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Antimicrobial Action of A Chinese Medicine Extract On E. Faecalis Biofilm

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ABSTRACT

This manuscript is to investigate the effectiveness of various irrigants and an aqueous extract of *Fructus mume* in combating *E. faecalis* biofilm. A mono-species biofilm of *E. faecalis* was cultivated for 3 days on Thermanox™ plates. Each biofilm specimen was subjected to 10 seconds of immersion in different irrigants: *Fructus mume* solution, citric acid, sodium hypochlorite or sterile saline. The amount of viable bacteria remaining on the substrate was quantified by LIVE/DEAD® *Bac*Light™ staining and confocal light scanning microscopy (CLSM). Then, the same biofilm was retrieved and processed for scanning electron microscopy (SEM). Images were obtained from 12 sites throughout the biofilm, which were grouped into four regions of concern: Bottom where it would be immersed in the solution for most of the duration of the experiment; Centre where it was struck by the stream of irrigant; Middle and Upper where the effect was due to splashing or vapour of the irrigant. Results of the amount of viable bacteria residual indicated that *Fructus mume*

showed no significant activity, with an effect similar to physiological saline or citric acid, and significantly inferior to sodium hypochlorite. Sodium hypochlorite (0.5%) solution was superior to citric acid, *Fructus mume* and physiological saline as an antimicrobial agent against *E. faecalis* biofilm.

Keywords: *E.faecalis*, *fructus mume*, dental irrigants, biofilm, traditional Chinese medicine

Introduction

The existence of bacteria contained by the root canal concerned in the commencement, proliferation and continuation of pulpal and periapical disease [1, 2]. The aim of root canal treatment is the elimination of infection and to produce an acceptable environment suitable for healing of periapical region [3]. Irrigation is one of the crucial methods to achieve disinfection of the root canal space during endodontic treatment. It involves the removal of vital, necrotic tissue remnants, debris, microbial toxins and microorganisms [4].

The advantages of using irrigants are wetting of canal walls and removal of debris by flushing, dissolution of organic substance [5], demolition of microorganisms [6] ,removal of smear layer, softening of dentine and cleaning the areas that are unreachable by mechanical method.

An ultimate irrigant should have an antimicrobial effect by destroying the microorganism [6] and their by-products without damaging the normal tissue [7], high detergent strength, a low surface tension that easily penetrate the dentinal tubule and canal periphery, able to be kept in a fluid state, easy to be handle,biocompatible, high tissue dissolving and proteolytic power. Sodium hypochlorite (NaOCl) has been acknowledged as the most effective antimicrobial agent used to date.

Fructus armeniaca mume (*wu mei*), also known as *Prunus mume* or Japanese apricot, has been tested *in vitro* to be effective against *Streptococcus mitis, Streptococcus sanguis, Streptococcus mutans* and *Porphyromonas gingivalis* [8]. *Fructus armeniaca mume* is a common fruit in Asia, can be eaten raw and may be used as a herbal medicine once it ripens and prepared properly.

The purpose of this study was to investigate the effectiveness of various irrigants: sodium hypochlorite, buffered citric acid, physiological saline and an aqueous extract of *Fructus mume* in combating *E. faecalis*.

Mono-species biofilm of E. Faecalis was selected as the test subject for comparing the action of various irrigants as mentioned above. This bacterium is a facultative an aerobic gram positive coccus [37]. It is a well –know endodontic pathogen that is commonly found in teeth with recurrent endodontic lesions [2,17]. These microorganisms have shown a 'promising' biofilm structure and are widely analyzed in laboratory studies [12,18,19,20].

Materials and Methods

Thermanox (Thermo Fisher Scientific, Creek Drive, Rochester, NY) is a particular type of polymer of the polyelfin polyester cover-slip that has been used in bacteriological studies [9]. The material was cut into a 10 x 13 mm rectangular shape, with a pair of scissors. An identification mark was cut at one corner of the rectangle to aid orientation while viewing the confocal laser scanning microscope (CLSM) (FluoView FV1000; Olympus, Shinjuku-ku, Tokyo, Japan) or scanning electron microscope (SEM) (XL 30 CP; Phillips, Eindhoven, The Netherlands).

The Thermanox discs were sterilized in an autoclave (SES 2000; Eschmann Equipment, West Sussex, England). A total of 48 discs prepared were used.

E. faecalis (strain ATCC 29212) was inoculated on blood agar and cultured anaerobically for 72 hours. A bacterial colony on the agar was scooped and added into a Peptone-glycerol (PG) broth supplemented with 2% glucose for preparation of

a suspension of McFarland standard 4, by monitoring the optical density 660 nm (0.380 = 1 x 107 cfu/mL) under a spectrophotometer (Beckman DU Series 500; Brea, California, USA).

Each substrate was placed in an individual well of a 24-well sterile culture plate (Corning Glass Works, New York, USA). The substrates were inoculated with about 900 µL of prepared suspension into each well. Aseptic technique was strictly adhered. The culture plate was covered with a lid, and sealed with a thin plastic film (Parafilm M ; Pechiney, Chicago, IL, USA). The plate was incubated in an anaerobic chamber (Forma Anaerobic System; Thermo Scientific, Shatin, HK) with the medium (PG broth) renewed every day.

After incubation, the substrate (with the biofilm attached) was gently waved in a 1 mL solution of phosphate buffer solution (PBS; pH = 7.2) for 2-5 seconds to remove the non-adherent bacteria.

There were four groups (n = 12 each) in this experiment: (a) 0.5% NaOCl; (b) 2% *Fructus mume* (pH = 2.6); (c) 2% citric acid buffered to pH 2.6; and (d) physiological saline (0.9% NaCl) which act as control.

The Thermanox plates were placed in the culture well at an inclined position; about 45 degrees from the horizontal (see Figure 1.0). The tip of the irrigation needle (28 gauge) were positioned 4 mm above the centre of the specimen plate, guided by a travelling microscope.

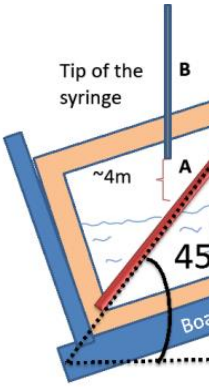

Figure.1.0. Diagrammatic representation of immersion model. Point A represented the tip of the needle, which was adjusted, aided by a travelling microscope, to be 4 mm from the surface of the substrate. Point B represented the body of needle that was clamped vertically to the bench.

All four groups were irrigated at the same time, with the four irrigants delivered simultaneously using a motorized syringe pump (MasterFlex L/S), calibrated to a flow rate of 0.3 mL per minute. The unit was flushed with 10 mL of distilled water before the procedure to remove any traces of irrigant in the system. One irrigant was used for one individual specimen and 1mL was injected onto the biofilm in each well until half of the specimen plate (about 6.5 mm from the bottom) was immersed in the solution. After irrigation, the specimens were left in the position immersed for 2 minutes (i.e. 5 minutes of total contact time with the all type of irrigant). Then, they were gently drained of excess irrigant and waved in another well containing a neutral izer, followed by PBS, before staining.

The specimens were stained using the LIVE/DEAD® *Bac*Light™ Bacterial Viability kit (Molecular probes, Eugene,OR, USA). It was used to quantify the viable cells in the biofilm. The kit consists of two different nucleic acid stains: Component A (SYTO®9) that is taken in by vital bacteria making them fluoresce in green ; and Component B (propidium iodide) that makes the dead bacteria fluoresce in red. Equal amounts of component A and B were added in a microcetrifuge tube (about 1.0µL each). Then, 0.88 mL of PBS was added and mixed evenly. The diluted mixture was applied to the wells containing the specimens. Then, the culture plate was wrapped in aluminium foil (to keep dark) for 30 minutes at room temperature, before examination under a CLSM equipped with filters (488 and 543 nm) to allow

picking up of signals from live (green) or dead (red) bacteria. Each specimen was placed on a glass slide (Vitromed, Basel,Switzerland) with two drops of LIVE/DEAD® BacLight™, onto the microscope stage. Independent images at 40x magnifications. To standardize the sites of measurements, a systematic of selecting viewing locations were implemented. Each specimen was divided into six regions, with its centre (position where the irrigating needle was aimed) designated as location “D” (Figure 2.0). Two randomly selected areas from each region were photographed for each substrate. The digital images were initially taken in the native OIB format (Olympus) and then converted and saved in Tagged Image File Format (TIFF) for later, quantitative analysis in softwares (ImageJ, National Institute of Health, Besthesda, Baltimore,MD; and Cell Profiler, Massachusetts Institute of technology, MA). Values for the amount of live and dead bacteria in the examination field were tabulated into a spreadsheet. Averages values and standard deviation in each group were computed. Data were analyzed using statistical analysis computer software (SPSS 17.0 for Windows, SPSS Inc., Chicago, USA).

After CLSM examination, the specimens were immersed in 0.5mL of PBS for 24 hours and prepared for Scanning Electron microscopy (SEM) preparation. All specimens were maintained at 4^0C prior to SEM examination. The specimen was pre-fixed in a mixture of 2% glutaraldehyde and 3% formaldehyde solution for two hours and dried. Then, they were immersed in 1% osmium tetraoxide for one hour, rinsed with deionized water for 10 second and soaked overnight in 70% ethanol. Dehydration using a series of ethanol up to 95% concentration was performed, before the specimen was mounted on aluminum stub using a carbon paint and

sputtered coated with palladium-gold (Ion Sputter JFC-1100;JOEL, Tokyo, Japan). SEM images (XL 30 CP; Phillips, Eindhoven, The Netherlands) were taken at five magnifications (20x,250x, 500x, 1000x and 2000x) for visualization of the biofilm appearance on the Thermanox sheet. One image of each magnification was taken for the upper and lower portion of the Thermanox plate (in relation to the C-D-E plane in Figure 2.0).

Results

A total of 576 CLSM images were obtained for all specimens. The six regions, A to F (Figure 2.0), were grouped into four "zones" for analysis: upper (which included A and B), middle (C and E), centre (D) and bottom (F), because there was no significant difference found between region A and B, and between C and E. The middle (region C and E) and upper (A and B) zones were exposed to splashing or vapour of the irrigant, respectively and two regions in the upper or middle zones were equivalent to each other for their relative position from the centre. Region F was immersed in the solution for most of the duration of the experiment; centre (region D) was affected by the stream of irrigant in addition to any antibacterial action.

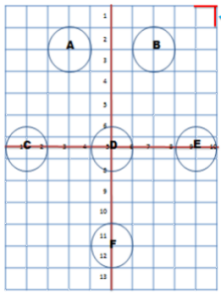

Figure.2.0. Location of 12 points of CLSM examination of each sample. Each point represent 2 point.

The amounts of live and dead bacterial cells were calculated separately as area percentages of red (dead) and green (live) signals from the CLSM micrographs (Figure. 3.0).

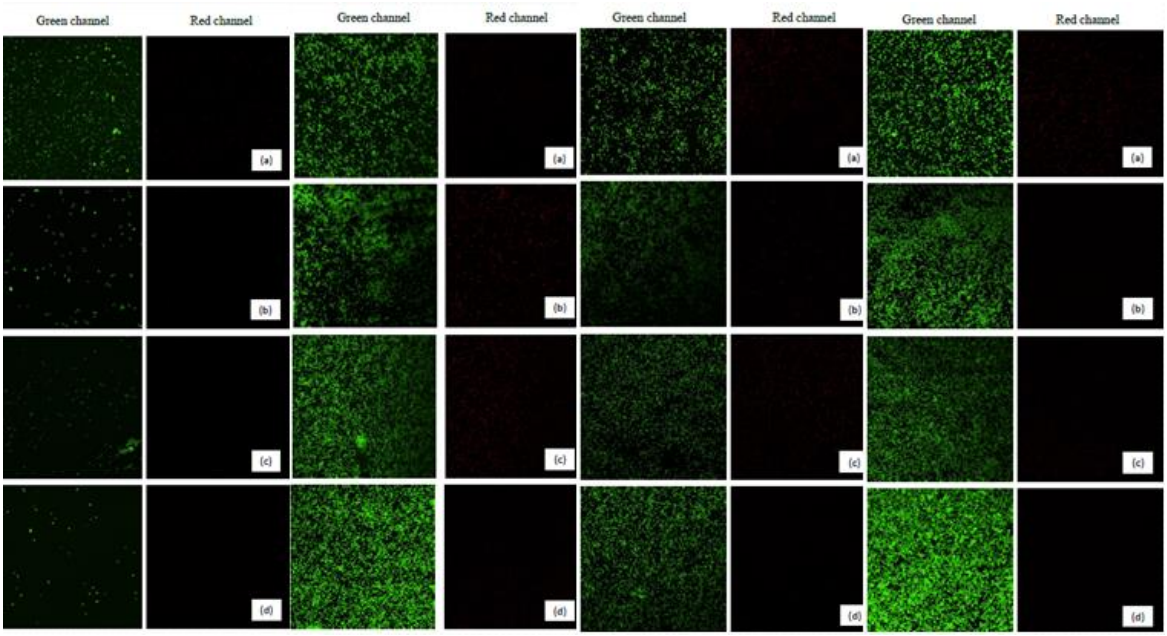

Figure.3.0 CLSM images (40x mag.) comparing four different irrigants; from left to right: 0.5 sodium hypochlorite, *Fructus mume*, buffered citric acid and physiological saline.

Four different zone are represented: a)upper, b) centre c)middle d)bottom

Then, the proportion of live cells among the total population was calculated using the formula below.

$$\text{Percentage of live bacteria} = \frac{\text{Live bacteria}}{\text{Total cell count (i.e live + dead bacteria)}} \times 100\%$$

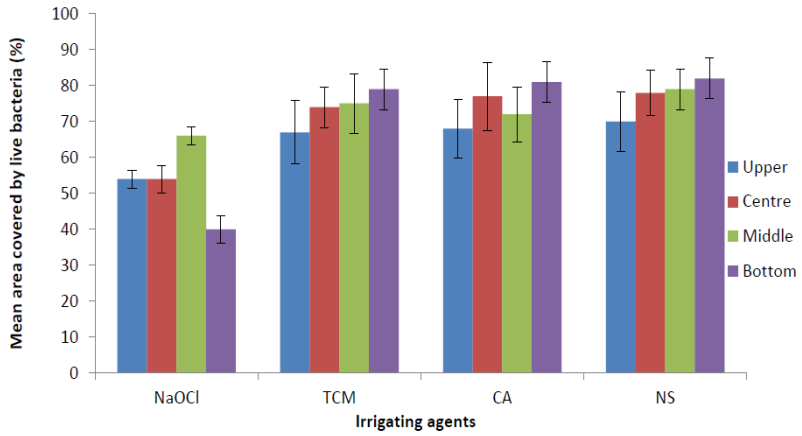

Figure 4.0. Bar chart comparing four different type of irrigants on the means area covered by live bacteria.

Results of the amount of viable bacteria residual indicated that *Fructus mume* showed no significant activity, with an effect similar to physiological saline or citric acid, and significantly inferior to sodium hypochlorite (Figure 4.0).

Scanning electron microscopy

High magnification views (2000x) at upper and lower region of specimen demonstrated the presence of cocciform bacteria attaching to the surface (Figure 5.0). Those in the upper regions showed dense bacterial population. For the NaOCl group, biofilm disruption could be noted compared with the other experimental groups (Figure 5.0).

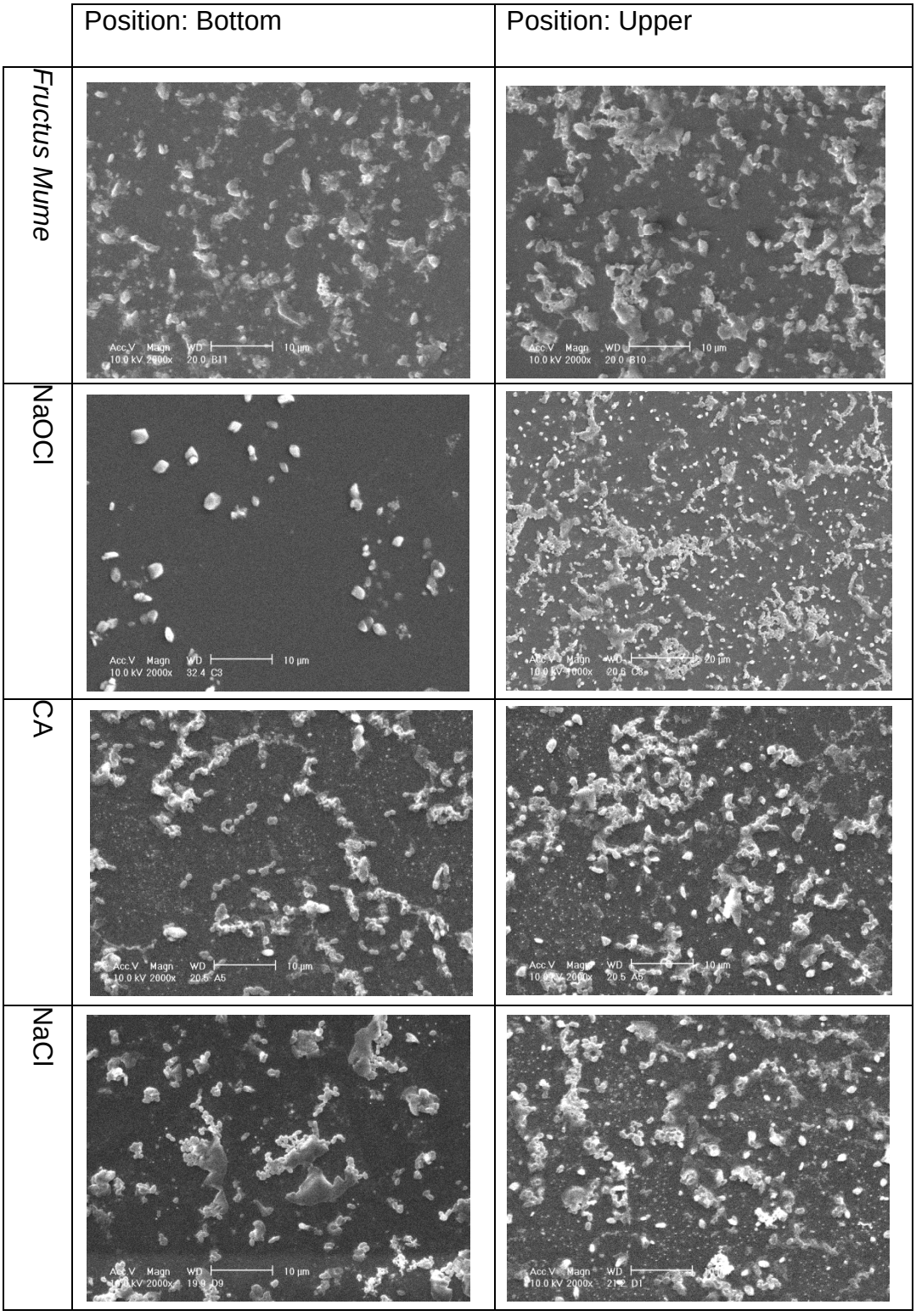

Figure 5.0 SEM images comparing four different groups. Images were obtained at 20 00x magnifications.

Discussion

The substrate was chosen based on a few factors. First, the material must be acquiescent for biofilm attachment and growth. It is well known that various bacteria can react differently and may produce different biofilm structures, depending on the interaction between the substrate material and the initial colonizers [7, 10-12].

Thermanox™ is a trademark of a particular type of polymer of the polyolefin polyester family. This material can be autoclaved without any deterioration. It comes in the form of a thin sheet and can be easily cut to shape by using scissors. Many researchers have used this as a substrate to harbour bacteria in their studies [9, 13]. According to the manufacturer, this substrate can be 'cell culture treated' for optimal cell attachment and growth. A simple method to determine which side of the coverslip has been treated is the "droplet" test. A drop of water or culture medium will spread evenly on the hydrophilic, treated side; the drop will form a bead on the untreated side. The material has been shown to be equal to, if not better than polystyrene or glass for cell attachment and growth [10, 14]. It seems to support a continuous, rapid biofilm growth up to 21 days, after which time the viable cell count fluctuated within 1 log unit [10]. With these purported advantages, Thermanox™ was chosen as the substrate for the main experiment in this study.

Mono-species biofilm of *E. faecalis* was selected as the test subject for comparing the action of traditional Chinese medicine (TCM; *Fructus mume*) and sodium hypochlorite in this study. This bacterium is a facultative anaerobic gram-positive coccus [15], and is a well-known endodontic pathogen that is commonly found in teeth with recurrent endodontic lesions [16, 17]. This microorganism shows a 'promising' biofilm structure and is widely analyzed in laboratory studies [12, 18-20]. It

was found to be resistant to root canal medicaments such as calcium hydroxide [21], antibiotics [22] and some irrigating agents[23]. However, the mono-species biofilm is not representative of the diverse polymicrobial infection encountered in the endodontic lesion[24]. Dental biofilm containing multiple species should be more relevant for studying the characteristic of antimicrobial action that will be conducted on the next part of this studies. *E. faecalis* has the ability to survive the 'aftermath' of root canal treatment [25], and overcome a prolonged starvation period and to revive successfully [9, 26].

For the purpose of this study, two methods were adapted in viewing the effect of the irrigating agent on the biofilm: scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) examination. The latter was for quantification measurement of the bacterial population within the biofilm. Scanning electron microscopy (SEM) allows a great depth of field and is able to show the surface topography of a structure in high resolution. To date, it remains as one of the most popular methods for illustrating the microstructure of the biofilm surface.

Confocal laser scanning microscopy (CLSM) is based on the principle of conjugate focus of an optical microscope. It is able to eliminate out-of–focus areas during observation and allows a process called optical sectioning, without destroying the specimen. Biofilm can be examined in this manner without any major alterations and disturbance. Although not applied in this experiment, CLSM is able to produce 3-dimensional reconstruction of multi-layer structures. With suitable fluorescent stains, both live and dead microorganisms can be recognized. Biofilm in this study was quantified in term of percentage area covered with bacteria for those selected

observation sites. All (digital) photographs were analyzed in a software, Cell Profiler (Massachusetts Institute of Technology, Boston, MA, USA) for the overall live and dead bacterial loads. The percentages of live and dead are related to each other, and only the percentages of live bacteria were reported. A reduction in the amount of live bacteria would indicate an antimicrobial effect of the irrigants. In this study, except for TCM, analysis of microbiological counts indicated that the data were normally distributed for the groups, which result was suitable for a parametric two-way ANOVA test

The Thermanox were placed in a well after biofilm inoculation as in the irrigation model (see Figure 1.0) During that time, electro-static interaction could be felt; this sensation appeared to agree with the suggestion that biofilms are able to generate electrostatic interactions especially for crosslinking between the matrix and the substrates itself [27]. Each specimen was subject to irrigation by one agent in its individual well. This model eliminated the variability and the influence due to the (complex) root canal system and allowed a fair comparison of the effect between agents used.

This protocol would be useful in identifying any minor effects due to the flow of the irrigant over the surface of the substrate and to the chemical actions of the agent. Notice that the central area of the specimen was subject to both the chemical (antibacterial) action and the “impact” of the solution that was directed to this area by the irrigating needle. The methodology stimulated the action of a single rinse with an irrigant to see the action of the agent on the biofilm.

This study evaluated the antimicrobial efficacy of *Fructus mume* (compared to citric acid [pH 2.6], 0.5% NaOCl and physiological saline) against a mono-species *E. faecalis* biofilm. Dunavant *et al*. (2006) compared the antibacterial effect of 1% versus 6% NaOCl, 2% of chlorhexidine and MTAD against *E. faecalis* biofilm grown in a flow-cell system, after immersion for 1 or 5 minutes, and showed that both NaOCl concentrations were significantly superior to other irrigants tested[22]. Direct contact of the agent with the biofilm is a method used to test the irrigant's capability to kill microorganisms [20]. However, it does not take into account the complex anatomy that can limit the accessibility of the agent to all areas of the root canal system. This is a limitation of direct-contact studies, such as this present one. The extent of bacterial reduction was not significantly different between *Fructus mume*, citric acid and physiological saline. Antimicrobial agents have been found to be less effective against bacteria within a biofilm [28].

One mechanism is the failure of antimicrobial agents to penetrate the entire thickness of the biofilm. Some antimicrobial agents seem able to permeate the depth of the biofilm [25, 29-31]. Even a thin biofilm can possess such resistance to killing by antimicrobial agents [32].

Both the contact time and concentration of the irrigants are a significant factor in eliminating the microorganisms [20, 33, 34]. In this present study, only half of the biofilm was exposed to (finally immersed in) the irrigating agent. For the 0.5% NaOCl group, the biofilm showed a 'total washout' appearance, indicating a reduction in the overall amount of bacteria present; indeed, this was reflected in total cell count. These studies indicated that NaOCl was the only irrigant which was able to eliminate the

biofilm in total [22, 34, 35]. In fact, NaOCl still remains as the irrigant of choice in most endodontic procedure.

Physiological saline was used as the control in this study to allow for the (mechanical) effect due to the irrigant flowing over the biofilm. Comparing the mean total number of cells covering the area after irrigation, a similar total number of cells was observed for physiological saline, citric acid and *Fructus mume*. This suggested an inability of physical flow of solution to disrupt and remove the biofilm from the substrate surface.

Buffered citric acid was included as a specific control for the low pH of *Fructus mume*, as an acid environment could be bactericidal to some organisms. Citric acid occurs naturally in the mitochondria and is generally used to inhibit the coagulation of blood *in vitro*. One study showed that a high concentration (50%) of citric acid was able to destroy *Bacillu*s in 5 minutes [36]. This concentration, however, was much higher than that used in this study (2.0%). The pH here was adjusted to that the TCM (*Fructus mume*) so that any net gain in antibacterial effect could be attributed to the TCM itself.

The limitation in this study is the methodology employed in this study was a simple model to demonstrate the effect of irrigant flowing on the (mono-species and three-species) biofilm. It does not take into account the velocity of the solution, especially in a root canal clinically, although the flow rate was kept constant for all groups. The complex anatomy of root canals will create a challenging environment to cleaning and shaping, which condition is not reproduced in this study. Conclusions from studies, such as the present one, of mono-species biofilm on standard

laboratory surfaces (glass, membrane or plastic) must be interpreted with caution. Only one concentration of the TCM and of NaOCl was tested. Future research maybe needed to determine if higher concentration of Fructus mume or even combinations with other materials may increase its antimicrobial effectiveness (and the possibility of smear layer removal).

Conclusion

Sodium hypochlorite (0.5%) solution was superior to citric acid, *Fructus mume* and physiological saline as an antimicrobial agent against *E. faecalis* biofilm. *Fructus mume* does not seem to show any antimicrobial effect, additional to the acidity of this agent.

Conflict of interest: None declared

Ethical approval: Not required

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