Efficacy of Cranberry Extract against established Endodontic Biofilm in an *In vitro* Tooth Model

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THESIS
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LIST OF ABBREVIATIONS

CDC	Center for Disease Control
CHX	Chlorhexidine gluconate
CFU	Colony forming unit
NaOCl	Sodium hypochlorite
$Na_2S_2O_3$	Sodium thiosulfate
NiTi	Nickle Titanium
PACs	Proanthocyanidins
PBS	Potassium phosphate-buffered saline
PPs	Paper points

SUMMARY

The primary goal of root canal therapy is to treat or prevent apical periodontitis. Microorganisms, both planktonic and those in a biofilm, are the primary etiology in the initial development of apical periodontitis as well as persistent disease. Successful treatment of pulp and periapical disease is dependent on the elimination of the infective microbiota from the root canal system. Antimicrobial irrigating solutions play a key role in the eradication of microbes by assisting in the removal of bacterial biofilms from un-instrumented surfaces, which have been shown to be 35% or more of the surface area due to irregular canal anatomy, isthmuses, fins, and accessory canals (Peters 2001). Currently, the standard endodontic irrigant is sodium hypochlorite due to its broad antimicrobial spectrum, ability to dissolve organic matter, and low cost. However, inadvertent introduction of sodium hypochlorite into the periapical tissues can cause pain and tissue damage due to its caustic and toxic properties. The aim of this study was to identify an endodontic irrigant with a broad antimicrobial spectrum, high efficacy against microorganisms in biofilms, and nontoxic to periodontal tissues.

The hypothesis is that cranberry concentrate wash is an effective antimicrobial endodontic irrigant against established intracanal multispecies biofilm in an *in vitro* tooth model.

Three cranberry extracts were prepared by Ocean Spray Cranberries, Inc., Lakeville-Middleborough, MA. The bacterial species used in the multispecies biofilm tooth model were *Fusobacterium nucleatum*, *Enterococcus faecalis*, and *Porphyromonas gingivalis*. Extracted human single canal teeth were collected and standardized to a length of 14.0 mm. Teeth were cultured in Schaedler broth with the 3 test bacteria strains for 21 days and then randomly

assigned to 6 treatment groups (saline, 5.25% NaOCl, 2% CHX, Cranberry Wash-1, Cranberry Wash-2, Cranberry Wash-3). The teeth were instrumented to size 35/04 and irrigated with 6 mL of the irrigants. Bacteria in the canals were sampled before (S1) and after instrumentation (S2). Bacteria in the dentinal tubules were sampled after instrumentation (S3) with a 40/06 file. Samples were plated in blood agar plates and CFUs were counted.

Results showed that all samples had comparable biofilm growth prior to instrumentation. Instrumentation and irrigation resulted in 90 - 99 % bacterial reduction in all groups. 5.25% NaOCl and 2% chlorhexidine were significantly more effective as irrigants when compared to the cranberry washes and saline. There was no statistically significant difference among cranberry washes and the saline control.

In conclusion, instrumentation and irrigation significantly reduced the bacterial loading inside the canals under current experimental conditions. Although cranberry extract has been reported to reduce supragingival plaque biofilm formation and adherence, under current experimental conditions, cranberry extracts did not demonstrate significant bactericidal effect as an effective endodontic irrigant against pre-established multi-species biofilm. The effectiveness of cranberry extract as an endodontic irrigant to prevent multi-species biofilm formation warrants further studies.

I. INTRODUCTION

A. Background

1. Microbiota and Endodontic Infection:

A major determinant of endodontic infection is the presence of pathogenic microbes in the canal system, as demonstrated in the renowned study by Kakehashi (Kakehashi et al., 1965). This infection of the root canal system can occur via several pathways. These pathways include near carious exposure that allows bacterial endotoxins to reach the pulp through patent dentinal tubules (Langeland, 1987), direct pulpal exposure due to caries or iatrogenic error, and direct exposure due to cracks or fractures from trauma or normal masticatory forces (Ricucci, 2015). Root canal infections are mixed and semi-specific infections with a predominance of obligatory anaerobic bacteria (Siqueira, 2004) and can exist in a planktonic state or form biofilms (Radcliffe, 2004). If pathogenic microorganisms such as *Streptococcus mutans, Streptococcus mitis, Streptococcus sanguinis, Streptococcus faecalis*, and *Streptococcus bovis* are removed at an early stage, a vital pulp can defend itself via the host defense system. However, if these microbes remain long enough, it can lead to irreversible pulpitis and/or pulpal necrosis (Langeland, 1987) requiring endodontic treatment to save the tooth. The elimination of pathogenic microorganisms is of the utmost importance during root canal treatment. Sjogren studied the influence of microbial infection at the time of endodontic treatment and showed a significantly higher rate of complete healing in teeth that had a negative culture prior to obturating (Sjogren et al.

1997). The presence of persistent microbiota, especially *E. faecalis*, has also been shown to play a significant role in endodontic treatment failures (Nair et al., 1990).

Primary endodontic infections are polymicrobial. They are predominantly *Bacteroides, Prophyromonas, Prevotella, Fusobacterium, Treponema, Peptostreptococcos, Eubacterium,* and *Camphylobacter* species (Neelakantan et al, 2017). *P. gingivalis* and *F. nucleatum*, obligate anaerobes, are commonly found in primary endodontic infections, each with incidences as high as 48% (Siqueira et al, 2008; Sundqvist et al, 1994). *E. faecalis*, a facultative anaerobe, is less likely to be found in primary endodontic infections than other endodontic pathogens but is significantly associated with endodontic treatment failures (Rocas et al., 2004). It is capable of surviving unfavorable environmental conditions and able to form biofilms, making it resistant to antimicrobial therapy (Nair, 1997).

2. Biofilms in Root Canal Systems:

Biofilms are a structurally complex sessile community of microbes that are tenaciously attached to a biological substratum. The development of a biofilm is a dynamic process. The phenotype of bacteria changes with the maturation of the biofilm. The stages for biofilm formation have been classified as attachment, irreversible binging, maturation and dispersion (Donlan and Costerton 2002). They are typically encased in the extracellular matrix comprised of polysaccharides, proteins, nucleic acids, and lipids which provides protection from environmental stresses such as pH shifts and desiccation. The quorum sensing, or cell to cell communication, ability of biofilms has also been shown to enhance their ability to

survive environmental stresses. Their structural complexity provides a protective mode of growth and they can exhibit an altered phenotype (Neelakantan et al, 2017). Biofilms that form on human tissue can release antigens that trigger the host's immune response, but the antibodies are not effective in killing the bacteria within the biofilm and often do damage to the surrounding host tissue. Bacteria in the biofilm are more resistant to antimicrobial agents compared with the planktonic cells. Antibiotics can reverse the symptoms caused by planktonic microbes but fail to kill bacteria in the biofilm. (Costerton et al, 1999). This ability for biofilms to resist antimicrobials has been attributed to quorum sensing and synergism that is present once a biofilm matures (Ceri et al, 1999). Root canal infections are biofilm mediated (Siqueira et al, 2002; Ricucci et al, 2008; Ricucci and Siqueira, 2010). The complexity and variability of the root canal system, along with the multi-species nature of biofilms, makes disinfection of the root canal system very difficult (Neelakantan et al, 2017). Among others, *P. gingivalis*, *F. nucleatum, and E. faecalis* have been commonly found to coexist in root canal biofilm infections. (Rocas et al, 2008).

3. <u>Chemomechanical Debridement</u>:

Non-surgical root canal treatment aims to eliminate microorganisms, both planktonic and biofilms, from the root canal system by chemomechanical preparation using specific instruments and disinfecting chemicals in the form of irrigants and/or intracanal medicaments (Neelakantan et al, 2017). Modern

chemomechanical preparation of the canals is typically accomplished using NiTi rotary files and antimicrobial irrigants. Antimicrobial irrigating solutions play a key role in the eradication of microbes by assisting in the removal of bacterial biofilms from un-instrumented surfaces, which have been shown to be 35% or more of the surface area due to irregular canal anatomy, isthmuses, fins, and accessory canals (Peters 2001). However, the irrigant can have difficulty reaching the un-instrumented portions of the root canal also. This can occur due to trapped air in the apical third of the canal causing a vapor lock which prevents the irrigant from flowing into the apical anatomy (Tay et al, 2010). And conventional needle irrigation can only deliver irrigant approximately 1.0mm beyond the needle tip (Munoz & Camacho-Cuadra, 2012). Therefore, the canals must be instrumented to a size that accommodates the diameter of the irrigation needle which is not always possible in narrow or curvy roots.

4. Endodontic Irrigants:

Numerous substances have been used as endodontic irrigants over the years. During World War I, Henry Dakin recommended sodium hypochlorite (NaOCl) as an antiseptic solution to irrigate wounds (Dakin, 1915). In 1918, the solvent action of NaOCl was demonstrated on necrotic tissue while it was noted that it was only mildly inflammatory to normal tissue (Austin and Taylor, 1918). NaOCl was found to be a more effective pulp tissue solvent than sulfuric acid, sodium-potassium alloy, sodium dioxide, sodium methylate, hydrochloric acid,

sodium hydroxide, and potassium hydroxide (Grossman and Meiman, 1941). An ideal endodontic irrigant should have a "broad antimicrobial spectrum, a high efficacy against anaerobic and facultative microorganisms organized in biofilms, the ability to dissolve necrotic pulp tissue remnants, the ability to inactivate endotoxin, the ability to prevent the formation of a smear layer during instrumentation or to dissolve the layer once it has formed, it should be nontoxic when in contact with vital tissues, noncaustic to periodontal tissues, and with little potential to cause an allergic reaction" (Kandaswamy and Venkateshbabu, 2010). Unfortunately, no ideal endodontic irrigant that exhibits all of the desirable characteristics has been identified. Although current irrigating solutions, such as NaOCl and CHX, possess exceptional antimicrobial activity, caustic and toxic effects to vital tissues have been noted. There remains a need for endodontic irrigants that are both antibacterial and/or anti-inflammatory while exerting minimal to no negative side effects on human tissue.

5. **Cranberries and Natural Antimicrobials:** The two most effective endodontic irrigants, NaOCl and CHX, also are the most toxic. If extruded beyond the apex into the soft tissue, NaOCl can cause intense pain, severe and diffuse swelling, bruising, necrotic tissue, and scarring (Guivarc'h et al, 2017). CHX, while not toxic to oral tissues, is cytotoxic to dental papilla stem cells and therefore not indicated for use during endodontic regenerative procedures (Trevino et al, 2011).

Plant-derived natural products represent a rich source of antimicrobial compounds, which have been incorporated into oral hygiene products. The continued search for an ideal endodontic irrigant has led researchers to investigate plant-derived natural agents such as berberine, tea, and cranberry juice extract.

Berberine is an alkaloid present in many medicinal plants that has been shown to have antimicrobial activity against oral pathogens while exhibiting low toxicity and low mutagenicity to human cells (Xie et al, 2012). Various teas contain polyphenols, which have been shown to protect against oral cancer, have a preventative effect against periodontal disease, and increase the antioxidant capacity of oral fluids (Petti & Scully, 2009). Cranberries also contain polyphenols, specifically the class known as proanthocyanidins (PACs). They have been shown to inhibit the colonization of *Streptococci* species (Sethi & Govila, 2011); reduce the formation of *S. mutans* biofilms (Koo et al, 2010); and inhibit the growth, enzymatic activity, and biofilm formation of *E. faecalis* (Wojnicz et al, 2016). But to date, we are unaware of any study that investigated the effect of cranberry polyphenols on established mature endodontic multispecies biofilms.

B. Statement of Problem and Significance

There are various endodontic irrigants in use today, but none meet all the criteria of an ideal endodontic irrigant. Cranberry juice concentrate has been shown to prevent the formation of both *Porphyromonas gingivalis* biofilms (Labrecque et al, 2006) and *Enterococcus faecalis* biofilms (Wojnicz et al, 2016). There are currently limited studies that investigate the use of cranberry juice concentrate or extract as an endodontic irrigant or on its ability to destroy established biofilms. Our goal was to test the efficacy of cranberry extract as an endodontic irrigant on established biofilms in the root canal system.

C. Hypothesis

Cranberry concentrate wash is an effective bactericidal endodontic irrigant against established intracanal multispecies biofilm in an *in vitro* tooth model.

D. Objective

To determine the efficacy of cranberry wash as an endodontic irrigant against established multispecies mixed culture endodontic biofilm in an *in vitro* tooth model.

II. MATERIALS AND METHODS

A. Materials

1. Irrigation Solutions:

- Saline – 0.9% sodium chloride irrigation solution, pH 5.5 (Baxter Health Care, Deerfield, IL, pH)
- Cranberry extract (Wash-1, 2, 3) – provided by Ocean Spray Cranberries, Inc. The operators were blinded to three different washes. No details of how the washes were extracted or their concentrations were provided. (Figure 1, Ocean Spray Cranberries, Inc., Lakeville-Middleborough, MA)
- Sodium hypochlorite - 5.25% NaOCl, pH 12 (James Austin Co, Mars, PA),
- Chlorhexidine gluconate – 2% CHX (Ultradent, South Jordan, UT)
- Phosphate-buffered Saline - 0.05M pH 6.8
- Sodium Thiosulfate– Sigma-Aldrich S-2929 (Sigma-Aldrich Co., St. Louis, MO)
- Lecithin – lecithin, 90%, soybean solid (Alfa Aesar, Haverhill, MA)
- Tween 80 – FisherBiotech BP338-500 (Fisher Scientific, Waltham, MA)

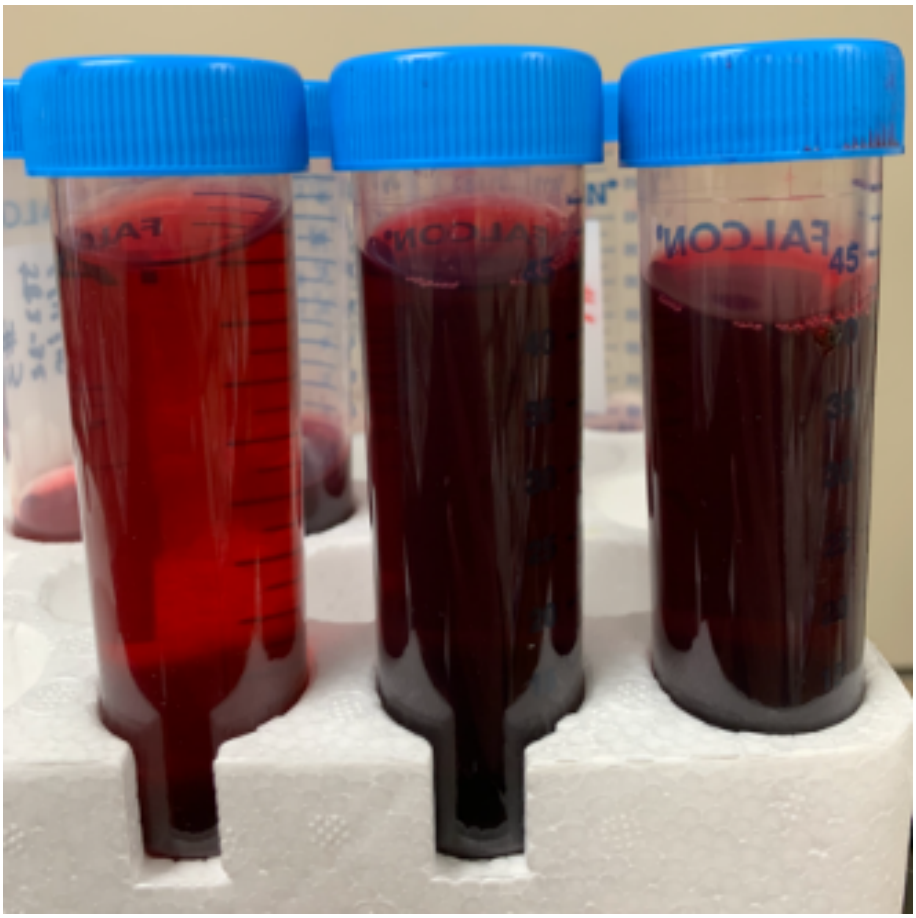

Figure 1. Cranberry extracts
Wash-3, Wash-2, Wash 1 (left to right)

2. Irrigation Delivery System:

- Conventional needle irrigation – 27 gauge open ended (Henry Schein, Melville, NY)

3. **Test Bacteria and Growth Media:**

- *Fusobacterium nucleatum subsp polymorphum* ATCC10593 (American Type Culture Collection, Manassas, VA)
- *Enterococcus faecalis* ATTC29212 (American Type Culture Collection, Manassas, VA)
- *Porphyromonas gingivalis* W83 (American Type Culture Collection, Manassas, VA)
- Blood Agar – CDC anaerobe blood agar (Becton, Dickson and Company, Sparks, MD)
- Schaedler Broth – (Oxoid, Basingstoke, MD)

4. Teeth:

Single canal extracted human anterior and premolar teeth were gathered from private practice dental offices and several of the dental clinics at the University of Illinois at Chicago College of Dentistry. All teeth were stored in 0.1% sodium thiosulfate solution or 1.3% NaOCl.

5. Endodontic Files:

Hand files – Stainless steel C-files (Roydent, Johnson City, TN)

Rotary files – ProFile landed NiTi files (Denstply Sirona, Charlotte, NC)

B. Methodology

The University of Illinois at Chicago Institutional Review Board determined that this protocol did not involve human subjects and therefore approval was not required. The flowchart of the methodology is depicted in Figure 2.

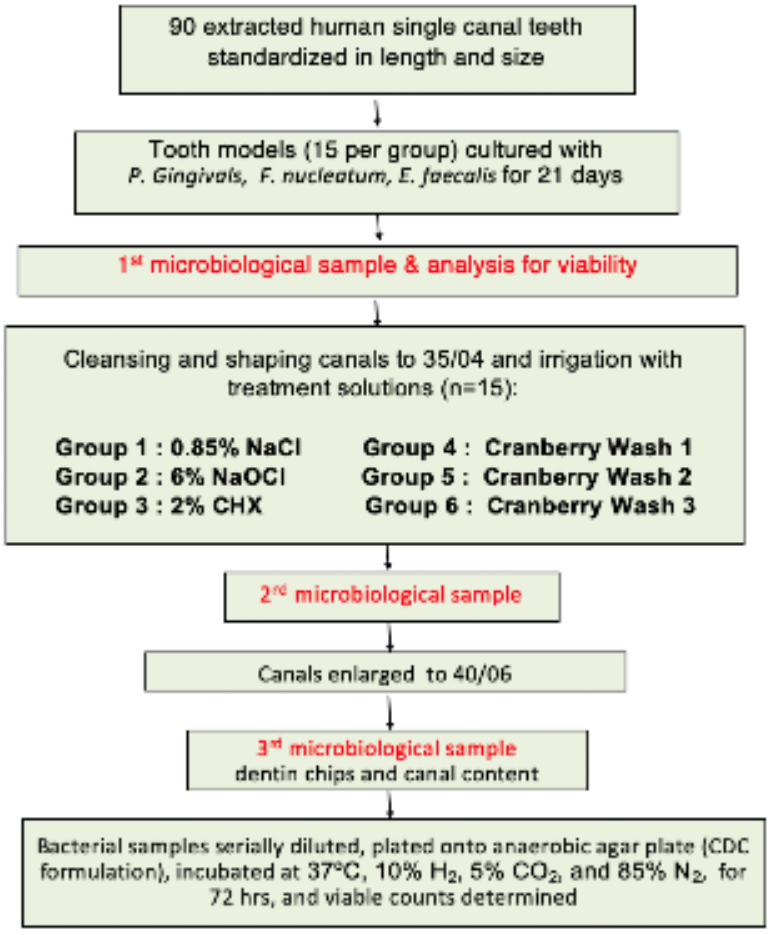

Figure 2. Flowchart of the methodology

1. *In vitro* Infected Tooth Model:

The infected tooth model used in this study was a modification of that previously described in a prior study (Xie et al, 2012). The model was shown to be reliable and reproducible and was consistent with the methods previously described in which bacterial sampling of dentinal tubules was accomplished by the serial shaving of 100-micron thick dentin zones (Haapasalo and Orstavik, 1987). Extracted human single canal teeth were collected, decoronated, and standardized to a length of 14.0 mm. The teeth were sterilized then cultured in Schaedler broth with *F. nucleatum*, *E. faecalis*, and *P. gingivalis* strains for 21 days for biofilm formation then were randomly assigned to one of the 6 test groups.

2. Extracted Teeth Selection, Storage, Preparation, and Sterilization:

Ninety permanent human single canal teeth were collected and stored in thymol solution until use. Inclusion/exclusion criteria included no root curvatures, and no root fractures. The exteriors of the teeth were debrided of any remaining bone, calculus, or soft tissue using a curette. The teeth were then decoronated, standardized to a length of 14.0 mm, and minimally instrumented to patency length using #8 and #10 C-files with tap water as the irrigant. The instrumented teeth were then coated on the outside with three coats of fingernail polish and the apical foramen were sealed with cyanoacrylate (Figure 3). The prepared root samples were placed in a glass jar with screw cap filled with dH2O and steam sterilized at 121 degrees C for 20 minutes. Before inoculation with the bacteria,

the human teeth were cultured with Schaedler broth for 72 hours at 37 ℃ to confirm the effectiveness of the sterilization procedure.

Figure 3. Prepared teeth prior to sterilization

3. <u>Biofilm Growth and Culture</u>:

The test bacteria were on blood agar under anaerobic conditions (Coy Chamber 5% CO2, 10% H2, 85% N2) at 37°C for 48 hours for strict anaerobic species. Viable cell counts were determined using standard curves equating absorbance (A660nm) with plate counts. Standard curves of colony forming units/mL (CFU/mL) versus absorbance at 660nm was determined for each organism with linear regression, by equating absorbance with number of viable cells. CFU/mL in samples were enumerated. Mixed culture bacteria suspensions were prepared containing *F. nucleatum*, *P. gingivalis*, and *E. faecalis* with

inoculation concentrations of *F. nucleatum* ($1x10^6$CFU/mL), *E. faecalis* ($1x10^4$CFU/mL), and *P. gingivalis* ($1x10^6$CFU/mL). Teeth samples then were randomly assigned to six different groups (n=15), place into sterilized jars with 200ml Schaedler broth and inoculated with 5ml mixed bacteria suspension. All jars were incubated at 37 ^oC under anaerobic conditions (Coy Chamber 5% CO_2, 10% H_2, 85% N_2) for 21 days to allow biofilm formation and maturation within the canals. Planktonic bacteria were sampled by gram stain every other day to check for contaminations. The 150 ml media was replaced with fresh media every other day.

4. <u>Test Groups</u>:

The teeth were randomly assigned to one of the following 6 treatment groups (n=15):

1 - sterile saline (7mL)

2 - cranberry extract-I (7mL, from here on designated as Wash-1)

3 - cranberry extract-II (7mL, from here on designated as Wash-2)

4 - cranberry extract-III (7mL, from here on designated as Wash-3)

5 - NaOCl (7mL 5.25%) + stop agent (3mL 5.0% sodium thiosulfate, $Na_2S_2O_3$)

6 - CHX (7mL 2.0%) + stop agent (3mL 0.07% lecithin + 0.5% Tween 80 detergent)

5. Instrumentation, Irrigation, and Sampling:

The teeth samples with covered biofilm in Schaedler broth were removed one at a time for instrumentation to minimize aerobic exposure. Prior to instrumentation, the canals were sampled for viable bacteria. This was accomplished by drying the canals with sterile paper points (Roydent, Johnson City TN) then rinsing the canals with 10μL of PBS (0.05 mol/L, pH=6.8). The canal walls were then scraped circumferentially with a size #10 C-file ten times to dislodge the biofilm. Four sterile paper points were consecutively placed into the canal to absorb the contents then transferred to a vial containing 900μL PBS (S1). The canals were then prepared with rotary instrumentation using size 25/04, 30/04 then 35/04. One mL of test irrigant was used between each file. After size 35/04, 5 mL of test irrigant was used. Additionally Group 5 (5.25% NaOCl) was irrigated with 3 mL 5% sodium thiosulfate (Na2S2O3) after the final irrigation with NaOCl and Group 6 (2.0% CHX) was irrigated with 3 mL of a mixture of 0.07% lecithin plus 0.5% Tween 80 detergent after the final irrigation with CHX in order to inactivate the NaOCl and CHX respectively. The canals were then dried with sterile paper points, rinsed with 10μL PBS, and the walls were once again scraped ten times circumferentially with a size #10 C-file to dislodge the bacterial biofilm. Four sterile paper points were consecutively placed into the canal to absorb the contents then transferred to a vial containing 900μL PBS (S2). Finally, the canals were instrumented to size 40/06 to obtain dentin chips in order to sample viable

bacteria in the dentinal tubules, and the entire file together with dentin debris was placed in a vial containing 900μL PBS (S3).

6. Viable Bacterial Counts Determination:

The samples from different stages of instrumentation (S1, S2, S3) were immediately serially diluted and plated on CDC anaerobic blood agar. Appropriate dilutions were pre-determined based on a pilot study. The plates were incubated at 37 °C for 48 hours anaerobically and then the total viable bacteria (CFUs) were counted (Figure 4). The percent reduction was calculated as 1-(viable CFUs in S2/viable CFUs in S1).

Figure 4. Viable bacterial colonies from serially diluted sample of Wash-2, S2

C. Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS, Chicago, IL). The statistical significance was set at $p<0.05$. The variables bacterial reduction and bacterial load in the dentin debris were expressed in both viable CFU/mL and percent reduction. A Kruskal-Wallis chi-squared analysis with 5 degrees of freedom, a Pairwise Comparison, and a Wilcoxon Exact test were performed. In addition, a Pairwise Two-sided Multiple Comparison Analysis using the Dwass, Steel, and Critchlow-Fligner methods was completed.

III. RESULTS

Results showed that all samples had comparable biofilm growth prior to instrumentation. (TABLE I, S1). Instrumentation and irrigation resulted in approximately 90-99 % viable bacterial reduction in all groups (Figure 5). NaOCl and chlorhexidine were significantly more effective as irrigants when compared to the cranberry washes and saline ($P < 0.05$). There was no statistically significant difference among cranberry washes and the saline control ($p > 0.05$) (TABLE I).

Intergroup analysis of bacterial loads in the canals before instrumentation and irrigation (S1) revealed no significant differences (TABLE I).

After chemomechanical preparation, the total viable bacterial counts (S2) in all groups were significantly reduced when compared with those prior to instrumentation (S1, TABLE I). When NaOCl and CHX were used as endodontic irrigants, these were significantly more effective than the control, Wash-1, Wash-2 and Wash-3 in reducing viable bacterial counts ($p < 0.001$; TABLE I and Figure 6). There were no significant differences observed among the groups irrigated with Wash-1, Wash-2, Wash-3 and the saline (TABLE III).

After irrigation with test agents, the Kruskal-Wallis Chi-squared test suggested a significant difference between the 6 agents (TABLE II). The viable bacterial counts in dentin (S3) were significantly reduced in NaOCl and CHX compared with the control group irrigated with saline (TABLE I). There was no significant difference among Wash-1, Wash-2, Wash-3 and the saline group (TABLE I). None of the test agents showed complete elimination of bacteria in dentin (TABLE I). NaOCl showed a significant difference in the bacterial load reduction compared to Wash-1, Wash-2, and Wash-3 (TABLE IV). There was no significant difference in

bacterial load reduction between NaOCl and CHX (TABLE V). A pairwise multiple comparison test analysis revealed the same results (TABLE VI).

TABLE I: THE EFFECT OF DIFFERENT IRRIGANTS ON THE TOTAL VIABLE BACTERIAL COUNT BEFORE AND AFTER INSTRUMENTATION AND IRRIGATION

	S1 ± SD (CFU/mL)	S2± SD (CFU/mL)	S3± SD (CFU/mL)
NaOCl	$1.58x10^{6} \pm 2.5610^{6}$ a	$1.07x10^{3} \pm 2.30x10^{3}$ b	$1.79x10^{2} \pm 3.34x10^{2}$ d
Saline	$6.93x10^{5} \pm 5.34x10^{5}$ a	$3.79x10^{4} \pm 5.33x10^{4}$ c	$1.76x10^{4} \pm 2.67x10^{4}$ e
CHX	$1.05x10^{6} \pm 1.14x10^{6}$ a	$3.95x10^{3} \pm 6.27x10^{3}$ b	$9.26x10^{2} \pm 1.14x10^{3}$ d
Wash-1	$3.87x10^{5} \pm 5.99x10^{5}$ a	$3.39x10^{4} \pm 5.50x10^{4}$ c	$3.39x10^{4} \pm 9.72x10^{4}$ e
Wash-2	$8.07x10^{5} \pm 1.07x10^{6}$ a	$8.30x10^{4} \pm 1.28x10^{5}$ c	$2.85x10^{4} \pm 5.30x10^{4}$ e
Wash-3	$6.86x10^{5} \pm 7.02x10^{5}$ a	$4.39x10^{4} \pm 7.63x10^{4}$ c	$2.28x10^{4} \pm 5.44x10^{4}$ e

S1 represents the viable bacterial count before instrumentation and irrigation, S2 represents the viable bacterial count after instrumentation and irrigation, and S3 represents viable bacterial count in dentin samples after instrumentation and irrigation. Different superscript letters indicate a statistically significant difference ($P < .05$).

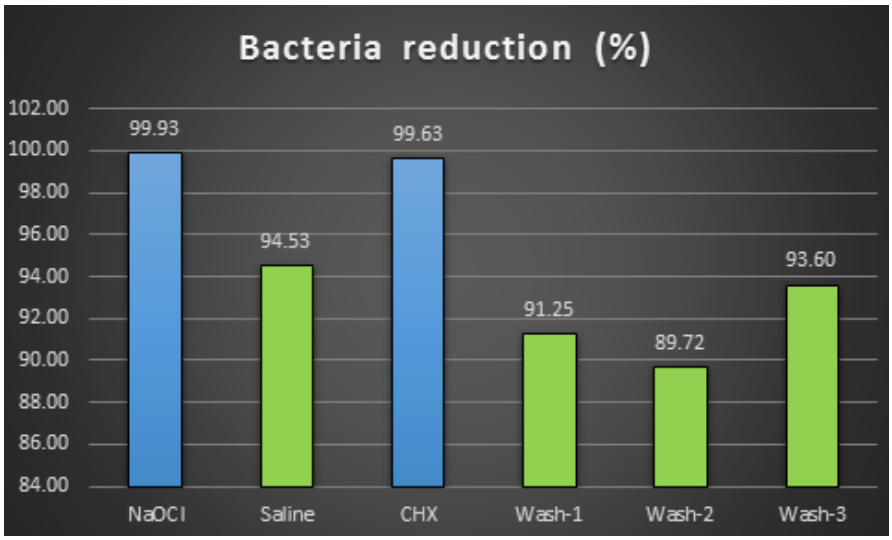

Figure 5. Bacterial reduction after instrumentation
Values represent percent viable bacteria reduction [1-(S2/S1)]

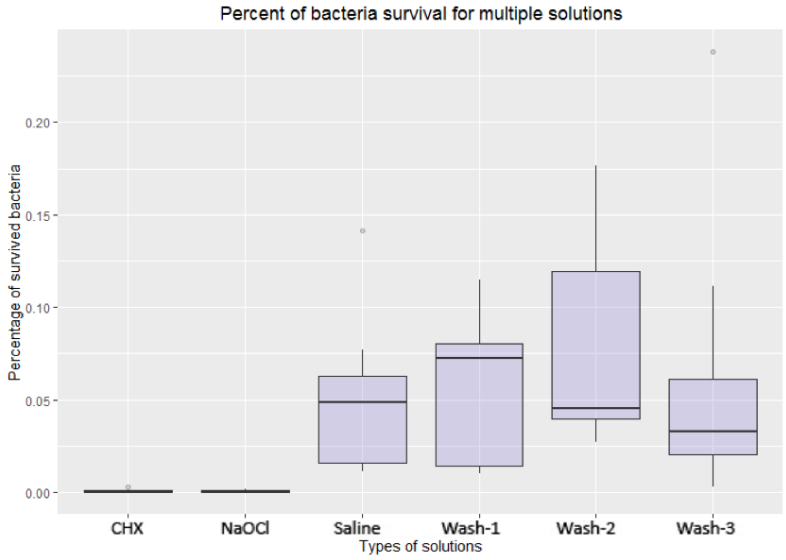

Figure 6. Boxplot for comparing percentages of viable bacteria after instrumentation using test irrigants

TABLE II: KRUSKAL-WALLIS CHI-SQUARED

Kruskal-Wallis chi-squared	Degrees of Freedom	P-value
32.451	5	4.836×10^{-06}

TABLE III: PAIRWISE COMPARISON BETWEEN SALINE AND WASH airwise 1, 2, & 3

Solution 1	Solution 2	P-value
Saline	Wash-1	0.6665
Saline	Wash-2	0.3562
Saline	Wash-3	0.9263
Wash-1	Wash-2	0.3562
Wash-1	Wash-3	0.8291
Wash-2	Wash-3	0.1083

TABLE IV: WILCOXIAN EXACT TEST

Solution 1	Solution 2	P-value
NaOCl	Wash-1	0.0001748
NaOCl	Wash-2	0.0001028
NaOCl	Wash-3	1.72×10^{-05}

TABLE V: NaOCl & CHX COMPARISON USING WILCOXIAN EXACT TEST

Solution 1	Solution 2	P-value
NaOCl	CHX	0.4231

TABLE VI: THE MULTIPLE COMPARISON TEST METHOD (DSCF).

Pairwise Two-Sided Multiple Comparison Analysis

Dwass, Steel, Critchlow-Fligner Method

Variable: psur

group	Wilcoxon Z	DSCF Value	Pr > DSCF
CHX vs. NaOCl	1.3596	1.9228	0.7512
CHX vs. Saline	-3.9116	5.5318	0.0013
CHX vs. Wash1	-3.9695	5.6137	0.0010
CHX vs. Wash2	-4.2162	5.9626	0.0004
CHX vs. Wash3	-4.0906	5.7851	0.0006
NaOCl vs. Saline	-4.1515	5.8711	0.0005
NaOCl vs. Wash1	-4.1515	5.8711	0.0005
NaOCl vs. Wash2	-4.2235	5.9729	0.0003
NaOCl vs. Wash3	-4.3652	6.1733	0.0002
Saline vs. Wash1	-1.5641	2.2120	0.6224
Saline vs. Wash2	-1.6984	2.4019	0.5328
Saline vs. Wash3	-0.4404	0.6228	0.9979
Wash1 vs. Wash2	-0.7764	1.0980	0.9715
Wash1 vs. Wash3	0.9608	1.3587	0.9302
Wash2 vs. Wash3	1.8234	2.5786	0.4505

IV. DISCUSSION

A. Study Findings

Plant-derived natural products represent a rich source of antimicrobial compounds, which have been incorporated into oral hygiene products. However, their application in endodontics is less well documented. Cranberry, a native North American fruit, has been reported to have anti-bacterial, anti-adhesion, anti-tumorigenic, anti-inflammatory and antioxidant activity (Côté et al., 2010; Bodet et al., 2006 & 2008; Wojnicz et al., 2016; Kim et al., 2015). Besides its broad-spectrum antimicrobial activity against microorganisms, cranberry extract has also demonstrated inhibition against biofilms formation of *E. faecalis* (Wojnicz et al., 2016). Unpublished data from a study conducted in Wu's laboratory (Wu, C., University of Illinois at Chicago, Department of Pediatric Dentistry) has shown that cranberry extract affected viability of several anaerobic gram negative periodontal and endodontic pathogens including *P. gingivalis* and *F. nucleatum*.

The present investigation focuses on the evaluation of antibacterial activities of cranberry extract against endodontic pathogens in a mature biofilm because of the multispecies nature of Endodontic infections (Deng et al, 2009). This study used an *in vitro* single canal tooth model to study the efficacy of three different cranberry washes when used as an endodontic irrigant against established multispecies biofilm. The most common Endodontic irrigants are NaOCl or a combination of NaOCl followed by CHX. We wanted to determine if using a natural product, such as cranberry extract, could be as effective an irrigant against microbes while avoiding the potential toxicity and caustic side effects of NaOCl and CHX. Our results show that all three of the cranberry washes used were no more effective than using saline as an endodontic irrigant. NaOCl and CHX were significantly more effective.

As previously stated, the development of biofilm is a dynamic process. The phenotype of bacteria changes with the maturation of biofilm. The stages for biofilm formation have been classified as attachment, irreversible binging, maturation and dispersion (Donlan and Costerton 2002).

The main component for cranberry extract is proanthocyanidins (PACs). Proanthocyanidin (PAC) is a naturally occurring plant metabolite widely available in fruits, vegetables, nuts, seeds, flowers and barks (Sarni-Manchado et al, 1999). As a bioflavonoid, it contains a benzene-pyran-phenolic acid molecular nucleus (referred to as flavin) as part of its much larger molecular structure. PACs are a mixture of monomers, oligomers, and polymers of flavan-3-ols (known as catechins), which are ubiquitous in plants. Widely used as natural antioxidants and free-radical scavengers, PACs have been proven to be safe in various clinical applications and as dietary supplements (Fujii et al, 2007; Yamakoshi et al, 2002; Xie et al, 2008). PAC from cranberry inhibited the surface-adsorbed gluocsyltransferases and F-ATP activities, and the acid production by *Streptococcus mutans* (Duarte et al, 2006). Wojnicz et al reported cranberry extract inhibits the growth, enzymatic activities of *E. faecalis* and limits the biofilm formation. Other studies have shown that PAC from cranberry inhibit bacterial adhesion, possible mechanism including affinity to bacterial adhesins (Tempiura et al, 2010). Vadekeetil et al reported that cranberry PAC inhibited the QS signal pathway of *Pseudomonas aeruginosa* by binding to signal molecules LasI (synthase gene) and LasR (receptor gene). QS-signal pathway is related to bacteria virulence gene activation, expression (production of rhamnolipid, pyocyanin, and violacein, etc) and biofilm formation (Vadekeetil et al, 2016). Thus, the majority of the reported inhibitory effect of cranberry seems related to the attachment, irreversible binding and maturation stage of the

biofilm. There's no clear evidence showing the inhibitory effect of cranberry to the mature, established biofilm. With the maturation of biofilm, the resistance to antimicrobial agent of bacteria increases (Singh et al, 2017). The extracellular polymeric matrix of the biofilm prevents the penetration of antimicrobial agent and host immune cells. Bacteria in the biofilm has a diversified growth make them more resistant to antibiotics. The multi-species biofilm model used in the current study were cultured for 21 days to allow the formation and maturation of endodontic biofilm (proved by previous study). This might be one of the reasons why cranberry washes were no more effective than the saline. The cranberry washes used in the current study were donated by Ocean Spray and we are not clear about how the washes have been processed which might also affects the result.

B. Study design

The polymicrobial nature of endodontic infections is well established. The intracanal microbiota in endodontically infected teeth exists both as a loose collection and as biofilm structures, consisting of cocci, rods, and filamentous bacteria. Many *in vitro* studies used planktonic cultures (bacteria in suspension) or monospecies biofilms. Although *E. faecalis* has frequently been isolated as the sole infectious microorganism in infected root canals, the fact that endodontic infections are typically polymicrobial is well accepted. By using molecular techniques, *E. faecalis* was reported to coexist with several other oral pathogens in root canal–treated teeth (Rocas et al, 2008). A study showed that the presence of a streptococcal biofilm on hydroxyapatite significantly increased the biofilm formation of *E. faecalis*, which emphasizes the importance to study the endodontic pathogens in a multispecies setting (Deng et al, 2009). In the

current study, we established an *in vitro* mixed-culture biofilm model system to investigate the bactericidal activity of selected antimicrobial irrigants. The polymicrobial characteristic of this model system was confirmed by sampling the culture suspension and by Gram stain. There were no significant differences in the baseline total bacterial load among the test groups (Table 1). This indicated the consistency and reliability of the experimental design.

We chose the single canal tooth model in the current study because it's easy to control the experiment conditions and reduce the anatomy difference between samples. However, the limitations are lack of anatomical variations.

We used sterile paper points to sample the bacteria in biofilms inside the canals and this may not sufficiently sample the total bacteria in the canal system, resulting in an underestimation of viable cell growth. Accurate detection of the viable bacteria may be problematic due to inaccessible residual bacteria growing inside the dentinal tubules or due to viable bacteria undergoing survival mode in response to environmental stress characterized by the absence of growth in the media (Munson et.al 2002; Cook et.al 2007).

C. Relevance of the Study

During endodontic treatment, rotary NiTi instrumentation and needle irrigation are used to remove the planktonic bacteria and biofilms attached to the canal walls. Irrigation is relied upon for the eradication of microbes in areas that instrumentation cannot reach, such as in isthmuses, fins, and anastomoses. However, current endodontic irrigants can be toxic and caustic and must be used with extreme care to avoid iatrogenic injury to the patient. Identifying a safe and effective irrigant is the ultimate goal.

Because natural products that contain polyphenols have been shown to be effective in preventing the formation of biofilms of selected oral pathogens, (Sethi & Govila, 2011; Koo et al, 2010; Wojnicz et al, 2016), we selected cranberry juice as a potential irrigant to reduce endodontic biofilms. However, our results indicated that the effectiveness of the cranberry washes were comparable to saline. Although numerical differences were observed between the cranberry washes and saline, they were not statistically significant. It has been reported that cranberry extract is effective against bacterial biofilm formation but less so against established biofilms. Because cranberry juice is edible, a positive result would help identify a safer and effective endodontic irrigant which could possibly improve results and reduce risks.

D. Future Studies

Because the cranberry washes were no more effective than saline, they cannot currently be recommended as an effective irrigant when used with standard needle irrigation based on our results. However, one weakness of the current study model was the different volume of irrigants used between the test groups and the control groups due to the use of stop agents. It is recommended that a 3 mL final saline rinse be added to the test groups to match the total volume or irrigants used in the control groups.

A consideration of the current study tooth model was the variability of the canal sizes amongst the teeth. The teeth were randomly assigned to the different groups and thus there was no control over the distribution of which group had the more regular, easy to clean anatomy. Therefore, our results should be interpreted with skepticism because it is not clear if the result of the cranberry washes was due to lower efficacy against mature biofilms or due to random tooth anatomy. Increasing the sample size should address this shortcoming and improve the

study outcome. Furthermore, it is recommended that the efficacy of cranberry extract against mature biofilm be tested in an agar plate model where the tooth anatomy variable is eliminated. If these results prove positive, an *in vitro* model with more consistent tooth anatomy and size may be utilized to repeat this study. It is also recommended that possible preventative uses of cranberry washes in dentistry be investigated. The potential of polyphenols being incorporated into toothpaste, mouth rinses, and dental sealants warrants further exploration.

E. Conclusions

Instrumentation and irrigation significantly reduced the bacterial loading inside the canals under current experimental conditions. Although cranberry extract has been reported to reduce supragingival plaque biofilm adherence and formation, it did not demonstrate significant germ-kill effect as an effective endodontic irrigant against a preestablished multi-species biofilm. The effectiveness of cranberry extract as an endodontic irrigant to prevent multi-species biofilm formation warrants further exploration.

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