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In-Vitro Study of Lerak Fruit Ethanol Extract (*SapindusRarak DC*) on the Adhesion of *Fusobacteriumnucleatum* and Prevent Root Canal Wall Porosity

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Abstract:- *Fusobacteriumnucleatum* (*F. nucleatum*) has been reported as a trigger for endodontic infections. This infection can be treated by cleaning and shaping procedures using EDTA irrigation and NaOCl. Both of these materials have some drawbacks. Lerak fruit (*Sapindusrarak DC*) inhibited the growth of gram-negative bacteria such as *F. nucleatum*. This study aimed to evaluate the potential of Sapindusrarak DC in inhibiting the development and formation of *F.* biomass concerning the porosity of the root canal walls. This study used *F. nucleatum* isolate ATCC 23726 and ethanol extract of Lerak fruit. Using SEM, the assessment was carried out by evaluating the biomass index, adhesion test, and root canal wall porosity. The results showed the results showed Lerak fruit extract with concentrations (6.25%, 12.5%, 25%) had similarities with 2.5% NaOCl solution + 17% EDTA (p>0.05) in preventing adhesion and inhibiting the development of F. .nucleatum and Lerak fruit extract 6.25%, 12.5%, 25% could prevent porosity better than 2.5% NaOCl irrigation solution + 17% EDTA (p<0.05). The ethanolic extract of Lerak fruit can prevent adhesion, inhibit the growth of F.nucleatum bacteria, and reduce excessive porosity in the tooth root canal wall.

Keywords:- *Adhesion, Fusobacteriumnucleatum, Irigant solution, Porosity, Sapindus Rarak DC.*

I. INTRODUCTION

The ability of microorganisms to cause root canal infection is influenced by virulence factors consisting of microbial products, the structure of cellular components, and strategies that contribute to pathogenicity. One of the bacteria in the biofilm formed in endodontic infection is *F. nucleatum*(Wong et al., 2021). It bacteria, including obligate anaerobic gram-negative bacteria, and rods, is often found in root canal infections with apical periodontitis, acute apical abscess, post-endodontic infection, and pulp necrosis (Meng et al., 2021). One of the virulence factors of *F. nucleatum* bacteria is adhesin which plays a role in cell attachment, colonization, and coaggregation. Bacterial adhesion plays a significant role in determining the invasion of the dentinal tubules (Bashir et al., 2015).

The microbiological point of view, endodontic infections can be treated by chemical and mechanical procedures cleaning, and shaping. Mechanical debridement is an essential step for tissue removal and should always be accompanied by irrigation to remove remnants of pulp tissue and dentinal debris from the root canal system (Dennis et al., 2021). Sodium hypochlorite (NaOCl) 0.5%-5.25% is an irrigating solution commonly used in endodontic treatment because it has antimicrobial activity and can dissolve the remaining pulp tissue. However, NaOCl can significantly reduce the Ca/P ratio of the root surface dentin and cause the dentin surface to be porous after being observed. Forty seconds resulted in a decrease in the mechanical properties of dentin by 75%9. NaOCl cannot be used as sole irrigation, and it is often combined with a chelating agent such as ethylenediamine tetra-acetic acid (EDTA) (Ok et al., 2015). Irrigation with 17% EDTA for one minute followed by a final rinse with NaOCl is the most often recommended8. However, the combination results in the opening of the dentinal tubules, destruction of intertubular dentin, reduction of dentin hardness, and substantial erosion of dentin (Afshan et al., 2020).

Due to the weakness of irrigation materials that are often used, natural materials are used as an alternative to root canal irrigation which can be expected to be better and more biocompatible. One of the natural ingredients that can be developed for root canal irrigation is Lerak fruit (*Sapindusrarak DC*)(Nevi Yanti and Prasetia, 2017). The pharmacological properties of Lerak fruit include antifungal, bactericidal, and anti-inflammatory properties. The main content of Lerak fruit is triterpenoidsaponins which are surface-active compounds that act as surfactants (low surface tension) so that they can flow to areas that are not reached by mechanical cleaning and act as detergents that can dissolve dirt, so it is related to the possible effect of Lerak fruit on the smear layer like organic and inorganic substances. An anti-adhesion test will be performed in this study to determine the potential of Lerak fruit extract in preventing bacterial

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adhesion to the root canal to inhibit the growth of *F. nucleatum*, as well as a biomass test to determine the interaction activity of *F. nucleatum* bacteria and an irrigation solution of Lerak fruit extract, as well as a porosity test. to observe the clinical outcome on the root canal wall

II. MATERIAL AND METHODS

A. *Extraction of Lerak fruit*

The Lerak fruit was washed under running water, weighed 940 grams, and the flesh was cut with a width of ± 3 mm, and dried in a drying cabinet at a temperature of ± 40˚C for one week.The dried fruit pieces were mashed, sifted, obtained powder, and then stored in a closed plastic container.Add 800 ml of 70% ethanol for maceration and then store in a closed container and allowed to stand for three h.The mass is put into a percolator, pouredwith 200 mL of 70% ethanol, and filtered with a layer of filter paper.Leave until the liquid drips, and the percolator is closed, left for 24 h,then evaporated with a vacuum rotary evaporator until a thick extract is obtained with a honey-like consistency(Soraya et al., 2020).

B. *Culture of Fusobacterium nucleatum*

Fusobacteriumnucleatum culture was performed on Chromagar VRE media.The petri dish is divided into three parts.Heat the oase needle and wait for it to cool, then take onecolony of pure culture to be inoculated in area 1 with zig-zag strokes.Then reheat the oase needle and wait for it to cool, followed by zig-zag strokes in area 2 perpendicular to the first stroke, then continued with zig-zag strokes in area 3. The petri dish was tightly closed and incubated for 24 h at 37°C in an anaerobic, then equalized with McFarland 0.5 or equivalent to a concentration of $1.5x10^8$ CFU/mL (Gani et al., 2015).

C. *Adhesion Assay*

Serial 96-well triple microplates were coated with 50 µL Mueller-Hinton Broth (MHB) for 15 min and aspirated. Then 50 µL of *F. nucleatum* was added and incubated for 15 minutes at room temperature, then 100 l of the test material from each group was added and incubated for 24 h, 48 h, and 72 h. Furthermore, all the test material in the microplate was aspirated, and each was given 50 l of 2% crystal violet for 5 min and washed with PBS (Phosphate Buffer Saline). Furthermore, Lugol's solution was given for 1 min and washed with PBS. The rest of the metabolism of cells that are not bacterial cells are dissolved in 96% alcohol for 20 sec. Then, 50 µL of safranin solution was given for 2 minutes and washed again with PBS. On a microplate basis, the anti-adhesion activity of irrigating solution against *F. nucleatum* cells was assessed by Spectrophotometry Elisa reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 620 nm (Mubarak et al., 2018).

D. *Biomass Assay*

Bottles are weighed first with an analytical balance before being given an irrigation solution or bacteria.Then 3 ml was taken in each group of irrigation solution and considered before incubation.This treatment was repeated in each group's irrigation solution, interacting with 50 l of F.nucleatum.Then it was incubated at 37°C for 24 hours, 48 h, and 72 h, then weighed again.The scale value (g/mL) became an indicator of biomass before and after interaction with *F.nucleatum*(Soraya and Alibasyah, 2021).

E. *Scanning electron microscope of porosity*

A total of 25 mandibular premolars were extracted (sample criteria: the crown of the tooth was intact, the tooth had one root and had one root canal, the tooth root was relatively straight, there were no caries in the root canal, there was no crack, in essence, the tooth apex was utterly closed, long teeth are selected between 15-18 mm). Then cleaned and put into a plastic container containing a saline solution. The prepared teeth were sterilized in an autoclave for 18 hours at 37°C. Then the access cavity was prepared using an endoaccess bur. 100 µL of BHI medium was added to each group and incubated for 1.5 hours, then rinsed with saline. Then, each group was injected with 25 µL of F.nucleatum and incubated in an aerobic atmosphere for 6 h.

They determined working length with the help of a caliper and root canal irrigation with K-file no. 10. Irrigation of the root canal using a 5 mL syringe with a two-side vented needle type and size of 30 G, according to each treatment group. Root canal preparation using Mtwo files from files #10.04 to #25.06 (VDW, Germany). After irrigation, then dry with paper points. They were then incubated for 24 hours, 48 hours, and 72 h. The prepared teeth were stored in glycerol solution, then rinsed with PBS solution for 10 seconds. The tooth's root was cut vertically using the carborundum disc in a mesial-distal direction. The porosity examination with Scanning Electron Microscope (SEM) - JEOL JSM-6390A (1000x). It is to observe the porosity distribution in one-third of the root canal. Furthermore, the porosity analysis using Image-J software was carried out computerized to obtain the results of the distribution of the total porosity

F. *Statistical Analyses*

The anti-adhesion test was analyzed based on the group of test materials with Kruskal Wallis and other statistics with the Mann-Whitney test while based on incubation time with one-way ANOVA and other statistics with LSD.The biomass test was analyzed based on the test material group with one-way ANOVA and advanced statistics with LSD, incubation time with Kruskal Wallis, and other statistics with the Mann-Whitney test. The porosity value was analyzed based on the test material group and incubation time with a one-way ANOVA

III. RESULTS AND DISCUSSION

Figure 1 shows that the strongest anti-adhesion activity occurred in the 25% Lerak fruit extract irrigation solution and 2.5% NaOCl irrigation solution at an incubation time of 72 hours with an OD of 0.114 adhesins. Meanwhile, the lowest anti-adhesion occurred in the 17% EDTA irrigation solution group at 48 hours of incubation, with an OD of 0.050 adhesins. The incubation time of 72 hours was the strongest anti-adhesion property against *F. nucleatum* in all irrigation groups. Kruskal-Wallis statistical test yielded a p-value > 0.05, which indicated no significant difference between all irrigation groups in preventing *F. Nucleatum* adhesion. In

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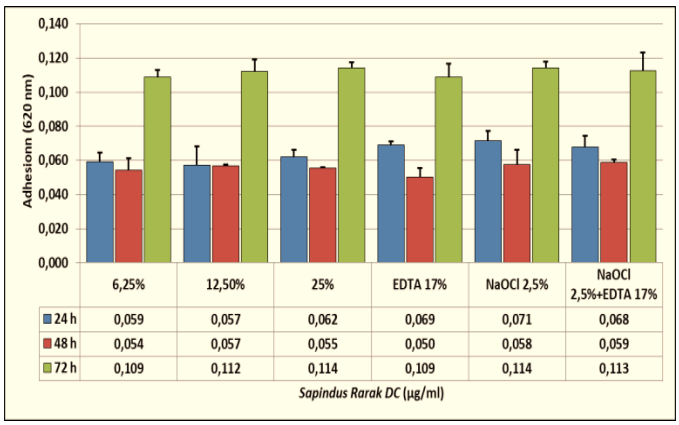
contrast, there was a significant difference between incubation time and the one-way ANOVA test ($p < 0.05$).

The incubation time was analyzed using the LSD test. The results showed that the incubation time of 24 hours significantly differed from those of 48 hours and 72 hours ($p<0.05$). The incubation time of 48 hours was also significantly different from the incubation time of 72 hours ($p<0.05$).

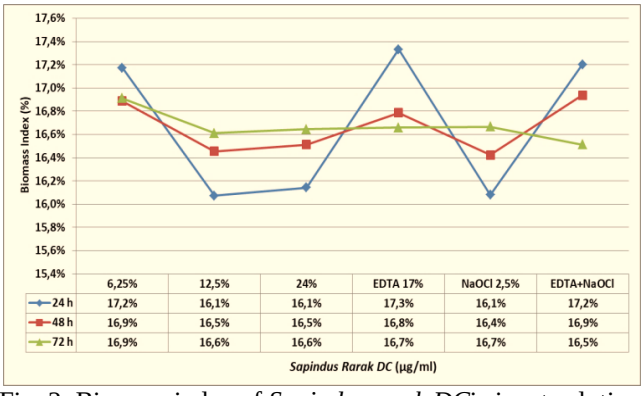


	6,25%	12,50%	25%	EDTA 17%	NaOCl 2,5%	NaOCl 2,5%+EDTA 17%
24 h	0,059	0,057	0,062	0,069	0,071	0,068
48 h	0,054	0,057	0,055	0,050	0,058	0,059
72 h	0,109	0,112	0,114	0,109	0,114	0,113

Fig. 1: Adhesion of *Sapindusrarak* DC to *F. nuleatum* in root canals

In Figure 2, it is reported that the best irrigation solution for inhibiting the development or activity of bacterial fermentation, which is characterized by a low percentage of biomass index, is the irrigation solution of 12.5% Lerak fruit extract and 2.5% NaOCl with a total biomass of 16.1% at 24 incubation. O'clock. Meanwhile, the highest percentage of biomass index indicating the weakest irrigation solution in inhibiting the growth of *F. nucleatum* was EDTA, 17%, with a total biomass of 17.3% at 24 hours of incubation.

One-way ANOVA test yielded a p-value > 0.05, indicating no significant difference between all irrigation groups in preventing *F. Nucleatum* adhesion. While between incubation time and the Kruskal Wallis test, there was also no significant difference ($p>0.05$). The incubation time was analyzed using the Mann-Whitney test. The results showed that the incubation time of 24 hours was not significantly different from those of 48 hours and 72 hours ($p>0.05$). The incubation time of 48 hours was also not significantly different from the incubation time of 72 hours ($p>0.05$).



	6,25%	12,5%	24%	EDTA 17%	NaOCl 2,5%	EDTA+NaOCl
24 h	17,2%	16,1%	16,1%	17,3%	16,1%	17,2%
48 h	16,9%	16,5%	16,5%	16,8%	16,4%	16,9%
72 h	16,9%	16,6%	16,6%	16,7%	16,7%	16,5%

Fig. 2: Biomass index of *Sapindusrarak DC*irrigant solution interacted with *F. nucleatum* in root canals

In Figure 4, it is shown that the combination irrigation solution group of 2.5% NaOCl + 17% EDTA had the highest porosity formation rate compared to the other test materials from all incubation times.They were followed by a 25% Lerak fruit extract irrigation solution, while saline irrigation solution had the lowest porosity level.The one-way ANOVA test resulted in a p-value <0.05, which indicated a significant difference between all irrigation groups in forming the root canal walls' porosity. Between incubation times, there was no significant difference ($p>0.05$). The incubation time was analyzed using the LSD test. The results showed that the incubation time of 24 hours was not significantly different from the incubation time of 48 hours and 72 hours of irrigation ($p>0.05$).The incubation time of 48 hours was also not significantly different from the incubation time of 72 hours ($p>0.05$).

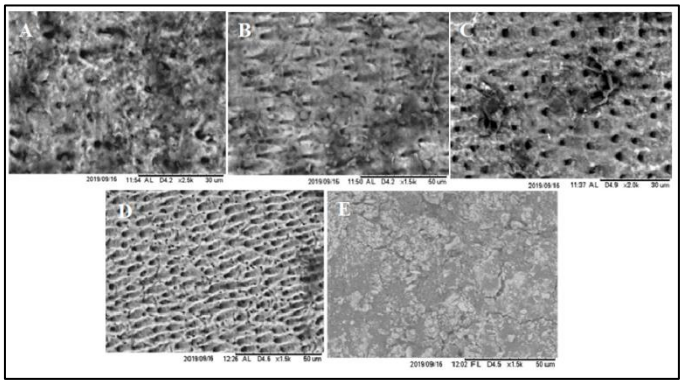
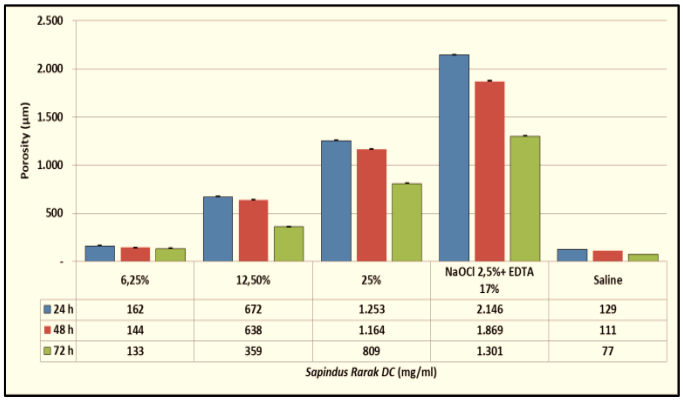


Fig. 3: The scanning electron microscope of porosity profile on the tooth root canal wall.In the SEM images, each treatment with Lerak fruit extract 6.25% (A), Lerak 12.5% (B), Lerak 25% (C), NaOCl 2.5% + EDTA 17% (D), Saline(E) with different incubation time variations after interaction with *F. nucleatum.*

Bacterial adhesion is an important virulence factor in pathogenesis and infection.The adhesion process is the initial stage of bacterial infection that plays a role in bacterial colonization on the surface of host cells1(Janoir, 2016).Adhesion of *F. nucleatum* to other bacteria and tooth surfaces is caused by fimbrial and non-fimbrial adhesions.Five adhesins are involved in biofilm formation by F.nucleatum, such as FomA, 300-350 kDaGalactose-binding adhesives, N-acetylneuraminic acid, and specific *F. nucleatum*adhesins(Ding and Tan, 2016).



	6,25%	12,50%	25%	NaOCl 2,5%+ EDTA 17%	Saline
24 h	162	672	1.253	2.146	129
48 h	144	638	1.164	1.869	111
72 h	133	359	809	1.301	77

Fig. 4:-The porosity of root canal walls after irrigation with ethanolic extract *of Sapindusrarak DC*and*F. nucleatum.*

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Based on the results of the Kruskal Wallis statistical test, it was found that there was no significant difference in the formation of anti-adhesion between the irrigation solution groups p>0.05 (0.904). The results of further statistical tests using the Mann-Whitney test also showed no significant difference (p<0.05) between one irrigation group and another. So it can be assumed that Lerak fruit extracts of various concentrations (6.25%, 12.5%, 25%) were as good as 2.5% NaOCl irrigation solution + 17% EDTA which became the gold standard in root canal irrigation in preventing adhesions. The presence of active components in the irrigating ingredients of the Lerak extract, such as flavonoids and tannins, can also affect the adhesion of bacteria. Flavonoids can deactivate adhesins from bacteria, thus affecting their adhesion ability. In addition, tannins can also bind to the adhesin protein possessed by bacteria, thereby damaging the surface availability of bacterial cell receptors, forming irreversible complex compounds with proline, thereby inhibiting protein synthesis (Roy et al., 2018). The results of this study are also in line with several previous studies, which showed that the antibacterial effect of the ethanolic extract of Lerak fruit ranged from 0.01% to 25%.

One way ANOVA statistical test based on incubation time showed a significant (p<0.05:0.001). The results of further statistical tests with LSD also showed a significant difference (p<0.05) between 24 hours, 48 hours, and 72 hours incubation. The incubation time of 72 hours had the strongest anti-adhesion activity and was significantly different from 24 hours and 48 hours in all groups of irrigation solutions. It can be illustrated that time is related to activity between biofilm formation as a cell adhesion pathway for *F.nucleatum* in host cells (Zhang et al., 2020). The 24- 48 hours are the highly proliferative phases of the development of *F.nucleatum* adhesion. It is related to the activity of *F.nucleatum*, which can maintain itself stable during interaction with experimental materials(Proença et al., 2018). Bacteria have an extraordinary ability to defend themselves from stress responses to the environment. When there is a lack of nutrients, they enter the stationary phase (resting phase)by developing resistance cells (Bertrand, 2019).

The results of the second study were regarding assessing the activity of the irrigation solution against F.nucleatum bacteria considered from the biomass index. This principle measures the potential of an irrigating solution in inhibiting the development or activity of *F.nucleatum* fermentation. The smaller the percentage of the biomass index, the stronger the role of the irrigation solution in preventing bacterial activity from synthesizing the active compounds contained in the test material. The one-way ANOVA statistical test showed an insignificant difference between the biomass index and the irrigation solution groups, p> 0.05 (0.051). Further statistical tests with LSD showed no significant difference between 6.25%, 12.5% , and 25% Lerak fruit extract irrigation solution and 2.5% NaOCl + 17% EDTA irrigation solution. So it can be assumed that Lerak extract in various concentrations (6.25%, 12.5%, 25%) is as good as 2.5% NaOCl + 17% EDTA, which is the gold standard for root canal irrigation in inhibiting fermentation activity and development of *F. nucleatum*.

Based on the results of the GCMS test on Lerak fruit extract, there are several active components in Lerak fruit extract, such as 6-Octadecenoic acid (27.46%), Dodecanamine N-Dimethyl (19.91%), and Hexadecanoic Acid (6.82%).These components play a role in inhibiting microbial growth by damaging the structure of cell walls and membranes.The active compounds in the Lerak fruit are 28% saponins, alkaloid compounds, polyphenols, antioxidant compounds and flavonoid groups, and tannins (Makarewicz et al., 2021).Polyphenols work as antibacterial by denaturing cell proteins and damaging plasma membranes.Alkaloids act as antibacterials with the mechanism of interfering with the arrangement of peptidoglycan in bacterial cells so that the formation of cell walls becomes imperfect, causing bacterial cells to become efficiently lysed and ending in bacterial death (Othman et al., 2019).

The results of the third study based on the one-way ANOVA statistical test showed a significant difference in the porosity value of the type of irrigation solution test material p <0.05 (0.001). 2.5% NaOCl + 17% EDTA had the highest porosity at all incubation times, as seen on the Scanning Electron Microscope (SEM). The results of further statistical tests with LSD showed that the porosity level of 2.5% NaOCl + 17% EDTA irrigation significantly differed from Lerak extract of 6.25%, 12.5% , and 25%. So it can be assumed that Lerak extract in various concentrations (6.25%, 12.5%, 25%) did not cause excessive porosity compared to 2.5% NaOCl + 17% EDTA, which became the gold standard for root canal irrigation. In addition, the porosity level based on incubation time showed no significant difference, p>0.05 (0.747) in both 24 hours, 48 hours, and 72 hours. It means that the type of test material in different groups determines the level of porosity, while the incubation time has no effect.

Demineralized dentin presents two types of porosity: the first is tubular porosity, and the second is porosity due to the collagen meshwork.Porosities due to these tubules have been reported to be 12–32% and 21%, with a mean tubular diameter of 3–3.5 mm for demineralized dentin (Shen et al., 2018).This study showed the formation of more porosity in the 2.5% NaOCl + 17% EDTA group in the form of a larger dentinal tubule size. There were several secondary tubules observed in the intertubular dentin.It is in line with research by Gorduysus et al. (2015), where erosion occurred around the dentinal tubules in the 17% EDTA and 2.5% NaOCl combination group.SEM images show excessive dentin degradation causing the intertubular dentin to disintegrate and the conjugation of two or more dentinal tubules, resulting in enlarged dentinal tubules with an irregular shape.

Hypochlorous acid contained in NaOCl, when in contact with organic tissue, will release chlorine combined with amino acid groups to produce chloramines in the chloramination reaction10.Sodium hypochlorite breaks down the peptide chain, and the protein chlorinate group results in N-chloramine being broken down into other parts, causing collagen and proteoglycan breakdown (da Cruz Nizer et al., 2020).EDTA can dissolve inorganic tissues by removing metal ions such as calcium and chemically binding them through two nitrogen atoms in the amino group and four oxygen atoms in the carbonic group, causing decalcification

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of dentin(Guo et al., 2019).The greater the volume concentration of protein, the significantly reduced impact of damage to the protein-mineral surface on dentin.Dentin becomes porous due to the loss of dentin minerals and denaturation of the collagen matrix (Abdallah et al., 2018).

The ability of the Lerak fruit extract to remove the smear layer is due to the presence of saponins which are the active components of the Lerak fruit extract which act as surfactants that can flow into inaccessible areas by mechanical cleaning and act as detergents that can dissolve dirt, so it is associated with the possible effect of Lerak fruit against organic and inorganic smear layers(Nevi Yanti and Prasetia, 2017). Hydrophilic (polar compounds) and hydrophobic (non-polar compounds) groups in Lerak fruit allow saponins to dissolve organic and inorganic components in tooth root canals. The smear layer, an inorganic layer of dentin that mostly contains calcium hydroxyapatite and tricalcium phosphate, a non-polar compound, will dissolve in the hydrophobic group (non-polar compound) of the saponins in the Lerak fruit (Pribadi et al., 2019). So from the SEM image, it can be seen that irrigation solutions derived from nature, such as Lerak fruit extract, can clean the smear layer without causing excessive erosion of the root canal walls.

IV. CONCLUSION

Lerak fruit extract in various concentrations (6.25%, 12.5%, 25%) was not significantly different from the 2.5% NaOCl + 17% EDTA solution, which became the gold standard in root canal irrigation of teeth in preventing bacterial adhesion and inhibiting growth*F.nucleatum.*While clinically Lerak extract in various concentrations (6.25%, 12.5%, 25%) was better because it did not cause excessive porosity at all incubation times compared to 2.5% NaOCl + 17% EDTA, which became the gold standard for irrigationroot canal

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