000 linear precision saw, Buehler, Illinois, USA) under air-water spray to standardize the total length of each root to 14 mm in length.

### *4.1. Instrumentation of the root canals*

Hand K- File #10 (Mani Inc. Tokyo, Japan) was introduced into the root canal till appear at the apex to ensure canal patency. The working length was established by subtracting 1mm from that length. All root canals were instrumented using Denjoy FF rotary Ni-Ti files (Denjoy Dental, Changsha, China)) to file size FF5 (#40/04). All canals were only irrigated with 3 mL distilled water after each file used using 3mL plastic syringe and 27-guage side vented needle introduced into the canal just before binding (2-3 mm short of the WL), then short (1–2 mm) in–and-out motions were applied to the needle during irrigation.

### *4.2. Coating of the specimens*

Samples were dried with gauze then two layers of nail polish were applied to their external surfaces. The apical foramina were sealed with composite resin (Meta Biomed, Cheongju, Korea)

### *4.3. Sterilization of the specimens*

Samples were placed into vials containing tryptone soy broth (Thermo Scientific™ Oxoid™

Hampshire, UK) and autoclaved for 20 minutes at 121°c.

## 5. Preparation of the tested irrigation solutions

Apple vinegar (Acetificio Andrea, Milano, Italy) extracted from apple fruit (*Mallus domestica*), dates vinegar (Rovan, Almafraq, Jordan) extracted from dates palm fruit *(Phoenix dactylifera)* and pomegranate vinegar (Acetificio Andrea, Milano, Italy) extracted from pomegranate fruit *(Punica granatum L.*) were purchased from a local market. The vinegars were used in full concentrations. NaOCl in 2.5% concentration was used (R&S, Paris, France). EDTA solution was used in 17% concentration (Prevest DenPro, Bari Brahmana, India). Sterile isotonic saline solution (0.9% NaCl) was also used.

### *5.1. Quality characteristics of the tested vinegars*

The tested vinegars were subjected to chemical analysis of the total phenol content (TPC) and the pH value. The total phenols were determined using the Folin-Ciocalteu assay and measured as mg GAE/L (GAE: gallic acid equivalent ) [(9)] .

## 6. Microbiological assay

### *6.1. Preparation of the bacterial suspension*

Pure culture of *Enterococcus. faecalis* (isolated clinical strain from urinary tract infection) on blood agar (Thermo Scientific™ Oxoid™ , Hampshire, UK) with 5% RBCs was obtained from the Microbiology Laboratory, Suez Canal University Hospital. Confirmation of bacterial species was done by colony morphology, gram stain and biochemical reactions (bile-esculin test and catalase test). Mid exponential bacterial suspension with concentration of $15 \times 10^9$ colony forming units/ milliliter (CFU/mL) was used[(10,11)].

### *6.2. Bacterial Susceptibility Assay*

To determine the optimum retention time of irrigants in the root canal that would achieve maximum percentage of bacterial growth inhibition, tube minimal inhibitory concentration test (MIC) was carried out at four contact times (5, 10, 15 & 20 minutes)[(12,13)].

### *6.3. Contamination of root canals*

Each root was placed in upright position in sterile Eppendorf tube ( Elkay, Shrewsbury, Ma, USA) using sterile forceps, then automatic micropipette (Dragon Lab Ltd, Beijing, China) was used to inoculate the root canal with 20 $\mu$l[14] of fresh bacterial suspension in the mid logarithmic phase. Then each Eppendorf tube was filled with sterile tryptone soy broth (Thermo Scientific™ Oxoid™ , Hampshire, UK) and incubated aerobically for 21 days at 37°C. The nutrient broth was changed with sterile broth on alternate days. After 21 days incubation period, two random specimens were sectioned longitudinally using chisel and hammer and subjected to scanning electron microscopy for confirmation of bacterial colonization and biofilm formation[15].

### *6.4. Specimens randomization and grouping*

The remaining 65 specimens were equally and randomly distributed into five groups according to the irrigant used (n=13). Random sequence generation was performed using a computer-generated number (www.randomizer.org). Group I: Apple vinegar, Group II: Dates vinegar, Group III: Pomegranate vinegar, Group IV: NaOCl / EDTA and Group V: Saline (control group).

### *6.5. First microbial sampling (S1)*

Microbial sampling from root canals was performed under strict aseptic conditions as previously described[16] with some modifications; Each root canal was rinsed with 1 mL sterile isotonic 0.9% saline solution (Fibco, Alexandria, Egypt) in order to remove the free bacterial cells then three sterile ISO size #40 paper points were successively placed into the canal at the WL. Each paper point was rubbed against the canal walls and kept for 1 minute. The three paper points were transferred to a glass tube containing 1mL sterile isotonic saline solution and vortexed for 60 seconds. The bacterial suspensions in the glass tubes were tenfold serially diluted up to $10^{-6}$ then 0.1mL[17] alliquotes were inoculated onto slanetz and bartley agar and incubated aerobically at 37 °C for 24 h. The total CFUs were calculated and transformed into actual counts based on the dilution factor. All bacterial counts were logarithmically transformed ($Log_{10}$ CFU)[18].

### 7. Antibiofilm assay

The irrigation protocol followed two stages system (main irrigation and final irrigation) according to previously described protocol[19] with some modifications. The 20 minutes retention time of the main irrigant was chosen based on the results of MIC test as it achieved the maximum % of bacterial growth inhibition.

***In groups I, II, III:***

Main irrigation with **6mL** of the corresponding vinegar for 20 min retention time followed by **2mL** saline. Final irrigation with **1mL** of the corresponding vinegar for 1min retention time followed by **2mL** saline.

***In group IV:***

Main irrigation with **6mL** NaOCl for 20 minutes retention time followed by **2 mL** of 5% sodium thiosulfate[20,21]. Final irrigation with **1mL** EDTA for 1 min retention time followed by **2mL** saline.

***In group V:***

Main irrigation with **6mL** saline for 20 min retention time followed by **2mL** saline. Final irrigation with **1mL** saline for 1min retention time followed by **2mL** saline.

The same flow rate (0.1 mL /sec) was used for all irrigants. All irrigation procedures were done by conventional needle irrigation using plastic syringe and 27-guage side vented needle (Ultradent Endo-Eze, Utah, U.S) introduced into the canal 2-3 mm short of the WL, then short (1–2 mm) in–and-out motions were applied to the needle during irrigation.

2 mm shorter than WL. All irrigation procedures were carried out by a single operator.

### *7.1. Second microbial sampling (S2)*

Samples were taken immediately after irrigation in the same way as S1.

### *7.2. Third microbial sampling (S3)*

After the second microbial samples, specimens were again incubated under the same conditions for one week and the nutrient broth changed on alternate days. S3 Samples were taken after one week in the same way as S1 and S2.

## 8. Smear layer removal assay by scanning electron microscopy (SEM)

Five roots from each group were selected randomly to evaluate the ability of tested irrigants to remove the smear layer. Chisel and hammer were used to split the roots buccolingually. The hemisected half with fewer irregularities and patent apical third was selected for SEM analysis[22]. Representative images were taken at coronal, middle and apical thirds at magnification 2000x and processed using ImageJ software (version 1.53a National Institutes of Health, USA). The total area of open dentinal tubules was calculated as % of the total image area using the following equation[23]:

Open dentinal tubules % =

$$\frac{\textit{Total area of open dentinal tubules } (\mu m)}{\textit{Total image area (um)}} \; X\ 100$$

Imaging and image analysis were performed by single operator who was blinded to the group allocation.

### Statistical analysis

Shapiro Wilk and Levene tests were used for testing the normality of data. Data were analyzed using the statistical software SPSS (version 25, IBM Co. USA). Statistical analysis for results was performed by applying the one-way ANOVA test followed by post hoc Tukey's test for intergroup and intragroup comparison and $p<0.05$ was considered statistically significant (95% significance level). Pearson correlation test was performed for correlation between antibacterial and smear layer removal actions of the tested irrigants and significance was set at $P > 0.05$

## RESULTS

Chemical analysis of the total phenol content revealed that dates vinegar had the highest content (265.5mg GAE/L), followed by pomegranate vinegar (254.25 mg GAE/L) and apple vinegar (209mg GAE/L). While for the pH value, it was found that apple vinegar has the highest pH value (2.8) followed by pomegranate vinegar (2.7) and dates vinegar (1.9). Biofilm formation in the root canals was confirmed as shown in Figure 1.

Results of the antibiofilm assay are shown in Table (1). Dates vinegar had the least mean Log10 CFU/mL after 20min of irrigation followed by NaOCl/EDTA with no statistically significant difference. However, after 1 week, NaOCl/EDTA achieved the least mean Log10 CFU/mL followed by dates vinegar with no significant difference. The mean Log10 CFU/mL of pomegranate vinegar group was less than apple vinegar group after 20min of irrigation with no significant difference. However, after one week, the mean Log10 CFU/mL of pomegranate vinegar was significantly less than apple vinegar group.

**Table (1)** *Comparative evaluation of mean $Log_{10}$ CFU/mL between all groups.*

| Group | S1 (Before irrigation) | S2 (After 20 min) | S3 (After 1 week) | P value intragroup comparison |
|---|---|---|---|---|
| **I** | 8.22±0.12$^{aA}$ | 3.27±0.13$^{bC}$ | 6.49±0.11$^{bB}$ | **≤ 0.001** |
| **II** | 8.31±0.22$^{aA}$ | 2.82±0.02$^{cC}$ | 3.94±0.03$^{dB}$ | **≤ 0.001** |
| **III** | 8.36±0.08$^{aA}$ | 3.32±0.09$^{bC}$ | 5.46±0.06$^{cB}$ | **≤ 0.001** |
| **IV** | 8.22±0.13$^{aA}$ | 2.91±0.02$^{cC}$ | 3.80±0.07$^{dB}$ | **≤ 0.001** |
| **V** | 8.30±0.07$^{aA}$ | 8.10±0.053$^{aA}$ | 8.12±0.08$^{aA}$ | **0.073**$^{ns}$ |
| **P value intergroup comparison** | **0.626**$^{ns}$ | **0.000** | **0.000** | |

*- ns: non-significant (p>0.05)* *- P value significant at P ≤ 0.05*

*-Means with different small letters superscript are statistically significant at columns.*

*-Means with different capital letters superscript are statistically significant at rows.*

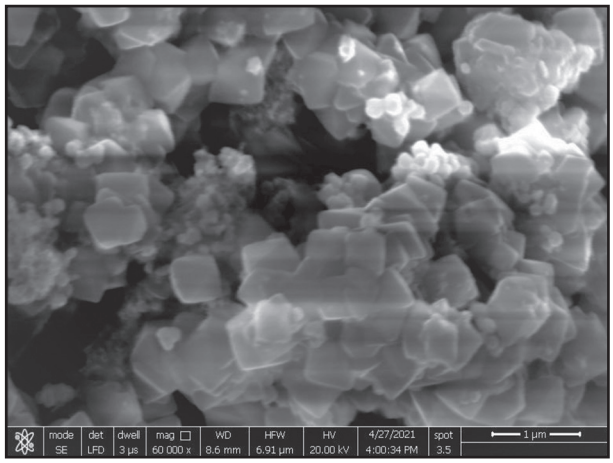


Fig. (1) Representative SEM micrograph (magnification 60000×) showing E. faecalis biofilm after 21 days of incubation.

Results of the smear layer removal assay by SEM are shown in Figure 2 and the image analysis results are shown in Table (2). NaOCl/EDTA showed the highest ability to remove smear layer followed by dates vinegar with no significant difference. The smear layer removal ability of pomegranate vinegar was statistically not significantly higher than apple vinegar.

In the coronal third: There was no statistically significant difference between dates vinegar and NaOCl/EDTA, there was no statistically significant difference between apple vinegar and saline. Pomegranate vinegar showed less ability to remove smear layer in the coronal third than both dates vinegar and NaOCl/EDTA which was statistically significant, on the other hand pomegranate vinegar showed higher ability to remove smear layer in the coronal third compared to both apple vinegar and saline and this difference was statistically significant.

In the middle third: There was no statistically significant difference between dates vinegar and NaOCl/EDTA, there was no statistically significant difference between dates vinegar and pomegranate vinegar as well as between dates vinegar and apple vinegar and there was statistically significant difference between Saline and all groups.

In the apical third: There was no statistically significant difference between dates vinegar and NaOCl/EDTA. There was no statistically significant difference between dates vinegar and pomegranate vinegar as well as between dates vinegar and apple vinegar and there was statistically significant difference between Saline and all groups.

Saline had significantly the least antibacterial action and smear layer removal ability.

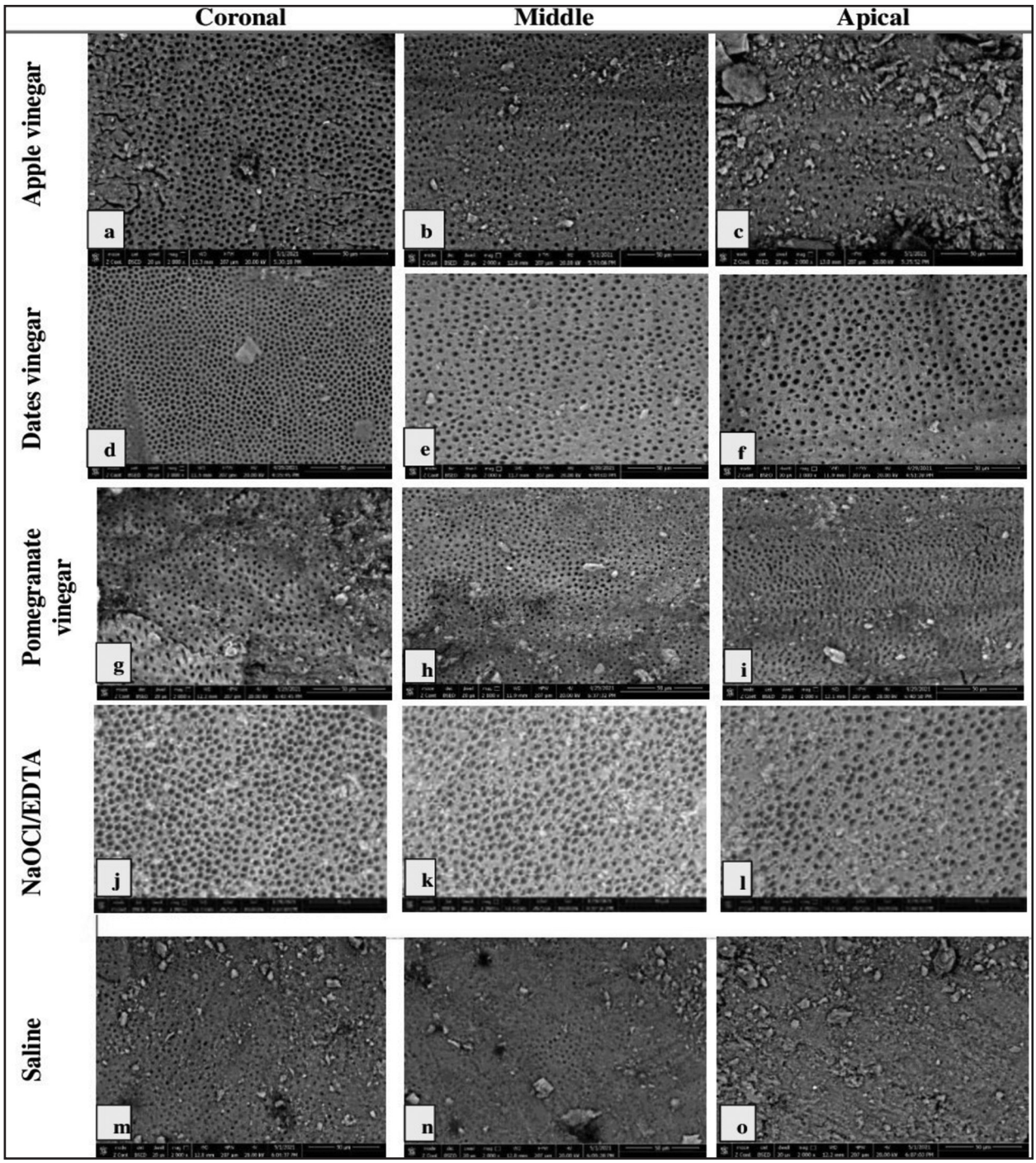


Fig. (2) Representative SEM micrographs (magnification 2000×) after smear layer removal by the tested irrigants. Both apple vinegar and pomegranate vinegar groups show partially opened dentinal tubules in coronal (a & g) and middle thirds (b & h) respectively, while the apical third of both groups (c & i) show heavy smear layer with nearly closed dentinal tubules. The coronal third of dates vinegar group (d) show a highly cleaned surface with completely open dentinal tubules, while the middle (e) and apical (f) thirds show partially open dentinal tubules. NaOCl/EDTA group show completely open dentinal tubules in the coronal third (j), while the middle (k) and apical (l) thirds show partially open dentinal tubules. Erosions of the peritubular and intertubular dentin are visible in NaOCl/EDTA group. Saline group show dense smear layer with completely closed dentinal tubules in all thirds

**Table (2)** *Comparative evaluation of mean percentage of open dentinal tubules in different thirds between all groups.*

| Group | Coronal | Middle | Apical | P value intragroup comparison | Overall means |
|---|---|---|---|---|---|
| **Apple** | 5.44±1.12$^{cA}$ | 5.01±1.32$^{bA}$ | 3.09±0.88$^{bB}$ | 0.035 | **4.51$^{b}$** |
| **Dates** | 14.79±1.43$^{aA}$ | 7.76±2.75$^{abB}$ | 5.82±2.44$^{abC}$ | 0.01 | **9.46$^{a}$** |
| **Pomegranate** | 9.44±1.37$^{bA}$ | 6.10±1.27$^{bB}$ | 3.43±1.35$^{bC}$ | 0.012 | **6.32$^{b}$** |
| **NaOCl/EDTA** | 15.44±1.28$^{aA}$ | 10.04±2.67$^{aB}$ | 8.92±2.58$^{aC}$ | 0.013 | **11.47$^{a}$** |
| **Saline** | 3.51±1.31$^{dA}$ | 1.06±0.91$^{cB}$ | 1.15±0.68$^{cB}$ | 0.53$^{ns}$ | **2.09$^{c}$** |
| **P value intergroup comparison** | **0.002** | **0.015** | **0.003** | | **0.001** |

*-Means with different capital letters superscripts are statistically significant at rows.*

*-Means with different small letters superscripts are statistically significant at columns.*

*The result of Pearson correlation test revealed that there was a positive correlation between the results of antibacterial and smear layer removal actions and this correlation was statistically significant (P > 0.05).*

## DISCUSSION

Mechanical instrumentation solely cannot eradicate microorganisms in the infected root canal. Moreover, it leaves smear layer covering the root canal wall. This layer contains bacteria, bacterial byproducts and necrotic tissues which act as substrate for bacterial survival and proliferation. Also, this layer limit deep penetration of disinfecting agents into the infected dentinal tubules. Moreover, it acts as a barrier between filling materials and the canal wall and therefore compromises the formation of a satisfactory seal. That is why using irrigants is an essential step in the biomechanical root canal preparation. Unfortunately, none of the recently available irrigants can simultaneously eradicate intracanal microorganisms and remove the resulting smear layer[22]. Moreover, growing resistance of the microorganisms to the antimicrobial agents and drawbacks of the recently available irrigants make an urgent need to search for herbal alternatives[7].

The current study tested the antibacterial action and smear layer removal abilities of three natural vinegars (apple vinegar, dates vinegar and pomegranate vinegar) compared to the alternating use of NaOCl and EDTA. In the current study, extracted human premolar teeth were used as a substrate for biofilm growth instead of nonbiological surface as this represents the biofilm's natural environment simulating the clinical condition.

In the current study, the root canals were mechanically prepared before bacterial inoculation then the contaminated root canals were only treated by passive irrigation using the tested irrigants to evaluate the absolute effect of the tested irrigants without any additional effect from the mechanical action of instrumentation[15,18].

Although root canal infection comprises multispecies biofilm, the single-species biofilm model was used to limit any variations that may be caused by microbial interaction. *E. faecalis* was chosen as test species as it is frequently found in failed endodontic cases, able to form a monospecies biofilm, invade dentinal tubules, resist harsh environmental conditions[15]. Moreover, *E. faecalis* is a facultative microbe that is non-fastidious (easy-to-grow) [24]

Although culture independent techniques are more sensitive than culture dependent techniques for microbial profiling, it has been stated that sensitivity of culture technique is fairly good and acceptable as long as it is carried out appropriately[25]. Also, for the non-fastidious bacteria such as facultatives, the sensitivity of culture techniques is considered high because theoretically only one bacterial cell is needed for growth[25]. Additionally, there was good correlation between results of culture based analysis and culture independent analysis for evaluation of *E. faecalis* biofilm[26].

The results of the anti-biofilm assay revealed that bacterial count in samples taken immediately after irrigation was significantly less than samples taken seven days after irrigation in all groups except for saline group. This can be explained by the bacterial regrowth after 1 week, in accordance with previous study which demonstrated that this bacterial regrowth was caused by remaining bacteria in the root canal even after irrigation[14].

NaOCl/EDTA showed high antibacterial efficiency against intracanal *E. faecalis* biofilm, in accordance with previous studies[27,28]. This can be explained by the ability of NaOCl to oxidize and hydrolyze cell proteins and its hypertonicity that enables it to osmotically draw fluids out of microbial cell[29]. Besides, EDTA has biofilm disrupting ability by sequestering of calcium resulting in weakening of the biofilm [26]. Similarly, dates vinegar showed high antibiofilm effect against intracanal *E. faecalis* biofilm. The results of dates vinegar were comparable to NaOCl/EDTA and the difference was statistically non-significant. On the other hand, pomegranate and apple vinegars showed significant more anti-biofilm effect than saline group, however this effect was significantly less than dates vinegar and NaOCl/EDTA. This may be explained by the higher polyphenol content and the lower pH value of dates vinegar compared to apple and pomegranate vinegars as shown in our results.

Plant polyphenols are large group of secondary metabolites that are formed within the plant in response to microbial attack[30]. These secondary metabolites are biologically active molecules that exert a biological effect on other organisms[31].

In general, there are four main mechanisms underlying the antimicrobial action of Phenolic compounds (PC) in the literature. Firstly, PC cause damage to the bacterial cell membrane which in turn lead to increased membrane permeability, depolarization and interruption of efflux pump. Secondly, PC cause down regulation of expression of important genes that are associated with certain virulence factors of the pathogenic bacteria leading to decreased auto-aggregation capacity, decreased adhesion and decreased biofilm formation ability. Thirdly, PC interfere with quorum sensing activity (cell to cell communication system between bacterial cells). Fourthly, PC have synergistic antibacterial effect when combined with common chemotherapeutics to which bacteria had developed resistance[6,32,33].

One advantage of using vinegar in the current study is that it acts as a matrix for polyphenols of the raw material allowing polyphenols to be in soluble state which makes them more biologically available [31].

In the current study, dates vinegar was superior to pomegranate and apple vinegars as antimicrobial agents. This may be explained by the following; Firstly HPLC analysis (high performance liquid chromatography) showed that the major phenolic compounds of dates fruits are phenolic acids and their derivatives[34]. Phenolic acids could exert a stronger antimicrobial effect than PC with larger molecular structure, this behavior is likely

associated with the small size of phenolic acids[32]. Secondly, It was assumed that dates fruit polyphenols exert prooxidant activity by generation of hydrogen peroxide causing oxidative damage within the bacterial cell[35]. Thirdly, the lower pH value of dates vinegar reaching 1.9 compared to 2.7 and 2.8 for pomegranate and apple vinegars respectively as shown in our study.

It was believed that *E. faecalis* can adapt to environments with a pH of 2.9–4.3 generated by lime extracts[36], the results of the current study was in agreement with this finding . However, it seems that the role of acidic pH value in the antibacterial activity of vinegars only comes secondary to the role phenolic compounds. This theory is supported by the results of the study of Shen *et al*, who found that phenolic-rich blueberry extracts with pH of 3.4 caused a greater reduction of viable bacterial count compared to solution of citric acid with the same pH [37].

Results of the antibiofilm assay in the current study are in accordance with previous study on dates fruit extract that was found to exert moderate antibacterial action against *E. faecalis*. The authors attributed this antibacterial effect to the phenolic compounds present in dates fruit extracts. Moreover, the results of the mentioned study revealed good correlation between the concentration of phenolic compounds and the antibacterial activity[34].

Regarding the smear layer removal assay in the current study, It was found that NaOCl/EDTA significantly removed the smear layer exposing the highest percentage of open dentinal tubules, this result coincide with recently published studies[38,39]. All tested vinegars in the current study significantly removed the smear layer compared to the saline group. The smear layer removal ability of apple, dates and pomegranate vinegars seemed to be pH dependent, this finding is in contrary to recently published study that showed that smear layer removal was independent of pH value[40]. So, it might be true that the smear layer removal ability of the tested vinegars in the current study was a function of their phenolic acid content rather than their pH value[40].

In the current study, the ability of apple vinegar to remove smear layer was significantly less than NaOCl/EDTA, in agreement with previous result[41]. However, this result was contrary to recently published study comparing 17% EDTA and apple vinegar applied for 1 and 3 minutes. In the mentioned study, there was no significant difference in the mean scores of smear layer among the tested groups either at the coronal or middle thirds[42]. This discrepancy might be due to NaOCl used in the current study which provided removal of the organic part of smear layer.

The ability of fruit vinegars to remove smear layer can be explained also by their content of organic acids (e.g. maleic, acetic and citric acid) and phenolic acids. Differences in smear layer removal abilities between the tested vinegars can be explained by their different content and concentration of organic and phenolic acids[43,44]. Better ability of NaOCl/EDTA group to remove smear layer than test vinegars might be attributed to the NaOCl that could remove the organic part of smear layer, low surface tension of EDTA that contribute for better wettability. Moreover, EDTA (17% solution) can remove not only Calcium ions but also water soluble non-collagenous proteins (NCP) at neutral pH. Thus not only Calcium ions but also calcium bonded to the extracted fractions of NCPs are removed by EDTA[45].

Results of the current study demonstrated that the removal of the smear layer was statistically less effective in the apical third of the root canal compared to coronal and middle thirds, regardless

of the irrigation solution used except for saline solution. This is in agreement with previous results and could be explained by less diameter of apical third, decreased permeability and presence of sclerosed dentin in the apical third which limits the chelating effect of any chelator[46].

The result of Pearson correlation test revealed that there was a positive correlation between the results of antibacterial action and smear layer removal ability of the tested irrigants and this correlation was statistically significant (P > 0.05(.

From the limitations of the current study is that microbial sampling was done with paper points which only provides sampling of the main canal lumen but the current study also involved further evaluation of the smear layer removal ability of the tested irrigants, so other methods to dislodge the attached bacterial such as filing or ultrasonics would affect the smear layer. So, future studies are strongly recommended to assess the presence of microorganisms in the depth of the dentinal tubules using the more advanced confocal laser microscopy followed by culture based or culture independent analyses. Moreover, the biocompatibility of dates vinegar with the periapical tissues and its effect on the physical and mechanical properties of dentin should be carefully evaluated. Within the limitations of this study, the null hypothesis was partially rejected.

## CONCLUSION

Dates vinegar can potentially be alternative for NaOCl/EDTA irrigation for reduction of intracanal biofilm as well as smear layer removal. Phenolic compounds are the primary factors associated with the antimicrobial and smear layer removal effects of plant-based vinegars.

## Declarations:

### *Ethics Approval*

All experimental protocols were independently reviewed and approved by the Research Ethics Committee (REC) of the Faculty of Dentistry, Suez Canal University (approval number 190/ 2019).

### *Consent for publication*

Not applicable

### *Data availability*

The datasets generated during this study are included in this article.

### *Competing interests*

No potential conflicts of interest.

### *Funding*

No funding was received.

### *Authors' contribution*

MS and MR contributed to the study conception and design, supervision, reviewing and editing. HE contributed to the irrigation procedures, writing the original draft. ES contributed to the microbiological procedures and data analysis. All authors read and approved the final manuscript.

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