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Original Article

Fructus mume aqueous extract's antibacterial effect on multi-species biofilm

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ABSTRACT

Aim: *The purpose of this study was to determine the efficacy of several irrigants and an aqueous extract of Fructus mume in eradicating three kinds of biofilm.* ***Materials and Methods:*** *A three-species biofilm of E. faecalis, P.gingivalis and F.Nucleatum were cultivated for one week on Thermanox™ plates. Each specimen was immersed for 8 minutes in various irrigants: Fructus mume solution, buffered citric acid, sodium hypochlorite and control (non-irrigated). LIVE/DEAD® BacLightTM staining and confocal light scanning microscopy were used to determine the number of viable bacteria left on the substrate. The same biofilm was then extracted and subjected to scanning electron microscopy (SEM).* ***Results:*** *Images were taken at 12 locations across the biofilm, which were then classified into four areas of concern: Bottom, which would spend the most of the experiment submerged in the solution; It was struck in the centre by the stream of irrigant.; Middle and Upper, where the impression was caused by the irrigant splattering or vaporizing. Results of the amount of viable bacteria residual indicated that Fructus mume showed no significant activity, with an effect like control and even buffered citric acid, and significantly inferior to sodium hypochlorite.* ***Conclusion:*** *Sodium hypochlorite (0.5%) solution was greater to buffered citric acid, Fructus mume and control group as an antimicrobial agent against three-species biofilm.*

Keywords: *E.faecalis,P.gingivalis , F.nucleatum fructus mume, dental irrigants, biofilm, traditional Chinese medicine,Japanese prune.*

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INTRODUCTION

In recent decades, there appears to have been a general trend toward growing usage of alternative medicines (Badakhsh et al., 2021; Barnes, Powell-Griner, McFann, & Nahin, 2004; Goldbeck-Wood et al., 1996). The World Health Organization heartens and cares efforts to reorient traditional medicine into national health planning. Augmented adverse effects of Western medicine (Yuan & Lin, 2000), steep new drugs, microbial resistance, lack of effective cure for chronic illness, and emerging new diseases are the rationale for public fascination towards complementary medicine (Humber, 2002). On the other hand, dentists are recommended only to apply for proven alternative medicine and carry the least risk (Little, 2004). The success of traditional Chinese medicine (TCM) is mainly on the experience of either the patient or practitioner. An evidence-based approach in complementary medicine is needed for us to find an alternative procedure and technique for enhancement or integration of conventional treatment.

In periapical periodontitis, microorganisms invading the necrotic pulp of teeth have been recognized as the cause of acute and chronic manifestations of the diseases. (Kakehashi, Stanley, & Fitzgerald, 1965; Möller A, Fabricius L, Dahlén G, Ohman A, 1981)·Root canal therapy aims to eliminate infection and promote periapical healing (Dag & Ford, 1998).Through endodontic treatment, irrigation is critical to disinfect the root canal space. Toxins, bacteria and critical necrotic tissue miscellanies are removed. (Grossman, 1943). In periapical periodontitis, microorganisms invading the necrotic pulp of teeth have been recognized as the cause of acute and chronic manifestations of the diseases. (Kakehashi, Stanley, & Fitzgerald, 1965; Möller A, Fabricius L, Dahlén G, Ohman A, 1981)·Root canal therapy aims to eliminate infection and promote periapical healing (Dag & Ford, 1998).Through endodontic treatment, irrigation is critical to disinfect the root canal space. Toxins, bacteria and critical necrotic tissue miscellanies are removed. (Grossman, 1943).

Flushing by irrigation removes dirt from canal walls and dissolves organic material (Byström A, 1985), demolishes microorganisms (Zehnder, 2006), softens dentine, removes smear layer, and cleaning places not accessible mechanically. Preferably, an irrigant should be antibacterial, have low surface tension, permeate the dentinal tubule and canal perimeter, be biocompatible, proteolytic, and have a substantial tissue dissolving effect.

Sodium hypochlorite (NaOCl) is the most widely antibacterial agent used today (Bloomfield & Miles, 1979). It has remained unchallenged until now despite numerous introductions of new products.

In different culture*s, Fructus Armeniaca mume* is identified *as oumae/maesil (Korea), ume/Japanese apricot (Japan) and wu mei (China)*(You et al., 2021). When ripe and cooked correctly, it can be used as a herbal treatment to alleviate coughs. Its efficacy against *Porphyromonas gingivalis, Streptococcus sanguis, Streptococcus mutans,* and *Streptococcus mitis* has been demonstrated *in vitro* (Wong et al., 2010).

This study aimed to determine the efficacy of an aqueous extract of Fructus mume, buffered citric acid, and sodium hypochlorite in removing three different biofilms. The null hypothesis is that the antibacterial of NaOCl, *Fructus mume* and buffered citric acid at the same pH value have no significant difference against a control group.

MATERIALS AND METHODS

Mirror-smooth polystyrene cover lid of 1.35 mm thickness was grooved into a 10 x 13 mm rectangle, using a sharp knife repeatedly until the break. 600-grit electro-coated, waterproof silicon carbide abrasive paper (Eagle Head, China).

All blank specimens (the rectangular polystyrene substrates) were sterilized by immersion in a beaker of 0.05% NaOCl solution in a stirrer-mixer (Cimarec SP131320-33) for 3 minutes. They were then washed with sterile distilled water for 10 minutes three times to remove any chemical traces. The passed polystyrene discs were reserved in clean distilled water until use.

Each specimen plate was incubated for one week with a biofilm composed of three different species (Enterococcus faecalis, Fusobacterium nucleatum, and Porphyromonas gingivalis). *Enterococcus faecalis* (Ef) (ATCC 29212), *Fusobacterium nucleatum* (Fn) (ATCC 25586) and *Porphyromonas gingivalis* (Pg) (ATCC 33277) were inoculated individually onto blood agar and cultivated anaerobically for one to two days. The colony of each species was used to produce a suspension of the individual bacteria to McFarland standard 4 (1 x 10^7cfu/mL).

The control measure was rigorously observed. A lid was placed on the culture plate and secured with a transparent plastic film (Parafilm M; Pechiney, Chicago, IL, USA). The plate was cultured in an anaerobic chamber (Forma Anaerobic System; Thermo Scientific, Shatin, Hong Kong) with a daily medium change (PG broth). Roughly 300 µL of suspension for each bacterium (to make a total of about 900 µL) was used for bathing the specimen in each well.

Forty-five rectangular plates were prepared and cultured for this experiment (n = 13 for each group, with six as controls). Each substratum (with biofilm attached) was tilted at about 45 degrees in a cell well for irrigation by the test solution. All irrigant solutions were prepared fresh, with their pH monitored.

One group was irrigated with 2% *Fructus mume* (pH = 2.6), the other with buffered citric acid to the same pH as the *Fructus mume*, one group was irrigated with 0.5% sodium hypochlorite (NaOCl), and the last six specimens were left not irrigated but were gently waved in the neutralizing solution and then in PBS, before LIVE/DEAD® *Bac*Light ™ staining.

All four groups were irrigated concurrently with a motorized syringe pump adjusted to 0.3 mL per minute (MasterFlex L/S: Pump Head System, Niles, IL, USA). The unit was thoroughly washed with 10 mL of distilled water to eliminate any remaining irrigant before the treatment. 1 mL of irrigant was injected into each well until half of the specimen plate (about 6.5 mm from the bottom) was submerged in the solution. Following irrigation, the samples were kept in place for 8 minutes (i.e., ten minutes in contact with the solution). After gently draining excess irrigants from them, they were waved in another pan containing a neutralizer and PBS prior to staining.

The specimens were stained with the LIVE/DEAD® BacLight™ stain according to a previously established CLSM and SEM studies procedure. A spreadsheet was used to tabulate the amount of living and dead bacteria in the examination field.

For all specimens, a sum of 540 CLSM pictures was acquired. The six sections, U to Z, were divided into four areas for analysis: upper (which included U and V), middle (which had W and X), central (Y), and bottom (Z), due to the absence of a substantial difference between sections U and V and W and Y. The middle (sections W and Y) and upper (sections U and V) areas were exposed to the irritant by wallowing or exhausts, respectively. Two sections in the upper or middle areas were comparable in terms of their distance from the centre. Section Z was deep in the solution for the majority of the experiment; the centre (section X) was irrigated in addition to any antibacterial effect.

Calculating the total number of cells and the percentage of live and dead bacteria cells allowed us to estimate the effect of each irrigant on the biofilm. The number of living and dead bacterial cells was determined independently using the area percentages of green (life)and red (dead) signals in CLSM micrographs (Fig. 1.0). Four distinct spots are represented: i) upper, ii) central, iii) middle and iv) bottom. Then, using the formula below, the quantity of living cells in the overall population was computed.

$$\text{Proportion of live bacteria} = \frac{\text{Live bacteria}}{\text{Overall population (i.e., dead + live bacteria)}} \times 100\%$$

The four locations (upper, centre, middle and bottom) were analyzed for the total amount of cells and percentages of live bacteria present. A test of equal variances (Levene's) and normality (Kolmogorov-Smirnoff) was performed. As the results for each group in this experiment did not follow a normal distribution, the data were analyzed using a non-parametric, two-way ANCOVA test. A post-hoc analysis utilizing a multiple range test (Student-Newman-Keuls) was performed to identify homogeneous subsets among the test irrigants. The level of significance was set at $p < 0.05$. In each group, the averages and standard deviations were calculated. Statistical analysis software was used to analyze the data (SPSS 18.0 for Windows, SPSS Inc., Chicago, USA).

RESULTS

Group	*Fructus mume* solution (TCM)		Buffered Citric Acid		NaOCl		Control	
Region	Green channel	Red channel	Green channel	Red channel	Green channel	Red channel	Green channel	Red channel
Upper								
Centre								
Middle								
Bottom								

Fig.1.0 CLSM imaging (40x magnification) comparing four groups: *Fructus mume* solution, buffered citric acid, NaOCl, and control (non-irrigated).

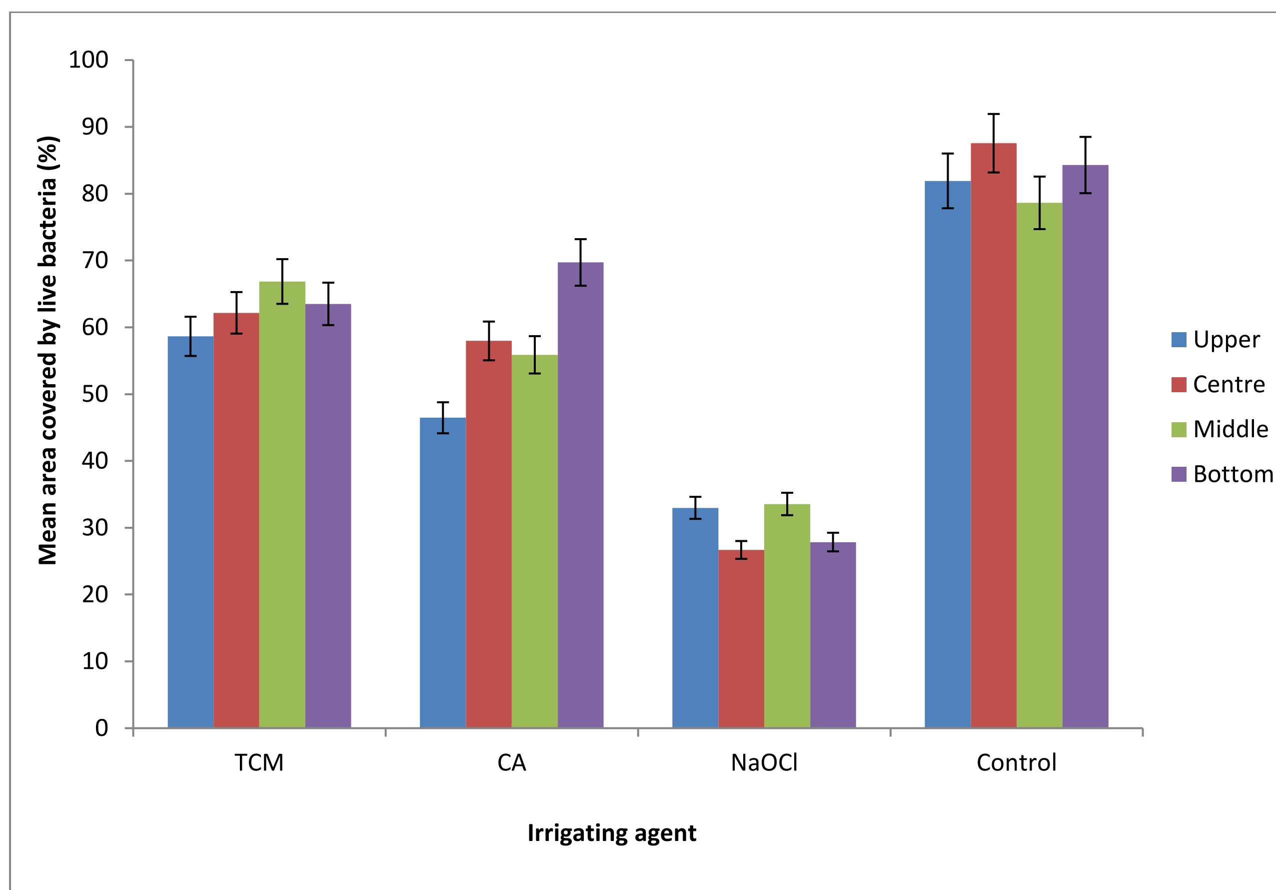

Fig.2.0 The mean total bacterial load (live and dead) at different locations.

The Kolmogorov-Smirnoff test verified the normal distribution of the data, and Levene's test confirmed the homogenous variance of the data. The parametric two-way ANOVA was used to compare the total bacterial load (live and dead) at different regions of the substrates between groups. The percentages of live bacteria at different regions of each experimental group varied from 59% to 67% for *Fructus mume*, 47% to 70% for buffered citric acid, 27% to 34% for sodium hypochlorite 79% to 88%. However, the difference between the four locations within all experimental groups was not statistically significant ($p > 0.05$). Since there is no significant difference in total load and the proportion of live cells between four locations (upper, centre, middle and bottom) of the substrate, the data were pooled for comparing the differences between groups (Fig. 2.0).

Table 1.0 The overall (mean) of bacterial loads and percentages of live cells.

Group	The proportion of live bacteria (%)		Total number of cell (live and dead) (cell/mm^2)	
	Mean	SD Value	Mean	SD Value
Fructus mume	63	10	2451	882
The buffered citric acid (pH 2.6)	58	6	1736	818
NaOCl	30	7	871	398
Control (Not irrigated)	83	10	2827	785

Student-Newman -Keuls test showed the total bacterial load of the buffered citric acid group was significantly lowered than control ($p < 0.001$). The difference in total bacterial load between *Fructus mume* and buffered citric acid was insignificant ($p > 0.05$). The same assessment also displayed that the percentages of live cells of Fructus mume and buffered citric acid were not significantly different from the control group ($p > 0.05$), but these groups were significantly higher than the sodium hypochlorite group ($p < 0.001$).

At relatively low magnifications (500x), the morphological of the three-species bacteria was not noted, but the resolution was not high enough to identify three different species solely. High magnification views (2000x) demonstrate the presence of cocci from bacteria on the specimen with no significant interruption, which bacteria appeared to be successfully attaching to the specimen (Fig. 3.0). This finding corroborated with high-power CLSM images (Fig. 1.0). All these groups did not confirm any considerable disruption of the biofilm. Compared to the other experimental groups, a zone of biofilm rupture could be identified in the NaOCl group. In both magnifications, it appeared that the biofilm had been eradicated.

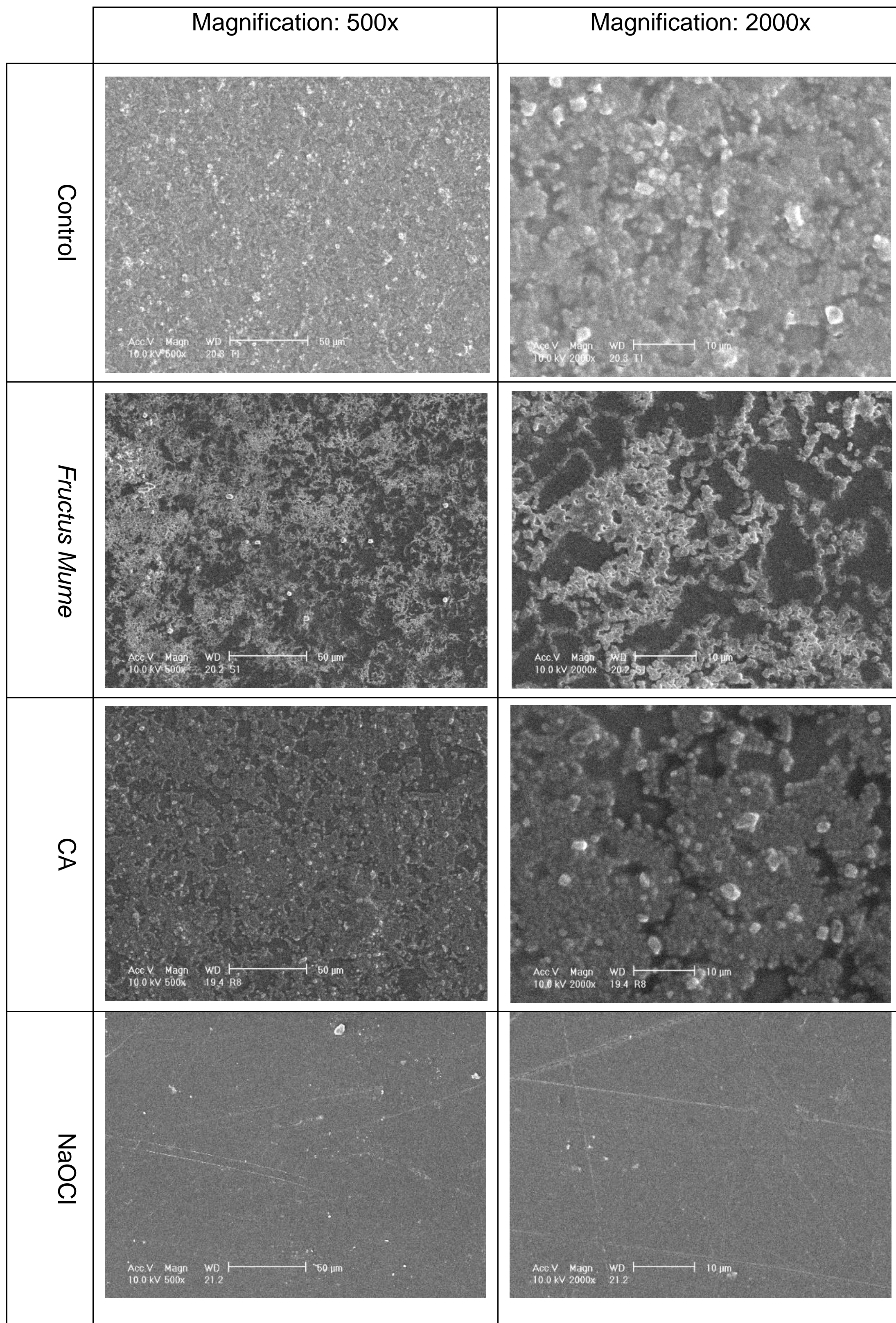

Figure 3.0 SEM photos demonstrate the comparison of four distinct groups. Images were taken at magnifications of 500x and 2000x.

DISCUSSIONS

Polystyrene is a transparent material generally used in laboratory and clinical settings. Numerous manufacturers make culture plates from polystyrene, and some products are treated with gamma radiation to render them sterile. We tested the router versus inner surface in a small pilot study (not described here) and found that *E. faecalis, P. gingivalis and F. nucleatum* built up a biofilm on either surface from one day to nine days. The disc samples were prepared from the (mirror-smooth) polystyrene cover lid into a standard rectangular shape. No surface roughening or polishing process was done to maintain its mirror-smooth surface. They were not autoclaved, as polystyrene would deteriorate at that temperature. For 'sterilization', the polystyrene disks were immersed in a beaker of 0.05% of NaOCl solution for 3 minutes, based on a previous report by Tse, 2010. The negative control, treated according to this method, showed no bacterial growth indicating that thorough disinfection has been achieved.

Dental biofilms, including numerous species, should be more critical for elucidating the antibacterial activity features. For this, a multi-species inoculation has been attempted in using three bacterial species: *E. faecalis*, *P. gingivalis* and *F. nucleatum* up to one week's incubation. Based on our pilot study, as the biofilm aged, *F.nucleatum* seemed to be bridging with various other cells. A series of coaggregations test between the three species has been conducted (not described here), as it would be challenging and techniques sensitive to produce a consistent and controlled three-species biofilm, it may be recommended to use a mono-species biofilm.

Two approaches were used to examine the influence of the irrigating agent on the biofilm in this study: scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). The latter was used to quantify the bacterial population contained within the biofilm. Other laboratory techniques have been employed to investigate different antimicrobial effectiveness of irrigating agents against microorganisms, such as quantification of colony-forming units (Abdullah, Ng, Gulabivala, Moles, & Spratt, 2005; Siqueira JF, Rocas IN, Favieri A, 2000), disc diffusion method (H. K. Haapasalo, Sirén, Waltimo, Ørstavik, & Haapasalo, 2000; Yamaguchi, Yoshida, Suzuki, & Nakamura, 1996), broth culture for the minimum inhibitory concentrations (MICs) (Kwon, Kwon, Kwon, & Lee, 2008; Wong et al., 2010) and real-time bioluminescent imaging (Nguy & Sedgley, 2006). CLSM allows the determination of the total bacterial content and the proportion of live or dead cells when used in conjunction with the viability staining kit.

SEM has a considerable depth of field and can display the external topography of construction in detail. It is still one of the most often used techniques for showing the microstructure of the biofilm surface. McComb & Smith (1975) have illustrated using SEM the cleanliness of the root canal wall after various instrumentation techniques and chemical (irrigant) treatments. The dental literature is replete with SEM examination of biofilms. If a high-vacuum SEM is used, this examination method requires the specimens to be dehydrated (using a graduating series of alcohol), which may distort biological (biofilm) structures. Hence, biofilms are not suitable for examination in a high vacuum. Conventional high-vacuum SEM may show a fibrous appearance for the biofilm instead of a gelatinous exopolysaccharide matrix surrounding the bacterial cells.

The confocal laser scanning microscopy (CLSM) technique is based on the conjugate focus value of the optical microscope. It can eliminate out of–focus spots during observation and enables a method known as optical sectioning without harming the specimen. This method allows for the examination of biofilms without causing significant disruption or modification. While not used in this work, CLSM can reconstruct multi-layer systems in three dimensions. Microorganisms, both alive and dead, can be identified using fluorescent stains.

Zaura-Arite et al. (2001) have combined fluorescent staining and CLSM to determine the structural characteristics, spatial distribution, and viability of the bacteria within the biofilm without disrupting the specimen. To minimize the noise in the CLSM image, Heydorn et al. (2000)(Dunavant, Regan, Glickman, Solomon, & Honeyman, 2006; Yamaguchi et al., 1996) proposed to use a fixed threshold value for all images taken. The operator can alter the setting for a similar grey level each time an image is taken.

Throughout this period, electrostatic contact could be sensed; this sensitivity seemed to support the hypothesis that biofilms can generate electrostatic communications, particularly for crosslinking between the matrix and the substrates. Every specimen was irrigated individually in its well by a single agent. This model reduced the unpredictability and impacted allied with the complexity of the root canal system, allowing for a truthful comparison of the effect of the various agents used both strength and drawback of this study yet.

This approach would help assess any mild impacts caused by the irrigants' flow over the substrate's surface and the agent's chemical activity. Observe how the core part of the specimen was subjected to both chemical (antibacterial) action and the "impact" of the solution directed to it by the irrigating needle. The approach examined the effect of a single rinse with an irrigant on the biofilm.

In the literature, an acidic environment (due to citric acid) appeared to have an antimicrobial effect (Dunavant et al., 2006; Yamaguchi et al., 1996). Yamaguchi et al. (1996) showed some antimicrobial effects of citric acid on twelve microorganisms: *Actinomyces naeslundii, Fusobacterium nucleatum. Staphylococcus aureus, Lactobacillus casei, Peptococcus niger, Peptostreptococcus anaerobic, Streptococcus sanguis Bifidobacterium bifidum, Porphyromonas endodontalis*, *Veillonella parvula, Propionibacterium avidum,* and *Eubacterium lentum.*

The antibacterial effectiveness of Fructus mume and citric acid against a three-species biofilm was investigated in this study. Unfortunately, the findings from this study indicated no significant difference in the amount of reduction in live cells between the *Fructus mume* and control group ($p > 0.05$). This demonstrated little if any antimicrobial effect for *Fructus mume* at the tested concentration.

Antimicrobials are ineffective against bacteria contained within biofilms. A few resistances possibility have been proposed in the literature. One possibility is the inability of antibacterial agents to infiltrate the biofilm to its full thickness. Certain antibacterial treatments appear to be able to permeate the biofilm's depth, and just a thin biofilm can exhibit this level of resistance to antimicrobial drugs. Another mechanism is that microorganisms may assume a 'starvation-like' physiological condition to lower their susceptibility to chemical agents. Dodds et al. (2000) further suggested that the age and thickness of the biofilm would decrease the susceptibility of bacteria therein to antibacterial effect.

The contact time and concentration of the irrigants significantly affect the microorganisms' elimination. Just half of the biofilm was exposed to (eventually immersed in) the irrigating agent in this investigation.

This investigation demonstrated that there was no significant difference between *Fructus mume* and citric acid in terms of total cell count and percentages of living bacteria. This is contradictory to the observation of Chen et al. (2011), who showed that *Fructus mume* (250%) possessed a more potent antimicrobial effect against *Streptococcus mutans* than citric acid (0.6%, pH 2.2). The difference could be explained by the fact that the TCM utilized in this trial was at a lower concentration. At a higher concentration, the solution could not pass through the irrigation needles.

This study used a basic model to demonstrate the influence of irrigant flow on the biofilm. (Ayoub & Cheung, 2018). However, it does not account for the solution's velocity, particularly in a clinical root canal, even though the flow rate was comparable in all groups. The complicated structure of root canals creates an environment that is difficult to clean and shape, which is not replicated in this work. Contents of the root canals can inhibit or neutralize the action of irrigating solution or intracanal medicaments (M. Haapasalo, Endal, Zandi, & Coil, 2005); this is another variable not considered here.

Another disadvantage of this study is the use of just one concentration of TCM and NaOCl. Additional study may be required to establish whether increasing the concentration of *Fructus mume* or combining it with other materials will enhance its antibacterial efficacy (and the likelihood of smear layer elimination).

CONCLUSION

Fructus mume had a similar antibacterial effect to citric acid buffered to the same pH against a three-species biofilm ($p > 0.05$). **Because of the acidity of the aqueous extract, it is unknown if *Fructus mume* has any different antibacterial properties.**

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