EFFICACY OF PROPOLIS AGAINST *FUSOBACTERIUM NUCLEATUM* BIOFILM

by

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INTRODUCTION

It is well-understood that the cause of apical periodontitis is due to bacteria within the root canal system.[1] Intracanal medication is an adjunct used in the treatment of endodontic infections. Published studies suggest more than one-third of root canals still have cultivable microorganisms present after the end of the first treatment visit.[2]

The currently used most common intracanal medication, calcium hydroxide, is sub-optimal due to its potential toxicity[2,3] lack of anti-bacterial efficacy[4,5] as well as its required length of time to be effective.[6] One of the drawbacks of using calcium hydroxide is the ability of dentin to buffer the pH of calcium hydroxide, thus decreasing its potential as an antimicrobial agent.[7] Haapasalo and Orstavik found that calcium hydroxide did not eliminate *E. faecalis*.[8] Safavi et al. found that even after relatively extended time periods calcium hydroxide was unable to disinfect dentin tubules.[5] A recent investigation has suggested calcium hydroxide dressing between appointments did not show the expected effect in disinfecting the root canal system and treatment outcome, indicating the need to develop more efficient inter-appointment dressings.[9] Siqueira showed that when calcium hydroxide was prepared as a mixture using saline it was inadequate in eliminating *Enterococcus faecalis* and *Fusobacterium nucleatum*.[10]

Estrela et al. performed a systematic review to conclude that currently used intracanal medicaments have a limited effect on bacterial biofilm.[11] Additionally, anti-biotic resistance is an increasing concern as *F. nucleatum* has shown reduced susceptibility or resistance to vancomycin, neomycin, erythromycin, amoxicillin, ampicillin, phenoxymethylpenicillin and tetracycline.[12] Regarding the need to develop a

better intracanal medication, propolis is being considered a potential medication due to its variety of favorable characteristics.

As previously stated, propolis has shown antimicrobial efficacy in many studies when tested against a variety of microorganisms. Recently, several studies have used *in-vitro* models to evaluate the anti-microbial efficacy of propolis against *E. faecalis*, a pathogen of secondary endodontic infections.[4,13,14] Several of these *in-vitro* studies have been performed under endodontic-relevant conditions within extracted teeth or dentin discs and compared with the most commonly used intracanal medicaments, calcium hydroxide and triple antibiotic paste. Propolis has been shown to be superior to both calcium hydroxide and triple antibiotic paste under these relevant conditions.

PURPOSE

A variety of *in-vitro* methods have been used to evaluate the anti-microbial effect of propolis including the agar diffusion or dilution method,[14] macrobroth dilution method,[15] Petri dish bioassay,[16] and the use of microtiter plates. The current study will use a combination of similar methods in order to investigate the antibacterial efficacy of propolis against biofilm of the primary endodontic pathogen *F. nucleatum*.

This study will use microbiological methods to investigate the antibacterial efficacy of propolis against the primary endodontic pathogen *F. nucleatum* in three phases: total biofilm plus planktonic combination, individual planktonic, and individual biofilm formations. This is of interest because *F. nucleatum* is one of the most common pathogens in the root canal system of teeth with primary endodontic infections.[17,18] Since endodontic biofilms may be more resistant to treatment with medicaments than

planktonic bacteria, the anti-bacterial potential of propolis as an intracanal medicament against primary endodontic infections and biofilm are of clinical interest.

HYPOTHESES

Hypothesis Ho

Propolis will not be inhibitory against *F. nucleatum* at low concentrations that could be applicable for use as an intracanal medication in endodontic infections as judged by its MIC/MBC/MBIC.

Hypothesis Ha

Propolis will have inhibitory activity against *F. nucleatum* at low concentrations that could be applicable for use as an intracanal medication in endodontic infections as judged by its MIC/MBC/MBIC.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Endodontic procedures have been recognized as being performed as far back as the first century A.D. Archigenes, a Greek, was credited with performing trephination into a pulp cavity, likely to establish pain relief.[19] Not so long ago, the empirical era existed prior to the practice of evidenced-based endodontics. Dental and endodontic treatments were described by people like Pierre Fauchard, "the founder of modern dentistry," who wrote the 1728 book, *The Surgeon Dentist*. He described endodontic anatomy, pathology, and treatment, offering the option of treating abscessed teeth with pulp extirpation by needle and a lead foil obturation. By the end of the 18th century, Frederich Hersch further described the pathosis and treatment of dental disease. He found that diseased teeth elicit pain on percussion (a clinical test still in use today).[20]

Koecker, the author of *Principles of Dental Surgery* in 1826, explored the connection between a necrotic pulp and periapical inflammation. He advocated the pulp capping procedure described as a means of retaining teeth to avoid necrosis of the pulp and the spread of inflammation and suppuration to the surrounding tissues.

The practice of using arsenic became common in the 1800s as a painless way to remove painful pulpitis.[20] Arsenic was the generic name given to drugs containing arsenious trioxide and was introduced by Spooner in 1836.[19] Many techniques at this time were aimed at performing pulpotomies. Alternative therapies such as boring a hole through the alveolar bone and root apex to drain suppuration or perforating the cervical part of a tooth to drain hemorrhage were attempts at early endodontic treatment.

Improvements in endodontic therapy included the invention of the broach by Edwin Maynard; the development of gutta-percha by Edwin Truman in 1847; the rubber dam by S. C. Barnum in 1864, and the suggestion of electric current pulp testing by Magitot in 1867. As evidenced by Barnum, establishing a state of asepsis was considered a potential factor in achieving success. In 1867 Joseph Lister documented his experience with antiseptic during surgery using carbolic acid. Around this time Leber and Rottenstein had proved the presence of *Leptothrix buccalis* and were describing that decay could lead to a gangrenous pulp. Following Lister's methods of antiseptic, in 1873 Witzel began using phenol in an attempt to sterilize pulps, and around this time Keyes described the use of iodoform as a pulp and periapical dressing.[20]

As the connections among microbial infection, asepsis, and treatment of pulpal and periapical disease were unfolding, Rogers published an article in *Dental Cosmos* suggesting that pathogenic organisms were the cause of pulpal disease and that destruction of these organisms was imperative to successful treatment. Tomes in 1879 and Underwood in 1882 began the change from the dead tooth theory to the septic theory. Their works not only made connections between necrosis and sepsis as contributing factors to pulpal disease, but they fueled a 30-year initiative to eliminate bacteria using caustic germicides. In 1895 chloropercha was developed by Dr. Bowman as a chloroform and gutta percha obturation method. Pulp stump fixation was practiced using the less caustic formalin method as a replacement from the arsenic method, and phenols and iodoform were still in use for disinfection. In 1894 John Wessler presented his method of pulp capping using oil of cloves which contained 80-percent to 90-percent zinc oxide and eugenol. Interestingly, zinc oxide and eugenol are still in use today as one of the most

common types of root canal sealer.[21] In 1915 Dakin[22] published a description of the use of 0.5-percent to 0.6-percent sodium hypochlorite to be used as a wound antiseptic.

In addition to the clinical methods being developed during this time, adjunctive tools for diagnosis were invented and incorporated in the early 1900s, such as the radiograph. William Roentgen is credited with the discovery of X-rays. Dr. Kells, a dentist who published many articles and developed many patented inventions related to radiographs, is credited as the first dentist to use radiographs to study root canals. Unfortunately, his extensive experimentation with X-rays is claimed to have caused his death by cancer in 1928.[21] The first dental X-ray unit was available in 1913 and in 1919 a conventional dental X-ray unit was commercially available.[23] Thomas Alva Edison even had an attempt to contribute to endodontics. In 1898 he attempted to use calcium tungstate to create a fluorescent dental mirror. By 1930 their use was discouraged due to excessive radiation potential. In 1900 Price coined the term "blind abscess" to describe a periapical radiolucent lesion with no clinically apparent drainage.[21] What he was describing would be described today as apical periodontitis. In 1908 Dr. Rhein developed a technique for measuring canal length and degree of obturation by taking a wire film.[24]

The use of local anesthetics was scant for many years even after the development of cocaine as a local anesthetic. Although many dentists used cocaine for approximately 20 years, its risk of toxicity made it less than desirable. In 1905 Einhorn developed procaine (novocaine), which lacked ideal efficiency in the office as it was complex to prepare for clinical use as an anesthetic requiring boiling and cooling prior to aspiration

into a syringe. H. S. Vaughn is credited as the first to use the local anesthetic infiltration technique for pulpal extirpation.[21]

The endodontic profession encountered challenges when Frank Billings focused attention on the connection between oral sepsis and bacterial endocarditis in 1904.[21] William Hunter contributed to this theory of focal infection in his historical lecture, "The Role of Sepsis and Antisepsis in Medicine."[23] These men theorized that systemic diseases were the result of foci of infection located in necrotic teeth. Rosenow contributed experimental work to support these theories that teeth and tonsils were the foci of infection for a variety of systemic diseases.[25] He was able to show that streptococci were present in a variety of organs and capable of traveling to distant sites in the body via the bloodstream.[23] Thankfully, opponents to this theory eventually prevailed to show there was no scientific basis for these claims. Reimann and Havens presented their position and published a review of literature to show that the "causative relationship of infections about the teeth and tonsils to systemic disease is unproved and that removal of teeth and tonsils in an effort to influence the course of systemic diseases is unjustified in the majority of cases."[25] Additionally, through the efforts of men within the dental community such as Coolidge and Johnson, the principle of preserving the pulpless tooth survived.[23] To further refute the focal infection theory, in 1937 Logan's work explained that the mere presence of microorganisms within tissues did not imply infection; that bacteria are often present within normal healthy tissues with no pathological significance.[23] Even today, the focal infection theory remains present among some clinicians although it continues to have no sound scientific evidential support. Regarding bacteremia, which is the closest thing to the modern focal infection theory, it is suggested

that only patients with the highest potential risk of complications from bacteremia, such as those at risk for infective endocarditis, immunosuppression, or those with an orthopedic prosthetic device be medicated with antibiotics before treatment. Even in these scenarios, the risk is considered unproven.[26]

Amidst the challenges of defeating the focal infection proponents, stricter attention was directed toward aseptic techniques, diagnosis, and culturing. In 1931 the hollow tube effect was described to explain the need to eliminate any void areas from root canal obturations. The belief was that any void could allow for apical percolation of tissue fluid and enzymatic breakdown within the canal to trigger a periapical inflammatory response. Soon after, silver points were developed as a means to obturate canals with a material that had the same size and taper as the instruments used for debridement. In addition, a cement of neo-balsam was used as a sealer with them.[23] Around the First World War, chloramine-T, which had been used in treating wounds, was incorporated into endodontic treatment as an intracanal medicament. Antibiotic use became a component of endodontic therapy in 1941 and 1944 when Fred Adams began using antibiotics within canals initially using sulfanilamide and subsequently using penicillin. At this time, Grossman was developing intracanal techniques to use penicillin and developed a method of using absorbent points with impregnated with penicillin. Several years later after more antibiotics became available and were used in combinations within the canal, Auerbach emphasized that asepsis could not be achieve solely by use of antibiotics and that the use of antibiotics combined with mechanical debridement was necessary.[23]

In 1959 Sargenti and Richter introduced N2, controversial because it contains contains paraformaldehyde and other unfavorable compounds.[23] Methods of pulpal treatment have varied throughout time. Coolidge explained that the purpose of obturation was to obliterate the canal space and to seal the apical foramen from ingress of tissue exudate.[27] Unique ideas were expressed as Ostby in 1961 proposed the possibility of instrumenting past the apex to stimulate a pseudo-pulp utilizing the growth potential of the periapical tissue.[27] In our current variety of techniques today, pulpal regeneration is a concept widely researched.

While all the discoveries of endodontic therapy were occurring, organized dentistry was developing speciality recognition in the field of endodontics. In 1943 the American Association of Endodontists was formed. Harry Johnston coined the term endodontia based on the greek terms "endon" (within) and "ho dontas" (a tooth). Additionally in 1956 the American Board of Endodontics was formed in Illinois.[23] Later, in 1963 endodontics was recognized by the American Dental Association as a specialty.[19]

FOUNDATION OF ENDODONTICS

"The ultimate goal of endodontic treatment is either to prevent the development of apical periodontitis, or in cases where disease is present, to create adequate conditions for periradicular tissue healing."[28] In the most well-known study in the field of endodontics, Kakehashi, Stanley, and Fitzgerald[1] in 1965 created surgical pulp exposures on gnotobiotic germ-free rats and conventional rats with normal microflora. They found that apical periodontitis developed in conventional rats but not in germ-free rats. From this information, they concluded bacteria is required to develop apical periodontitis. Through history this has been reconfirmed. Moller et al.[29] in 1981 used monkeys to conclude that

reactions in the periapical tissues were induced by microorganisms that established themselves within the necrotic pulp from the mouth. Of the teeth that were aseptically necrotized, those that were then inoculated with bacteria developed periapical inflammation, whereas teeth that were necrotic but remained uninfected had no periapical inflammatory reaction. These studies provide the classic foundation of knowledge that apical periodontitis is caused by microorganisms within the canal. Current research has also shown other microorganisms such as archaea, fungi, and viruses to be associated with endodontic infections but evidence is equivocal as to their involvement.[30-33] One study "confirmed that cytomegalovirus or Epstein-Barr virus active infections are detected in more than 90 percent of granulomas of symptomatic and large periapical lesions."[31]

Considering how to provide adequate endodontic therapy, Stewart[34] described the critical components: chemomechanical treatment, debridement, microbial control, and adequate obturation. He considered the chemomechanical preparation step to be most critical. An additional description of the goal of endodontic treatment is said to be total elimination of intracanal microbes.[35]

ANATOMY

Root canal anatomy has been shown to be extremely variable. Much discussion has been attributed to the complex interlacing network within the canal system, accessory canals and roots as well as the anatomy of the apical foramen. A classic study by Kuttler[36] in 1955 described the variability of the apical foramen. He explained there is a distance between the minor and major foramen and a funnel shape between them that disallows a hermetic seal if the root canal were to be treated past the minor foramen to

the major foramen. This distance was found to be 0.5 mm and is part of the foundation of knowledge to advocate debridement and obturation of roots to 0.5-mm coronal to the apical terminus. Contrary to this, in 2011 Meder-Cowherd and Williamson et al.[37] studied palatal roots of maxillary molars and found that most (65%) of canals did not demonstrate an apical constriction and that the apical region was variable. In 2008 using mandibular premolars, Hassanien[38] found the CDJ and apical constriction are not the same point; the apical constriction was always found coronal to the CDJ. It was also found that the Root ZX (J Morita Co, Tokyo, Japan) brings the file tip closer to the CDJ. Green[39] in 1960 found that approximately 50 percent of the major foramina terminate at a position up to 2 mm eccentric to the apex of the root.

The anatomy of the mesiobuccal root of maxillary molar teeth has been extensively studied as the appearance of two canals has been determined to be very common.[40,41] Gilles and Reader[42] found a second mesiobuccal canal (MB2) in 90 percent of maxillary first molars and 70 percent of maxillary second molars. Verma and Love[43] agreed with this confirming the presence of a second MB canal in 90 percent of maxillary first molars using micro CT. Additionally, de Pablo[44] described the mandibular first molar having complex anatomy with variable canal configurations and variable number of roots.

Weine et al. [40] described three canal configurations in their study of maxillary molars:

Type 1: A single canal from pulp to apex.

Type 2: Two canals that merge before the apex.

Type 3: Two separate canals with two distinct apical foramina.

Type 4: Splits from one main canal to two separate canals with two distinct orifices was later described and is referenced in many studies.[44]

Vertucci et al.[45] described canal morphology in a more extensive manner. They provided a breakdown of eight canal configurations:

Type 1: A single canal from pulp to apex.

Type 2: Two separate canals that join and exit as one.

Type 3: One canal that splits into two and rejoins as one prior to exiting its foramen.

Type 4: Two separate canals with two distinct apical foramina.

Type 5: One canal that splits into two separate canals with two distinct orifices.

Type 6: Two separate canals that join within the root and then split to exit as two distinct orifices.

Type 7: One canal starts, splits to two, rejoins as one, and splits to exit as two distinct orifices.

Type 8: Three separate canals that stay separate.

CHEMOMECHANICAL DEBRIDEMENT

In 1968 Dudley Glick[27] described the microbiological aspects of chemomechanical debridement. It was accepted that mechanical debridement or intracanal medication alone were insufficient by themselves to render a canal sterile. There was emphasis on using both mechanical and chemical disinfection to achieve asepsis within the canal. Much like today's attempts to disinfect teeth with triple antibiotic paste, there was a variety of drug combinations to place within the canal, and each type of drug had its own limitations.

While many methods of chemomechanical debridement have been evaluated over time, few chemicals are consistently in use for the chemical disinfection of the root canal system. To list a few of the commonly investigated irrigation solutions: sterile water, saline, sodium hypochlorite (in a variety of concentrations), chlorhexidine (in a variety of concentrations), iodine potassium iodide, camphorated parachlorophenol, formocresol (formaldehyde and cresol) and hydrogen peroxide. In 1975 Spangberg et al.[46] showed that 0.5-percent sodium hypochlorite was antibacterial, had the ability to dissolve necrotic tissue, and had a decreased toxicity due to dilution, yet it was unable to kill *Staphylococcus aureus* (whereas 1.0-percent sodium hypochlorite was able to do so). They claimed that in vital cases, the objective of irrigation solutions in cleaning the canal was aimed at removal of pulp and dentin, and the antimicrobial effect was a secondary goal. This was based on their understanding that in vital pulp cases, bacteria are not usually present in the canal. A study by Bystrom and Sundqvist[47] in 1981 provided evidence that through mechanical debridement with saline, they were able to decrease the bacterial count within canals 100-fold to 1000-fold, and in some cases higher. Although this supports mechanical debridement of the canal, they concluded that the supportive action of antimicrobial solutions would be necessary for further elimination of the bacteria that remain. They followed this research with further experimentation in 1983 that concluded the use of 0.5-percent sodium hypochlorite is more effective than saline as a root canal irrigant. They showed that more canals with no bacterial growth were found in the teeth treated with sodium hypochlorite.[48] Harrison and Hand[49] in 1981 showed that 5.25-percent sodium hypochlorite was the most effective antimicrobial solution when compared with saline, 0.5-percent sodium hypochlorite, and a mixture of 3.0-percent

hydrogen peroxide mixed with 5.25-percent sodium hypochlorite. They additionally concluded that dilution of sodium hypochlorite from 5.25-percent to 0.5-percent decreased its antimicrobial efficacy. In 2008 Mohammadi[50] conducted a review of the literature on sodium hypochlorite and explained several of its properties. It is a good choice for endodontic use against bacteria, biofilm, and fungi and has tissue-dissolving properties. Additionally, the authors described the *in-vitro* ability to enhance the bactericidal efficacy by heating NaOCl; the toxicity and complications with clinical use of sodium hypochlorite; the examination of sodium hypochlorite accidents with extrusion beyond the apex, or accidental introduction into the eye. Current literature supports using higher concentrations as well as longer exposure times for the greatest effect when using sodium hypochlorite. Retamozo et al. [51] found the most effective concentration and length of time to eliminate *E. faecalis* was 5.25-percent sodium hypochlorite at 40 minutes.

One of the additional properties of sodium hypochlorite of significant benefit is its ability to dissolve necrotic tissue. In 1978 Hand et al. [52] confirmed the effectiveness of 5.25-percent sodium hypochlorite as a necrotic tissue solvent and simultaneously confirmed the ineffectiveness of saline, distilled water, and 3.0-percent hydrogen peroxide, 1.0-percent sodium hypochlorite, and 0.5-percent sodium hypochlorite. Current literature confirms the ability of 2.5-percent sodium hypochlorite to completely dissolve pulp tissue, and the ability of 17-percent ethylenediaminetetraacetic acid (EDTA) to have minimal pulp tissue dissolvent action.[53]

Generally accepted as the most effective chelating agent, “EDTA has prominent lubricant properties and is widely used in endodontic therapy. It is used to enlarge root

canals, remove the smear layer, and prepare the dentinal walls for better adhesion of filling materials."[54] Baumgartner and Mader[55] described the ability of alternating sodium hypochlorite and EDTA for maximal tissue dissolution. Sodium hypochlorite dissolves the organic component of the pulp, and EDTA is able to dissolve the inorganic component. Calt and Serper[54] advised using EDTA no longer than one minute for smear layer removal due to its ability at longer time frames to excessively erode intertubular and peritubular detin.

In recent decades, the reputation of chlorhexidine (CHX) has risen as a potential intracanal irrigation solution. Ohara et al.[56] found that 0.2-percent CHX displayed the most effective antibacterial activity when compared with 5.25-percent NaOCl, 3.0-percent hydrogen peroxide, and 17-percent EDTA tested under laboratory conditions against six common endodontic pathogens (*Peptococcus magnus, Propionibacterium acnes, Veillonella parvula, Lactobacillus fermentum, Porphyromonas (Bacteroides) gingivalis, F. nucleatum*. Other studies have shown limited benefit from using CHX rather than NaOCl as the primary intracanal medicament of choice.

Several other relatively new medicaments have come onto the market as potential irrigation solutions such as BioPure MTAD (Dentsply Tulsa). MTAD consists of a mixture of doxycycline, citric acid, and a detergent (Tween 80).[57] Several studies show a lack of beneficial results in comparison with NaOCl for improvement in intracanal disinfection. A review of the literature regarding MTAD stated the initial studies show good antimicrobial activity against *E. faecalis*, but later studies that simulated clinical conditions showed a lesser antimicrobial effect of MTAD.[57,58]

Many studies have shown medicaments cannot completely eradicate bacteria from the root canal system.[59] Although we may not be able to completely eliminate all bacteria from the root canal system, irrigation solutions such as NaOCl and chlorhexidine appear to decrease bacterial counts favorably to encourage healing.[60] Debridement with proper irrigation solutions and techniques are an important part of the one-versus-two appointments debate. In 2012 Siqueira[61] found that in using 5.0-percent NaOCl, they did not completely eliminate bacteria from the teeth treated in one visit, and that only two cases were bacteria-free in the two-appointment group. This finding exemplified the complexity of the root canal system and the inability to completely eradicate bacteria from teeth.

An informed use of these medicaments is essential because current literature has shown potential interactions among irrigation solutions. Basrani et al.[62] using mass spectrometry presented the interaction between sodium hypochlorite and chlorhexidine, which produces a precipitate considered to be para-chloroaniline (PCA). PCA is known to be cytotoxic through formation of methemoglobin and was shown to have carcinogenic potential in animal studies. Further investigation has shown the chemical composition of the products may appear to be different depending on the methodology of chemical evaluation. Nowicki and Sem[63] used nuclear magnetic resonance (NMR) spectroscopy to determine that the chemical composition of the precipitate contains fragments of chlorhexidine, which are chlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH) and parachlorophenylurea (PCU) and not PCA. Additionally, a study by Thomas and Sem using a NMR analysis found no measurable quantity of PCA.[64] Several studies have suggested an intermediate rinse in between the use of NaOCl and chlorhexidine to

prevent the formation of PCA. Krishnamurthy et al.[65] found that absolute alcohol eliminates all formation of the apparent precipitate between NaOCl and chlorhexidine and that saline or sterile water will decrease precipitate formation, but not eliminate it. A study by Mortenson et al.[66] comparing citric acid, EDTA, and saline showed that these three medicaments decrease, but do not eliminate the formation of PCA. From these studies, it appears most prudent to use absolute alcohol as an intermediate rinse in cases utilizing NaOCl and CHX. Given EDTA is often used prior to the CHX rinse, it may be most logical to use the EDTA, followed by alcohol to enable two intermediate flushes between the NaOCl and the CHX.

OBTURATION

In 1967 Schilder[67] described the ultimate goal of root canal therapy is "to eliminate the root system as a source of infection and inflammation to the apical periodontium after irreversible pulp pathosis." He described that the final product should be an obturation of the canal system in three dimensions.[68] In 1940 Grossman described the criteria for an ideal endodontic filling material:

1. Easily introduced.
2. Liquid or semisolid and becomes solid.
3. Seals both apically and laterally.
4. Does not shrink.
5. Impervious to moisture.
6. Bacteriostatic.
7. Non-staining.
8. Non-threatening to periapical tissues.

9. Easily removed.

10. Sterile or sterilizable.

11. Radiopaque.

He additionally described the need to properly clean and shape the canal system in order to achieve a successful obturation.[69]

Historically, various methods have been used to attempt to obturate the canal. Silver points were once a common practice, but due to their inability to create an adequate seal as well as their tendency to corrode, they are no longer advisable.[70] Sargenti paste, or "N2" was a mixture of antibiotics, hydrocortisone, paraformaldehyde, and zinc-oxide-eugenol cement.[71] Its use is no longer recommended due to its high potential for cytotoxicity and inflammatory reactions. Newton, Patterson, and Kafrawy[72] found that monkey teeth with pulpitis treated with this technique developed reactions of apical periodontitis and osteomyelitis at six- and 12-month follow-up histologic examinations.

Another more recent obturation material on the market is Resilon. Resilon is promoted as a resin-based obturation material used with a resin-based sealer that supposedly creates a monoblock seal. Although this sounds favorable, untoward properties of this material have been researched, such as polymerization shrinkage; when the material sets and shrinks it leaves gaps between the obturation material and dentin where microorganisms could inhabit.[73] Additional criticisms of this material have been made due to its susceptibility to alkaline hydrolysis and enzymatic hydrolysis.[74,75] The polycaprolactone component of Resilon has been described as being biodegradable by bacterial and salivary enzymes such as cholesterol esterase and lipase PS.[75,76] Clinicians

have described finding root canals obturated with Resilon to develop apical periodontitis in cases that previously had no apical periodontitis.[77]

The most commonly used obturation material is gutta-percha. Friedman et al.[78] described that gutta-percha is composed of 20- percent gutta-percha (rubber), 66-percent zinc oxide (filler), 11-percent barium sulfate (radiopacifier), and 3-percent wax (stabilizer). Schilder[79] described the phase transitions that occur with heating gutta-percha. Gutta-percha exists in the beta-semicrystalline phase until it undergoes a phase transition to the alpha phase upon heating. Senia et al.[80] described the property to rapidly sterilize gutta-percha cones prior to their use with a 1-minute application of 5.25-percent sodium hypochlorite. Gutta-percha cones have been shown not to cross-react immunologically with natural rubber latex; thus, gutta-percha can be presumed to be safe for use in patients even if they have a latex allergy.[81,82]

Warm vertical condensation, cold lateral condensation, warm lateral condensation, and warm thermoplasticized gutta-percha are commonly practiced methods of gutta-percha obturation. A meta-analysis by Peng[83] et al. found that warm gutta-percha obturation methods were more likely to demonstrate overextensions of obturation materials than the cold lateral technique. Otherwise, they found post-operative pain, long-term outcomes, and obturation quality were similar between warm and cold gutta-percha methods. Reader et al.[84] compared warm techniques to cold lateral and found more lateral canals obturated with gutta-percha in the warm techniques. Whatever method is used, Schaeffer, White, and Walton[85] conducted a meta-analysis indicating that a better success rate is achieved when obturation is completed at or short of the apex in comparison with past the apex.

INTER-APPOINTMENT MEDICATION

In addition to medicaments used during the appointment, inter-appointment medication has been suggested to promote success of root canal therapy. One of the primary goals of inter-appointment medication is to increase the ability to eradicate bacteria from the canal. Hermann introduced the use of calcium hydroxide as an antibacterial dressing in 1920. In 1985 Bystrom, Claesson, and Sundqvist[86] compared the effects of calcium hydroxide, camphorated paramonochlorophenol, and camphorated phenol as inter-appointment medicaments. They found better antimicrobial success with calcium hydroxide and suggested its use for intracanal medication. Intracanal medication is an adjunct used in the treatment of endodontic infections as studies suggest more than one-third of root canals still have cultivable microorganisms present after the end of the first treatment visit.[2]

An extensive review of the properties of calcium hydroxide was done by Farhad and Mohammadi[7] in 2005 describing its variety of properties and applications including its use as an inter-appointment medicament. They describe that when the calcium and hydroxyl ions dissociate, there is a high pH environment created by the hydroxyl ions. Bacteria are unable to grow or survive at this high pH. There are several mechanisms by which these hydroxyl ions are antibacterial. The bacterial cytoplasmic membrane is disrupted by the peroxidation reactions occurring between hydroxyl ions and lipids within the phospholipid membrane. Additionally, cellular protein denaturation occurs because enzymatic activity is disrupted by the high pH. Bacterial DNA is disrupted by the hydroxyl ions and potentially even fatal mutations occur by the free radicals formed. Clinically, Nerwich et al.[35] showed that with intracanal dressing of calcium hydroxide,

the pH of outer root dentin began to rise between 1 day to 7 days and peaked at 2 weeks to 3 weeks. These findings support that hydroxyl ions diffuse through dentin during calcium hydroxide dressing, although other authors like Fuss et. al.[87] questioned the ability of a pH change at the outer root dentin. They showed that at seven days there was not a noticeable pH change in a medium surrounding the outer root surface when examining intra-canal calcium hydroxide *in vitro*.

Several authors have evaluated the optimal time to leave calcium hydroxide within the canal as an inter-appointment medication. Sjogren and Sundqvist[6] supported a seven day medication to eliminate bacteria. Shuping[88] exemplified efficient antibacterial effect at seven days. The study by Nerwich and Messer would suggest a 2-week to 3-week application could have optimal benefit.[35] Andreasen suggested using calcium hydroxide up to 1 month to avoid weakening root dentin.[89]

One of the properties of calcium hydroxide that appears to be unique is its ability to detoxify endotoxins such as lipopolysaccharide (LPS). Sodium hypochlorite and chlorhexidine have not shown the ability to detoxify LPS, making this a unique property in the additional use of calcium hydroxide in endodontic treatment.[90] Safavi and Nichols[91] concluded that calcium hydroxide hydrolyzed the lipid moiety of bacterial LPS, resulting in the release of free hydroxy fatty acids, thus having the potential clinical benefit of detoxifying LPS.

Antibiotics as intracanal medications have been used as individual and combination formulations. Triple antibiotic paste (TAP) is a common term used to describe a mixture of ciprofloxacin, minocycline, and metronidazole. Hoshino, Sato and colleagues[90,91] have shown this combination has excellent efficacy as a potential

intracanal medication to penetrate dentin and kill bacteria. The use of TAP has been reported clinically and is often considered in cases with trauma to immature permanent teeth.[92] Banchs and Trope[93] have described the use of TAP as part of their 2004 potential treatment protocol in the revascularization of immature permanent teeth. Furthermore, double antibiotic paste containing ciprofloxacin and metronidazole shows clinical efficacy and has been suggested to avoid the discoloration that minocycline may cause.[94-96]

A recent *in-vivo* clinical study presents an alternative to using calcium hydroxide as an inter-appointment dressing. The authors examined the use of "limewater" slurry, which is calcium hydroxide slurry, as an incorporated rinse during single-visit disinfection. They found the calcium hydroxide slurry decreased endotoxin by 99.18 percent, whereas use of intracanal medication of calcium hydroxide for 14 days without a final rinse of limewater or polymyxin B decreased endotoxin by 99.2 percent.[97] These results may have clinical implications for the use of limewater slurry to be used near the end of an appointment prior to a final rinse rather than an inter-appointment medication.

In 2012 Vera, Siqueira, Ricucci and colleagues[61] found their "2-visit protocol by using an inter-appointment medication with calcium hydroxide resulted in improved microbiological status of the root canal system when compared with the 1-visit protocol." They concluded that the use of inter-appointment medication is necessary to maximize bacterial reduction. Similarly, Shuping et al.[88] found that with instrumentation using sodium hypochlorite, 61.9 percent of canals were rendered bacteria-free and placement of calcium hydroxide for at least one week rendered 92.5 percent of the canals bacteria-free. They suggested using calcium hydroxide to more predictably eliminate bacteria.

Additionally, Siqueira[98] in 2012 found that although there are ways to optimize single visit disinfection, there is a lack of consistent clinical evidence to predictably reduce microbes without the use of an inter-appointment medication.

PROPOLIS

Propolis is a resinous substance that honeybees produce and use for protective effects to seal their hives. It has been used for a variety of purposes in folk medicine for centuries. The chemical composition of propolis is very complex and includes organic compounds such as phenolic compounds, esters and many different known flavonoids and flavonones, such as pinocembrin, pinostropin, isalpinin, pinobanksin, quercetin, naringenin, galangine, and chrysin.

As a potential intracanal medicament, Propolis is antimicrobial,[16,99-104] anti-inflammatory,[105] anti-osteoclastic,[106] anti-quorum sensing,[107] anti-biofilm,[103] and immunomodulatory. The antibacterial activity has been linked mainly to the flavonoid content and to the phenolic compounds, terpenes, and aromatic acids and esters that have been found in propolis.[108] Although both propolis and honey are produced by bees, propolis is different from honey and has a higher antimicrobial effect.[99] The chemical composition of propolis is also highly variable and depends on the local flora at the site of collection.

Propolis has been shown to have antimicrobial effects against various microbes such as *Bacillus subtilis*, *S. aureus*, methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *Enterobacter aerogenes*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Micrococcus luteus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *C. albicans*, *Candida tropicalis*, *Salmonella typhimurium*,

Prevotella nigrescens, *F. nucleatum*, *Actinomyces israelii*, and *Clostridium perfringens*.[14,16,99-103,109-111] Several commercial formulations of propolis were examined and it was concluded that flavonoid content above 1.0 percent has antimicrobial activity.[101] Propolis from different sources was also shown to have differing antimicrobial effects and is often described as ethanol extract of propolis (EEP). Propolis from Turkey, Iran, Tunisia, Brazil, Poland and others were shown to have antimicrobial properties.[16,99,100,109,112]

As described, many *in-vitro* studies have shown propolis to be an effective anti-microbial medication against bacteria of endodontic origin.[4,13,15,108,113] Additional appealing properties of propolis as a potential intracanal medication are its ability to promote healing, to reduce cytotoxicity compared with current medicaments, to inhibit osteoclastogenesis as well as osteoclast maturation and osteoclast activity, to affect inflammatory cytokine expression, and to reduce apoptosis and to enhance periodontal ligament cell metabolic activity and proliferation.[106,114-120] It also has been shown to have a faster anti-bacterial effect[4] and has shown physical properties that would make it suitable for placing as an inter-appointment dressing.[121]

Neiva et al. found that propolis reduced LPS-induced inflammatory cytokines from pulp cells and osteoclasts.[114] Harris described the property of propolis to block osteoclastogenesis and osteoclast activity downstream of COX activity.[106] Pileggi published a study finding that propolis inhibited osteoclast maturity and fusion of osteoclast precursors.[120] These properties are intriguing as having implications toward a potential role in the prevention and healing of resorption and apical periodontitis.

Clinicians are aware of the potential of propolis and clinical studies and case reports are published stressing its potential induction of healing and anti-bacterial effects.[115,122] A case report regarding the retreatment of two previously non-surgically and surgically treated teeth involved the same type of propolis that will be used in our experimental model. They used 100-percent propolis powder mixed with propylene glycol.[122] Since 2011, there have been over 280 research publications with the word "propolis" in their title.

F. NUCLEATUM

F. nucleatum is in the genus Fusobacterium, which belongs to the family Bacteroidaceae. The name Fusobacterium originates from *fusus*, meaning a spindle, and *bacterion*, meaning a small rod; hence it is a small, spindle-shaped rod. Additionally, the term *nucleatum* stems from the nucleated appearance often seen in light and electron microscope preparations due to the presence of intracellular granules. *F. nucleatum* is non-spore-forming, non-motile, and gram-negative. Although it is considered an anaerobe, it may grow in the presence of up to 6.0-percent oxygen.[12] LPS found on *F. nucleatum* is typical of gram-negative bacteria with its typical lipid A component and O-antigen polysaccharide. Clinically, "*F. nucleatum* is one of the most common oral species isolated from extra-oral infections, including blood, brain, chest, lung, liver, joint, abdominal, obstetrical and gynecological infections and abscesses."[123] It has also been associated with pregnancy complications and pre-term, low- birth-weight babies.[123]

One of the most discussed aspects of *F. nucleatum* is its ability to have an additive or synergistic effect with other bacterial species in co-infections. *F. nucleatum* possesses outer membrane proteins that are of interest in regards to coaggregation,

antimicrobial susceptibility, and cell nutrition. FomA is considered the major outer membrane protein of *F. nucleatum*.[124] It has been speculated that FomA, an outer membrane protein in the form of a porin may contribute to the antimicrobial susceptibility of *F. nucleatum*.[12] FomA has been suggested to participate in the coaggregation process. It has been shown that FomA forms trimeric, water-filled channels in lipid bilayer membranes, acting as non-specifc pores.[124] Research has shown neutralization of FomA considerably eliminated the enhancement of co-aggregation, biofilms, and production of volatile sulfur compounds mediated by an inter-species interaction of *F. nucleatum* with *Porphyromonas gingivalis*.[125]

F. nucleatum also contains adhesins. These outer membrane proteins are utilized in adhesion and co-aggregation to create multi-species biofilms. One of the adhesin proteins on *F. nucleatum* is called RadD. RadD has been shown to be a critical component in inter-species adherence and biofilm architecture.[126] Additionally, the adhesive properties of *F. nucleatum* are not restricted to bacteria. This microbe is able to attach to epithelial cells, erythrocytes, and immune cells. Hemagglutinins, which are adhesins on *F. nucleatum*, are utilized in hemagglutination and have shown to adhere to a variety of cells and collagen.[12]

Outer membrane proteins of *F. nucleatum* are involved in more than co-aggregation and biofilm formation. Fap2 and RadD are outer membrane proteins that have been shown to induce human lymphocyte cell death. This is a unique property because a majority of mechanisms of cell death induction are based on protein and toxin transfer from bacteria to host cells.[127] *F. nucleatum* also has the ability to secrete serine

proteases, which degrade extracellular matrix proteins such as fibronectin and fibrinogen in addition to their ability to degrade collagen type I and IV.[123]

F. nucleatum has been shown to be one of the most common microbes found in primary endodontic infections. Rocas et al. found *F. nucleatum* to be one of the most prevalent species found in asymptomatic primary endodontic infections using reverse-capture checkerboard DNA-DNA hybridization.[128] Siqueira et al. found *F. nucleatum* to be the most frequently detected species in symptomatic primary endodontic infections of acute apical abscesses using reverse-capture checkerboard DNA-DNA hybridization.[129] Using a culture technique Gomes et al. found *F. nucleatum* to be one of the most prevalent types of bacteria involved in primary endodontic infections.[130] Molecular methods have enhanced the ability to detect specific microorganisms and to provide a quantitative analysis of the different species. Using checkerboard DNA-DNA hybridization, Sassone et al. found *F. nucleatum* to be one of the most common microbes found in primary endodontic infections in both unexposed and exposed canals.[131] Siqueira and colleagues found *F. nucleatum* to be prevalent in the apical third of canal spaces in 6 of 23 teeth (26%) using a nested polymerase chain reaction assay[132] and as one of the most prevalent species in the apical root canal as detected by reverse capture checkerboard DNA-DNA hybridization.[17] Baumgartner et al. found variability in the presence of *F. nucleatum* based on geographical region. *F. nucleatum* was much more prevalent in acute apical abscesses in the US when compared with Brazil.[133] Therefore, the prevalence of *F. nucleatum* in primary endodontic infections in the US is well established.

BIOFILM

"Biofilm can be defined as a sessile multi-cellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polysmeric substance (EPS), usually polysaccharide."[134] Another author describes biofilms as "matrix-enclosed microbial aggregations that adhere to biological or non-biological surfaces."[135] This description has been seen in additional articles.[136] Biofilms represent specific bacterial communities that may have a polymicrobial makeup. The ability to form biofilms is regarded as a virulence factor.[134,136] Biofilms have specific structural, physical, chemical, and biologic features. They are not randomized assortments of bacteria; rather, they are an aggregation of bacteria that interact and position themselves for specific reasons. One of the features of biofilms that are advantageous to bacterial survival is their ability to afford protection to certain bacteria within the biofilm from host defenses, other competing microorganisms, environmental factors and antimicrobial agents.[134] This feature has great relevance to our study of testing an antimicrobial agent against *F. nucleatum* biofilm.

"Biofilms are controlled by a process called quorum-sensing triggered by species-specific, small, diffusible auto-inducers and small peptides."[137] Quorum-sensing is how microorganisms communicate within biofilm and maintain their homeostasis, structure and function. It is interesting to mention that "several herbal, animal and microbial extracts possess quorum-quenching activity."[137] This is of interest because inhibition of quorum-sensing could be a way that propolis acts in a biofilm-inhibitory manner.

MATERIALS AND METHODS

BACTERIAL STRAINS, MEDIA, AND PROPOLIS

A loop containing colonies of *F. nucleatum* (ATCC 10953) from a 24-h to 48-h blood agar plate (see Figure 4) was picked and passed into 5 ml of Brain Heart Infusion broth (Difco, Detroit, MI) containing 5 g of Yeast Extract/L supplemented with 5.0-percent (v/v) hemin/vitamin K solution (BHI-YE; Difco) (see Figure 5) and incubated anaerobically for 24 hours at 37 ºC in a GasPak jar (see Figure 6).[138] The propolis (Ecuadorian Rainforest LLC, Belleville, NJ) used is a previously studied[115,139] commercial formulation powder (see Figure 1) that was dissolved in dimethyl sulfoxide (DMSO) to create a stock solution of 50,000 ug/ml propolis. DMSO was the chosen solvent.[140,141] The positive control contained BHI-YE (with 5.0-percent v/v hemin/vitamin K) inoculated with *F. nucleatum*. There will be a negative control for each test concentration. One negative control group containing BHI-YE (with 5.0-percent v/v hemin/vitamin K) with no addition of *F. nucleatum* will be used. Additional individual negative controls followed the same concentration of propolis in DMSO: BHI-YE (with 5.0-percent v/v hemin/vitamin K) as compared with their test group with the exception of having no addition of *F. nucleatum*. An additional control plate with quadruplicate well evaluation will contain dilutions of DMSO to verify its lack of antimicrobial efficacy at the concentrations being used.

The dilution of propolis dissolved in DMSO that shows good solubility will be tested as a whole, although microscopic fragments of propolis may appear to be insoluble. The extent of whether the active antibacterial components of propolis are

soluble or insoluble is not being tested. The propolis in toto will be used, because the aim of this study is not to determine precise components of propolis that are antibacterial, or whether they are retained in the soluble or microscopically insoluble portions. Previous studies have shown a variety of antibacterial components of propolis, and the focus of this study is to determine the antibacterial efficacy of a specific commercially available propolis powder.

MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC)

MIC is the lowest concentration of propolis that is required to inhibit growth of *F. nucleatum*. MBC is the lowest concentration of propolis required to kill *F. nucleatum*. MIC and MBC were tested using concentrations within a range of previous study findings[109,111,140-143] and adjusted as needed to exemplify a range of possible concentrations using a two-fold dilution method. An overnight culture of *F. nucleatum* grown in 5 ml of BHI-YE (with 5.0-percent v/v hemin/vitamin K) will be treated with 50,000 μg/ml, 25,000 μg/ml, 12,500 μg/ml, 6250 μg/ml, 3125 μg/ml, 1562.5 μg/ml, 781.25 μg/ml, and 390.625 μg/ml of propolis in BHI-YE (with 5.0-percent v/v hemin/vitamin K) for 48 hours in sterile 96-well flat-bottom microtiter plates (Fischer Scientific, Newark, DE) (see Figure 10).[142-146] Propolis dissolved in DMSO to its stock concentration of 100,000 μg/ml will be further diluted using the same two-fold dilution method with BHI-YE (with 5.0-percent v/v hemin/vitamin K) to achieve these concentrations (see Figure 8). The experimental test groups will be inoculated with *F. nucleatum* in volume ratios of 190:10 μl BHI-YE/propolis:*F. nucleatum*. Each test concentration will have four wells to enable quadruplicate evaluation per each trial. Each

test concentration of propolis will have its own quadruplicate negative control wells containing 200-µl BHI-YE/propolis with no addition of *F. nucleatum*. Three trials were conducted for this study.

The optical density values of the bacterial cultures will be measured at 595 nm in a spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA) (see Figure 11) on the setting with no pre-mix for the total (biofilm and planktonic) reading. To determine the MIC the lowest concentration of propolis that provided no significance in total growth was reported. To determine the MBC *F. nucleatum* cultures from the wells with propolis concentrations equal to or higher than the MIC will be transferred onto blood agar plates and incubated for 48 hours. The MBC will be determined as the minimum concentration of propolis that is bactericidal after 48 hours of incubation.[138]

MINIMUM BIOFILM INHIBITORY CONCENTRATION (MBIC)

The MBIC is the lowest concentration of propolis that is required to inhibit visible *F. nucleatum* biofilm formation. The quantification of microbial biofilm grown in 96-well microtiter plates has been described as an approach to studying biofilm performed in previous studies[138,141,144,147-150] as well as in the endodontic literature.[151] It has been a published method of *F. nucleatum* biofilm evaluation in the oral microbiology literature.[144] *F. nucleatum* biofilm will be formed within 96-well microtiter plates[146] by allowing bacterial growth and biofilm adherence to well walls.[145] Propolis will be added simultaneously at the time of inoculation of the wells[142] to evaluate the influence of propolis on inhibition of new biofilm formation within the wells. *F. nucleatum* will be treated as previously mentioned in the MIC/MBC description. Once the total (planktonic and biofilm) reading has been performed by the spectrophotometer, 10 µl samples from

each concentration will be taken to be used for the MBC test and 120 μl will be transferred to a nonsterile 96-well plate to read the planktonic values with the spectrophotometer at 595 nm on the 5-second pre-mix setting. The remaining 80 μl will be discarded followed by washing the wells twice with 200 μl of sterile saline and fixed with 200 μl of 10.0-percent formaldehyde. After a 30-minute period of fixation the wells will be washed three times with deionized water and stained with 200 μl of 0.5-percent crystal violet for 30 minutes. After 30 minutes the wells will be washed three times with deionized water. Next, crystal violet will be extracted from the biofilm cells by incubation for 30 minutes with 200 μl of 2-propanol. The absorbance will be read at 490 nm on the 5-second pre-mix setting with quadruplicate wells of 2-propanol used as the blank control.[138]

STATISTICAL ANALYSES

Four wells on each plate were used for each study group (positive control and 8 propolis levels) and for negative controls for each group (media with no bacteria with the corresponding level of propolis). The average of the four wells for the negative controls was subtracted from the average for four wells for each group. The experiment was repeated three times. Comparisons for each group against its corresponding negative control were made using paired t-tests. Group comparisons were performed using one-way ANOVA, followed by pair-wise comparisons among groups using Tukey's method to control the overall significance level at 5 percent.

RESULTS

The results of the total containing biofilm and planktonic components were as follows (see Graph 3): Positive control, Propolis 390.625 μg/ml, Propolis 781.25 μg/ml, Propolis 1562.5 μg/ml, and Propolis 3125 μg/ml had significantly higher absorbance than their negative controls. Positive control, Propolis 390.625 μg/ml; Propolis 781.25 μg/ml; Propolis 1562.5 μg/ml were significantly higher than Propolis 12,500 μg/ml; Propolis 25,000 μg/ml; and Propolis 50,000 μg/ml ($p < 0.01$); while Propolis 3125 μg/ml and Propolis 6250 μg/ml were significantly higher than Propolis 50,000 μg/ml ($p \leq 0.01$).

The planktonic results were as follows (see Figure 4): Propolis 25,000 μg/ml and 50,000 μg/ml had significantly lower absorbance than their negative controls. There were no significant differences among groups ($p = 0.11$).

The biofilm results were as follows (see Figure 6): Values for the propolis 390.625 μg/ml and 781.25 μg/ml groups had significantly higher absorbance ($p < 0.05$) than their negative controls. There were no significant differences among groups ($p = 0.28$).

The results show that the MIC of the total (biofilm + planktonic) appears to occur at a concentration of 6250 μg/ml. The MBIC appears to occur at the concentration of 1562.5 μg/ml. The planktonic results exhibit no significant difference in test and control wells that would imply bacterial growth; rather, they merely exhibit this at the highest concentrations of propolis. The additional 10 μl of propolis-containing media rather than 10 μl of bacterial suspension may cause a more dense solution. There was no MBC at any

of the test concentrations. The propolis appears to inhibit bacterial growth and biofilm formation, but does not appear to be bactericidal at any of the tested concentrations.

FIGURES

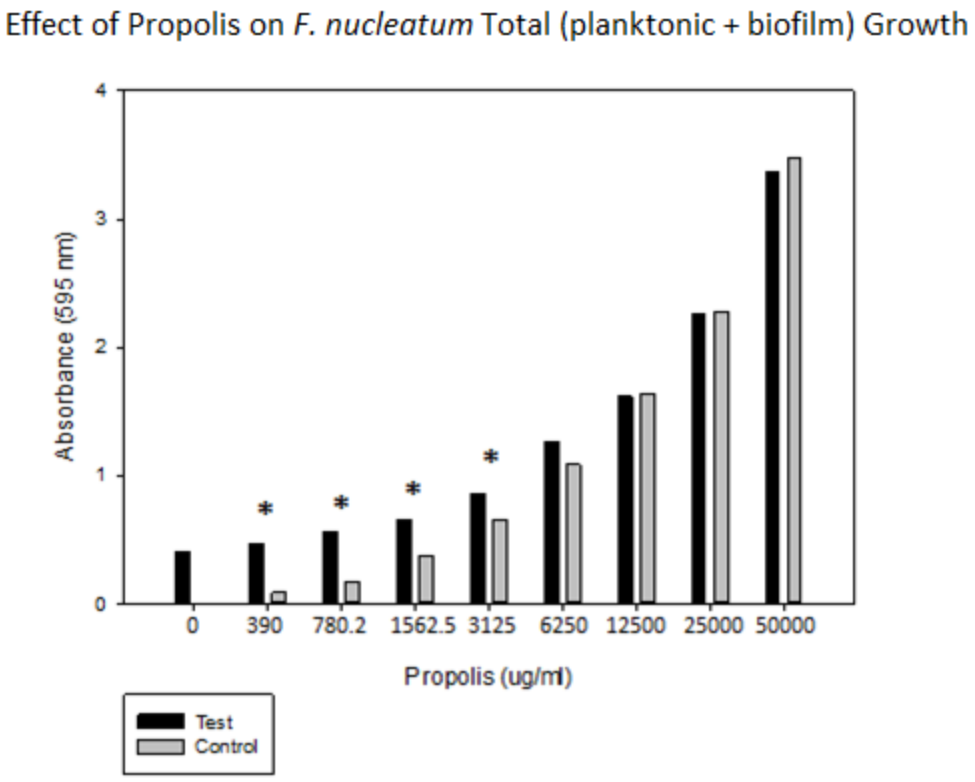

FIGURE 1. Average absorbance of total (planktonic + biofilm) test (propolis with *F. nucleatum*) and control (propolis with no *F. nucleatum*) wells. These values were used to compare the differences in absorbance. Note the wells with *F. nucleatum* show significant bacterial growth at 0, 390, 780.2, and 1562.5 and 3,125 µl. These values represent the mean of quadruplicate wells in each of three trials.

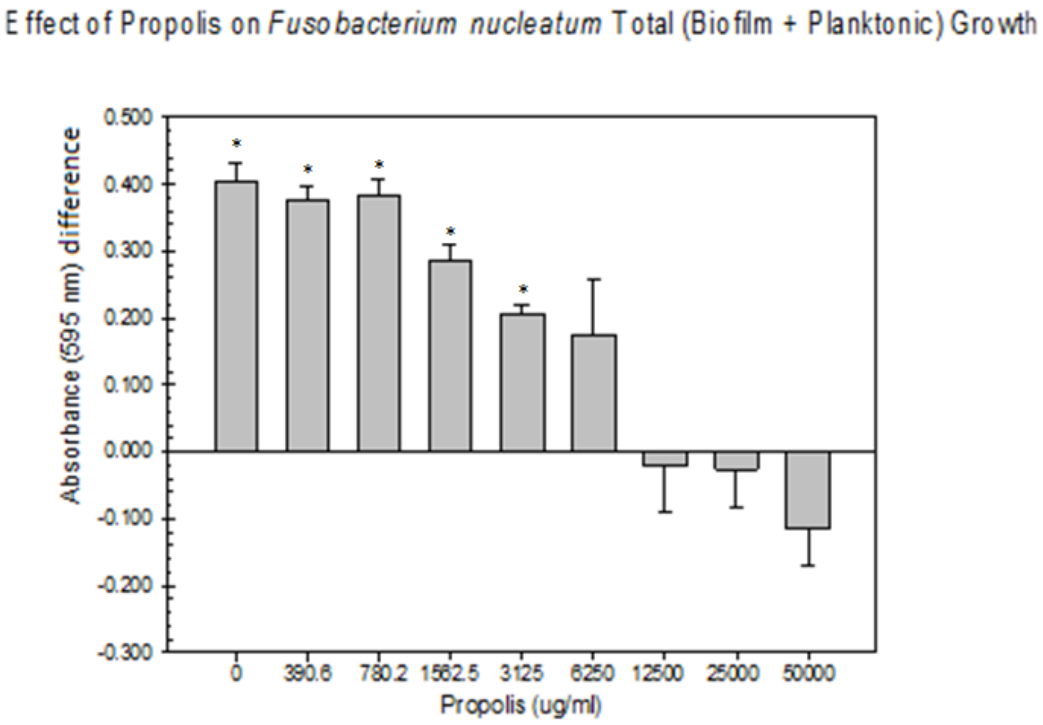

FIGURE 2. Effect of propolis on Fusobacterium nucleatum total (biofilm + planktonic) growth. Absorbance is shown as the differencbetween absorbance of the test wells with *F. nucleatum* and that of the negative control wells with propolis. The MIC was determined to be 6250 µg/ml as concentrations below this showed significant bacterial growth. The * symbol indicates significant difference in absorbance (significant bacterial growth).

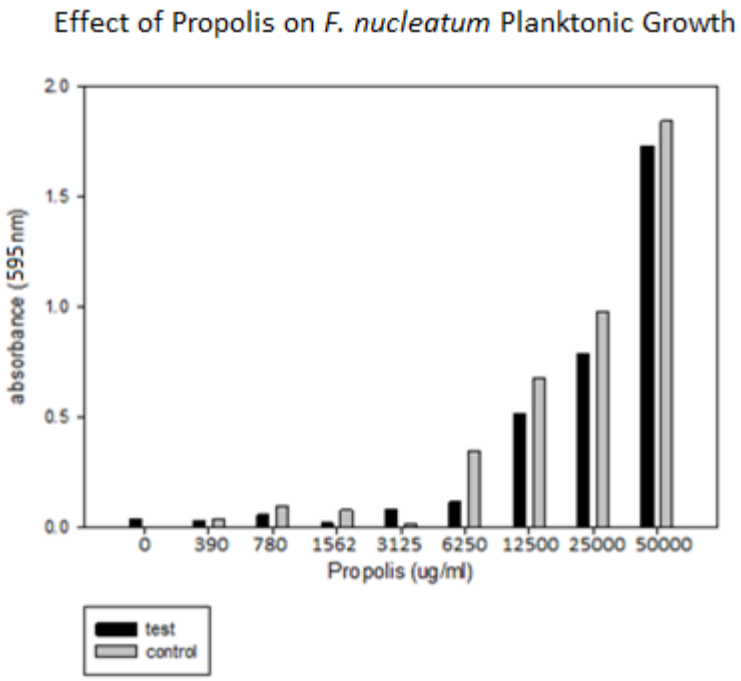

FIGURE 3. Average absorbance of planktonic test and control wells. Note the absorbance of the test wells with *F. nucleatum* in the planktonic form show less absorbance than the control wells.This was attributed to the variation of 10 µl of bacteria present in the test wells versus an additional 10 µl of the diluted propolis solution in the control wells (propolis contributes greater absorbance). There were no significant differences in absorbance between test and control wells. These values represent the mean of quadruplicate wells in each of three trials.

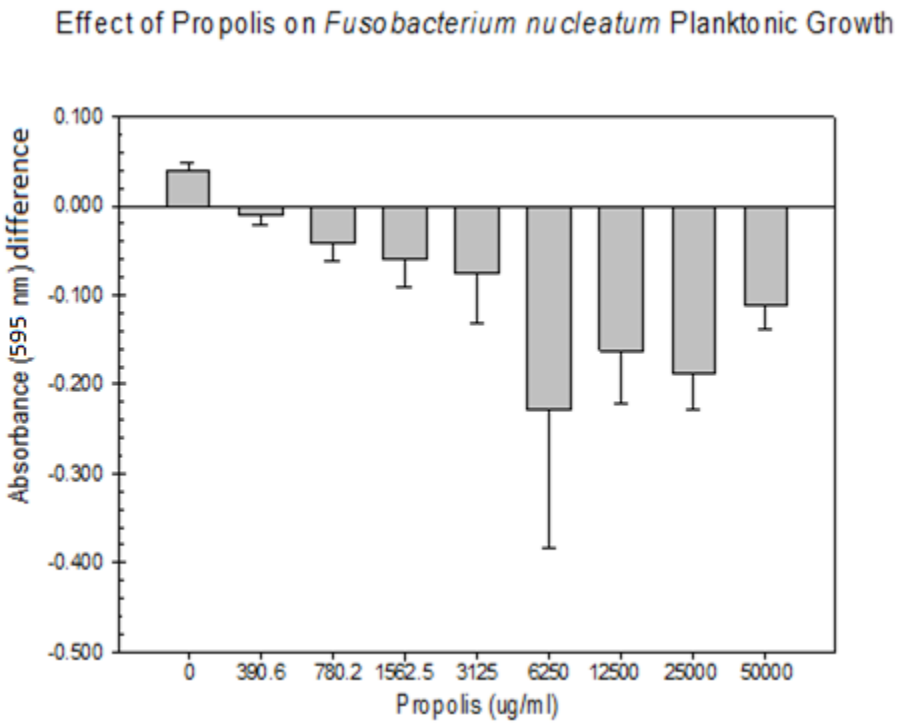

FIGURE 4. Effect of propolis on Fusobacterium nucleatum planktonic growth. Absorbance is shown as the difference between absorbance of the test wells with *F. nucleatum* and that of the individualized negative control wells with propolis. No MIC was found for the planktonic component as no test wells showed significantly greater absorbance when compared to their individualized control wells. The absorbance difference appears as negative values presumably due to the greater absorbance of the additional 10 µl of propolis in the control wells versus the 10 µl of bacteria in the test wells.

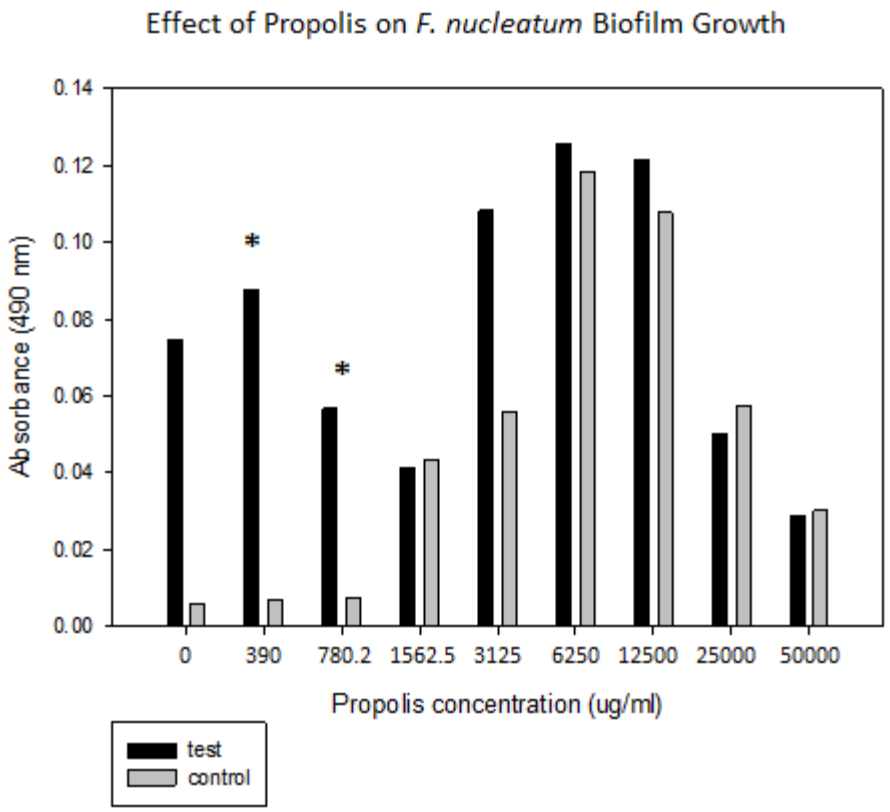

FIGURE 5. Average absorbance of total (planktonic + biofilm) test and control wells. Note the wells with *F. nucleatum* showed significant bacterial growth at 0, 390 and 780.2. The higher absorbance at 3125 µg/ml was due to an outlier at this concentration that was included for comprehensive statistical analysis. These values represent the mean of quadruplicate wells in each of three trials.

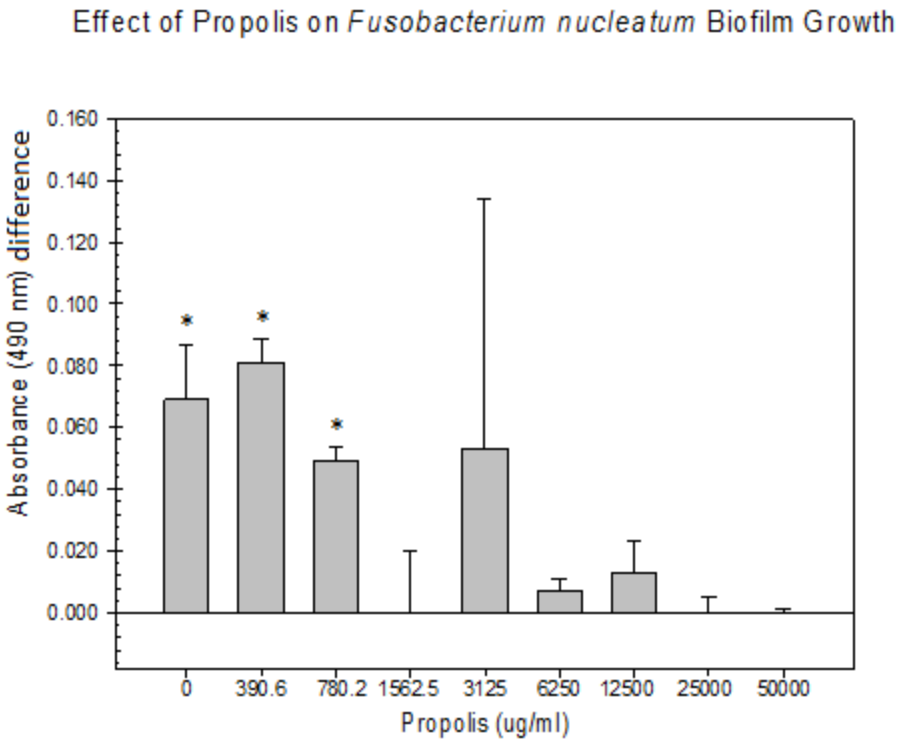

FIGURE 6. Effect of propolis on *F. nucleatum* biofilm growth. Absorbance difference is the difference between absorbance of the test wells with *F. nucleatum* and that of the individual negative control wells with propolis. The MBIC was determined to be 1562.5 µg/ml as concentrations below this showed significant bacterial growth. The * symbol indicates no significant difference in absorbance (no significant bacterial growth).

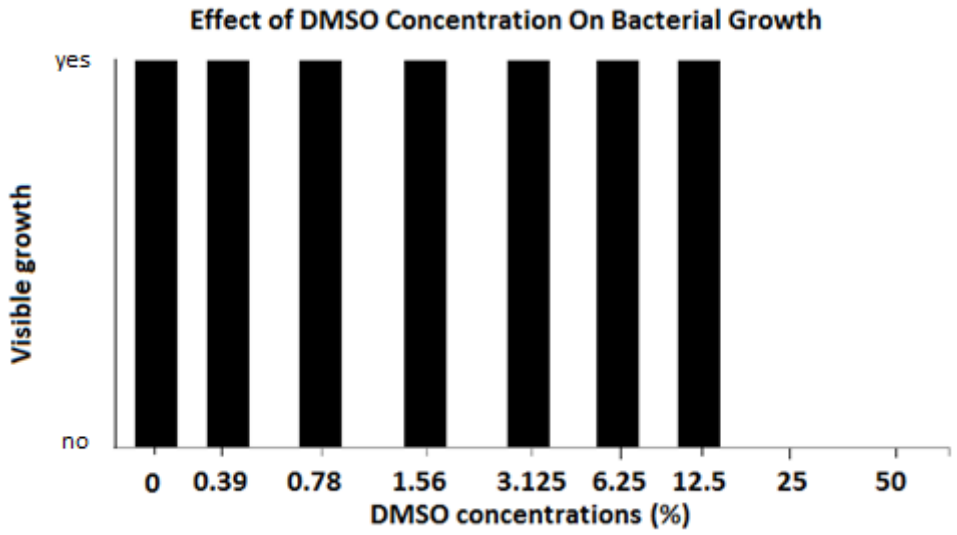

FIGURE 7. Lack of effect of DMSO on bacterial growth. All or nothing visible turbidity was examined. Turbidity (bacterial growth) was visible up until 25% DMSO. This DMSO concentration coincided in our study with the 25,000 μg/ml of propolis solution. The occurrence of the propolis MIC and MBIC at concentrations of DMSO that did not show bacterial inhibition exemplify the antimicrobial effect of propolis.

FIGURE 8. Commercial propolis from Ecuadorian Rainforest L.L.C.
Note: 500-g bag this study utilized <2 g of propolis (0.25g/25ml for 10,000 μg/ml solution and 1.0g/10 ml for 100,000 μg/ml solution).

FIGURE 9. All pipette work performed adjacent to Bunsen burner on bench top to minimize bacterial contamination; also used to flame cervical area of containers prior to removing solutions with pipette.

FIGURE 10. Pipettes – 200 µl and 1000 µl.

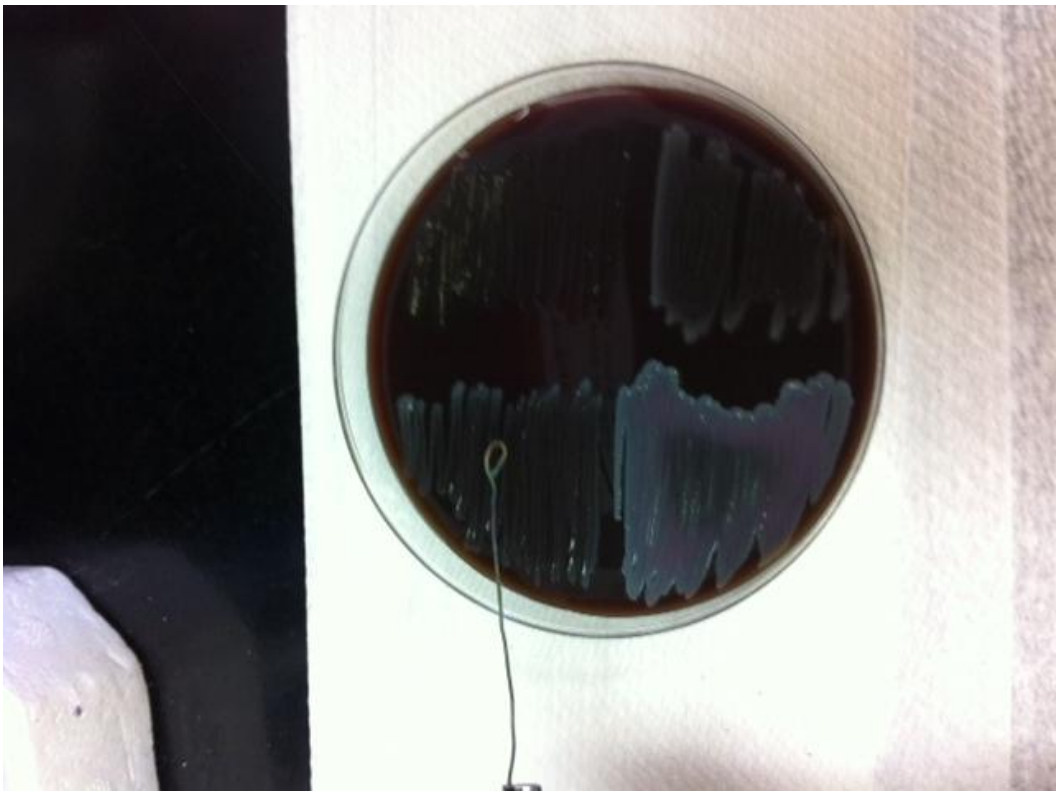

FIGURE 11. *F. nucleatum* plate and loop.

FIGURE 12. Gaspak Jar with *F. nucleatum* growing in sterile tube between day one and day two.

FIGURE 13. *F. nucleatum* turbid tube shows growth after 24-h incubation at 37^{o}C in BHI/Vit. K/hemin media.Ten µl from this tube was transferred to each test well and the positive control wells.

FIGURE 14. Day two: propolis dilution tubes – dilutions from 50,000 to 390.625 µg/ml.

FIGURE 15. Incubator – 37^{o}C with Gaspak jars inside.

FIGURE 16. Gaspak jar with propolis and *F. nucleatum* in 96-well microplate incubating between day two and experimental day three (48 hours later).

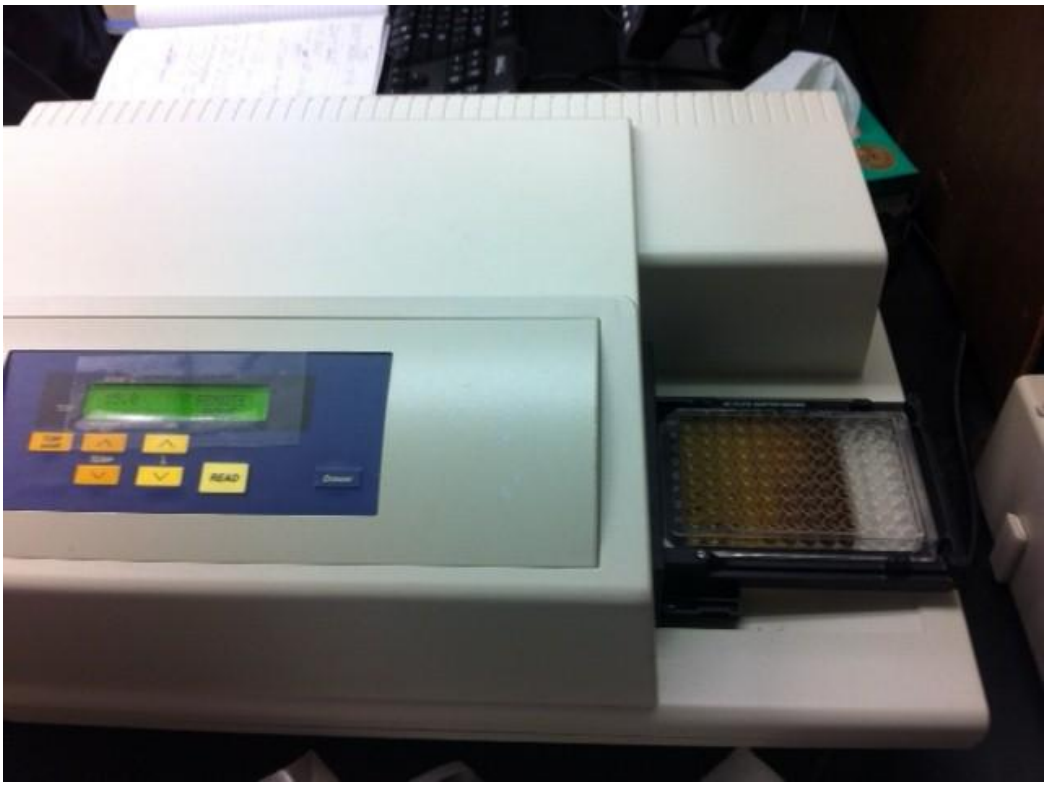

FIGURE 17. Spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA).

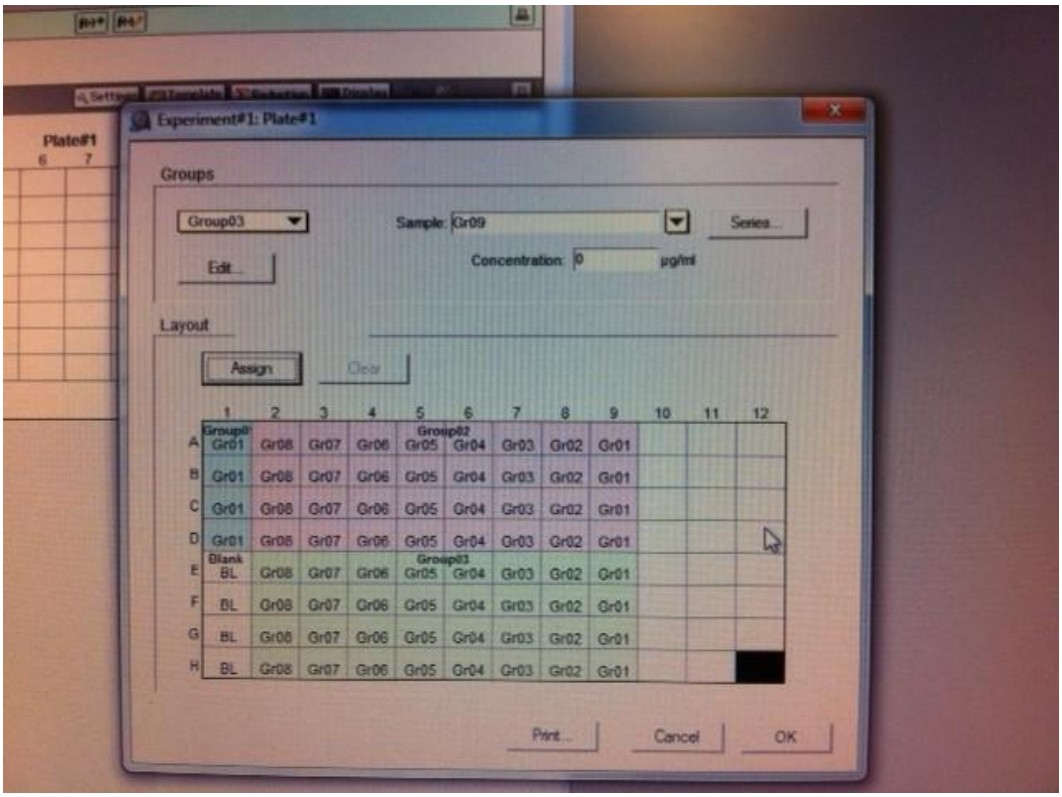

FIGURE 18. Template setup to match the 96-well plate solutions.

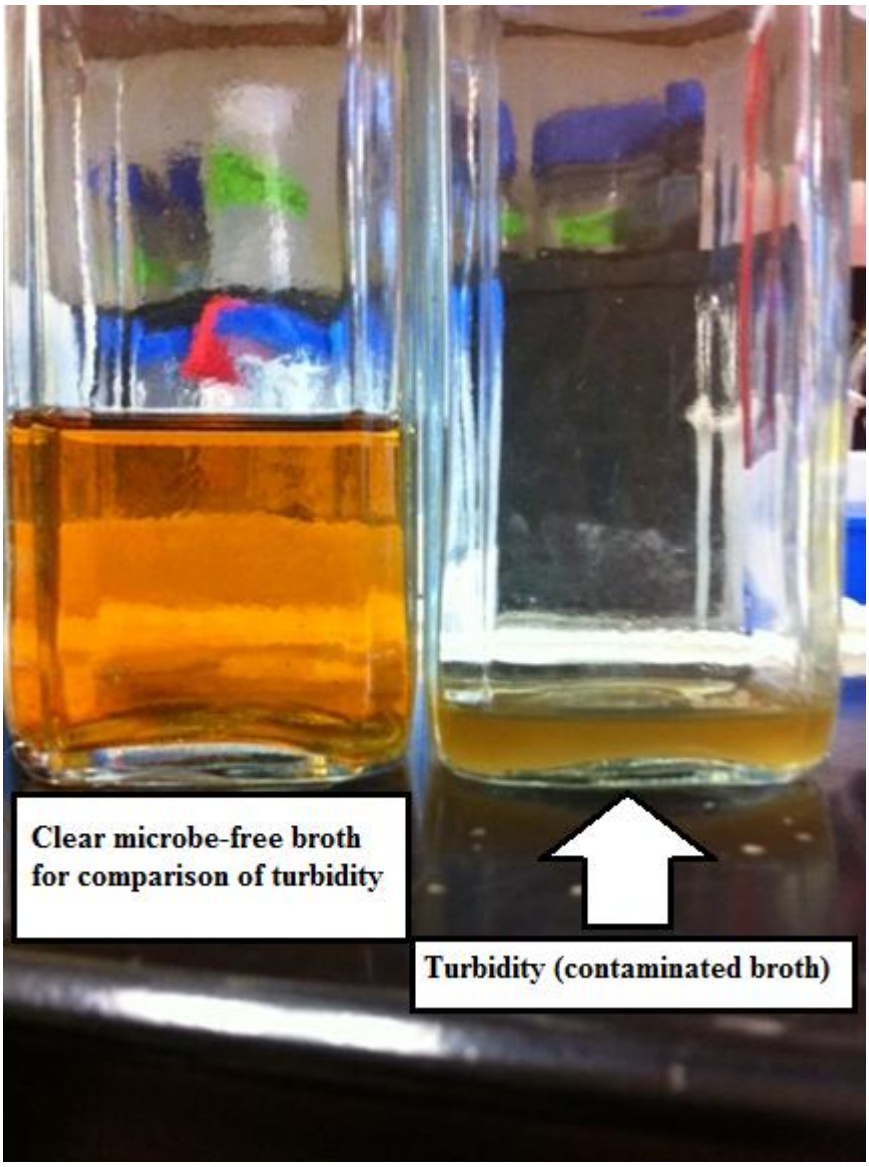

FIGURE 19. The bottle on the right shows contaminated BHI/Vit.K/hemin from experiment four (turbidity in the bottle on the right compared to the bottle on the left with no turbidity).

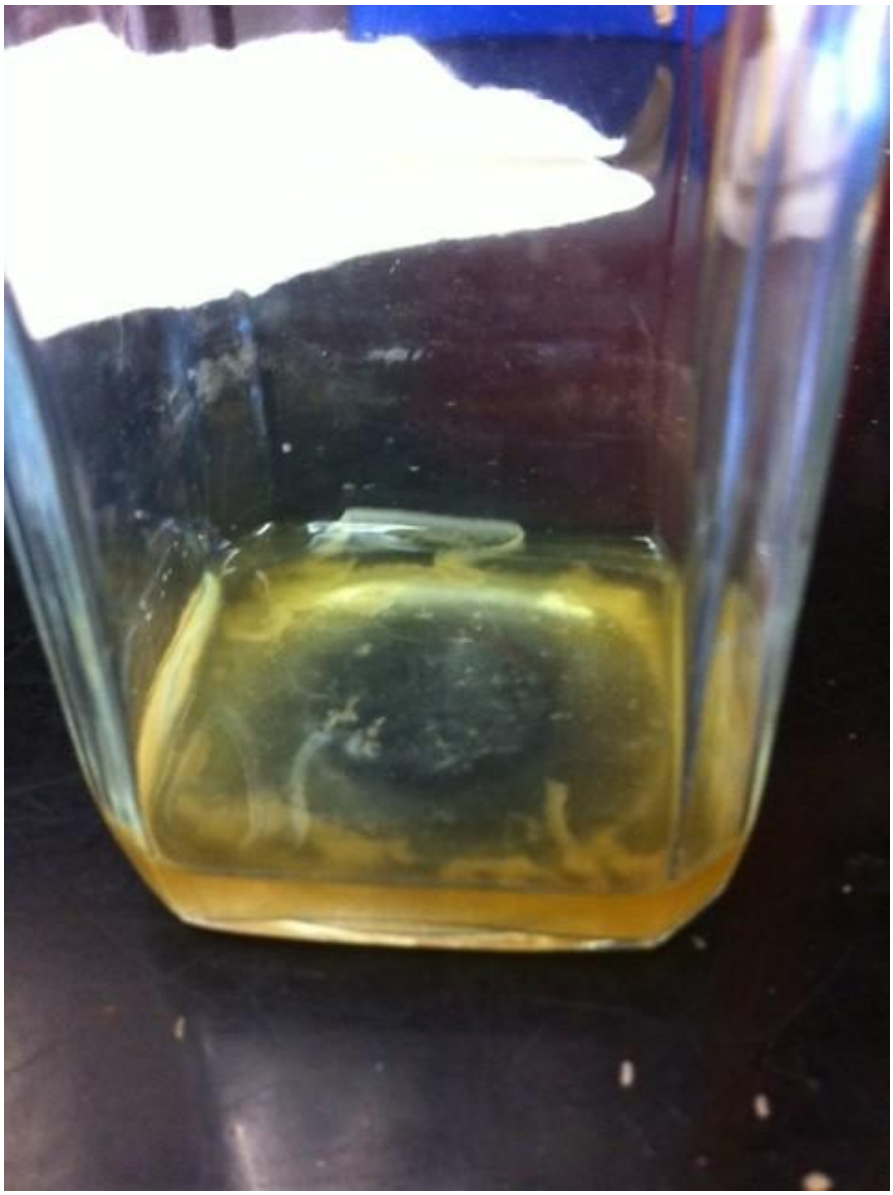

FIGURE 20. Closer examination of the turbidity shows bacterial colonization in contaminated BHI/Vit.K/hemin from experiment four.

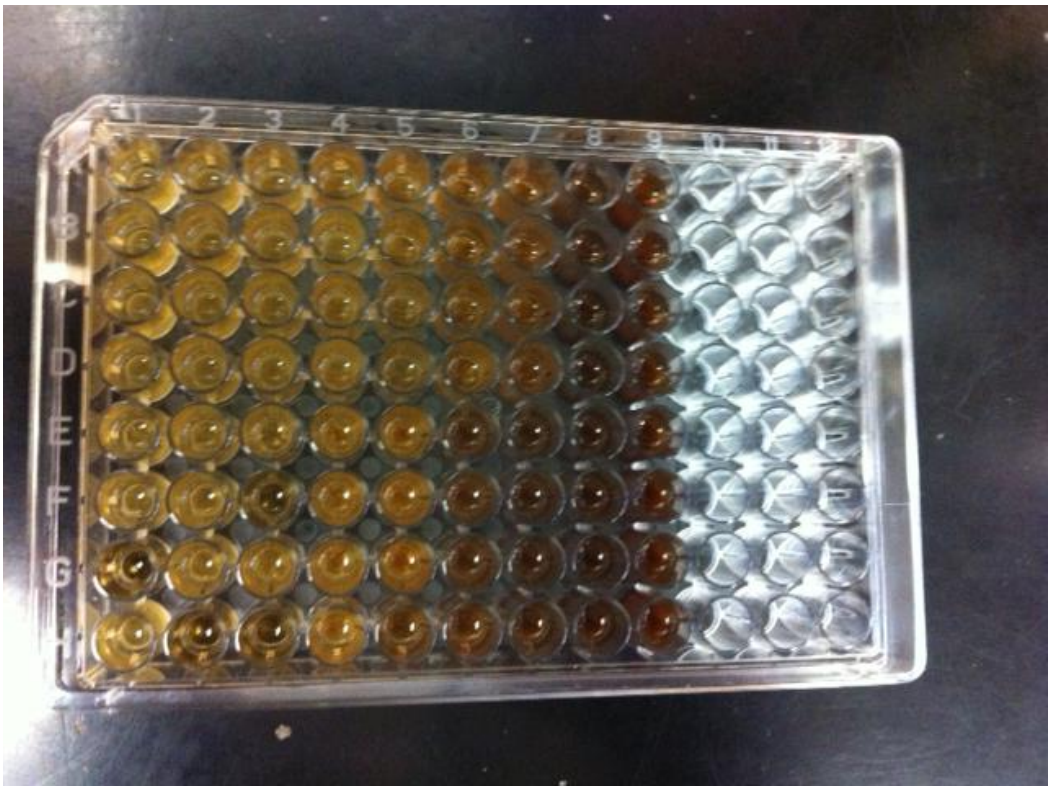

FIGURE 21. Contaminated experiment four 96-well plate. Almost all wells show turbidity representing contamination (including most of the negative controls containing propolis and the negative control of BHI-YE/vitK/hemin broth alone – exemplifying contamination of the broth).

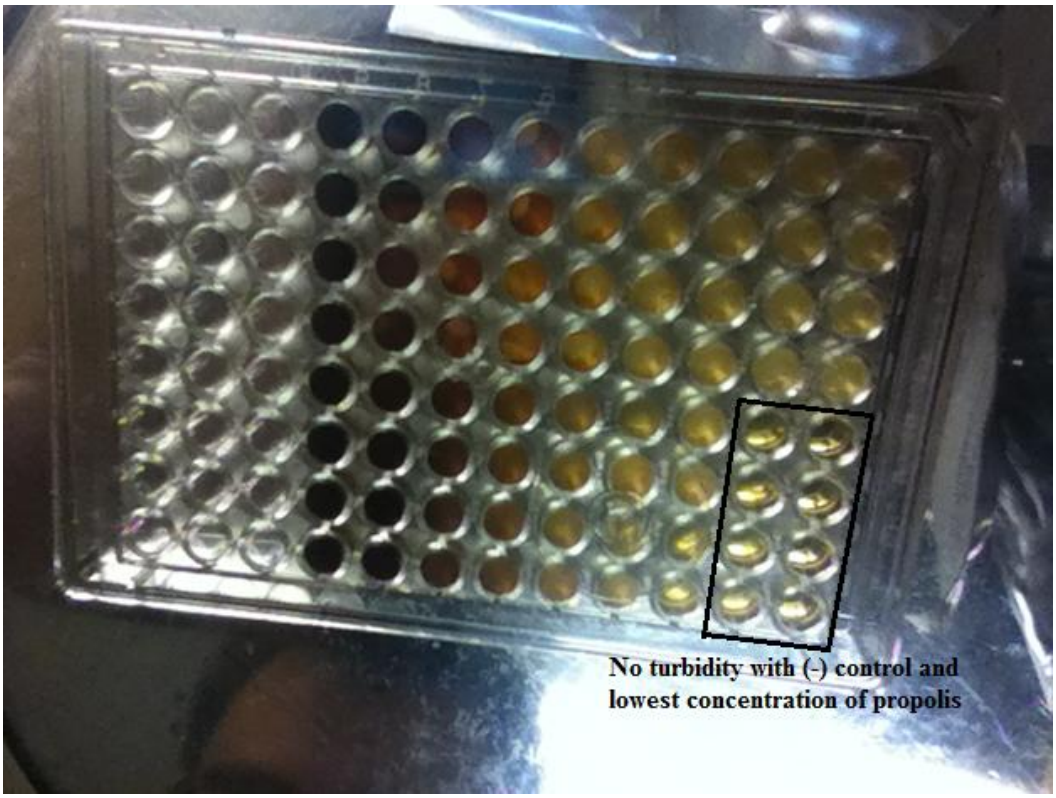

FIGURE 22. Contaminated experiment five – the negative control wells and lowest concentration of propolis wells show no turbidity – confirming no contamination of the broth and exemplifying contamination of the propolis stock solution.

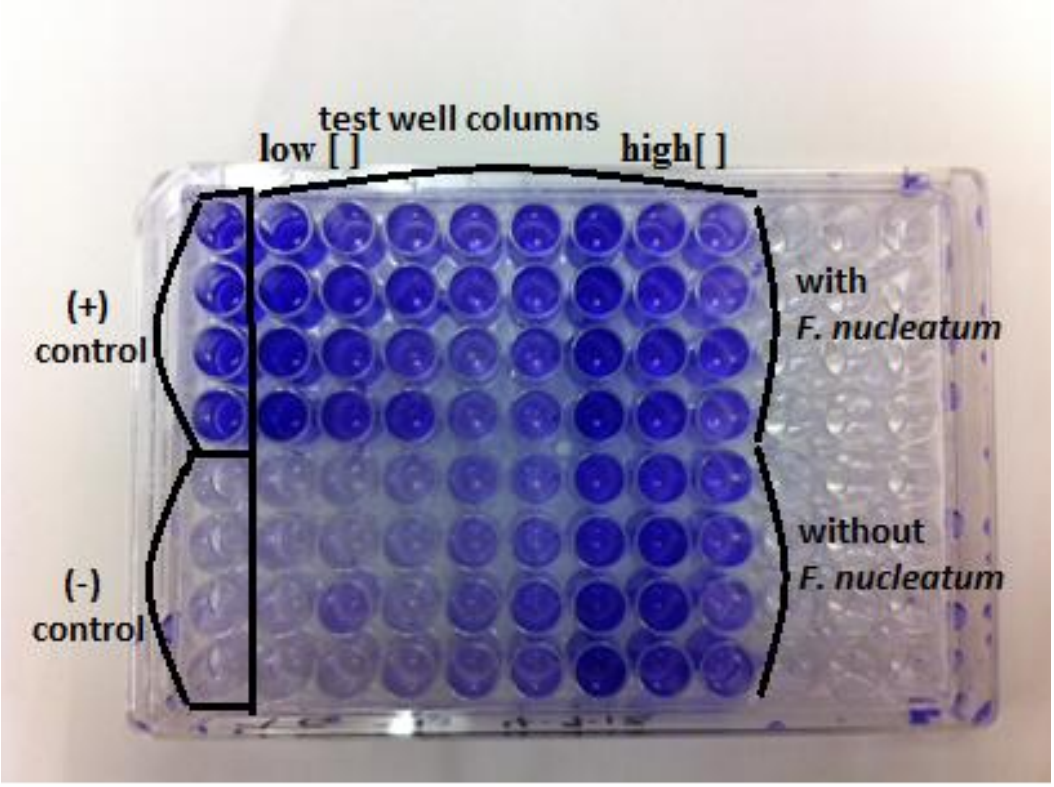

FIGURE 23. Experiment nine – successful controls and bacterial growth as expected (A1-D1 positive control; E1-H1 negative control; A-D:2-9 test wells; E-H:2-9 control wells). Propolis concentrations were as follows: column 2: 390.625μg/ml; column 3: 781.25 μg/ml; column 4: 1562.5 μg/ml; column 5: 3125 μg/ml; column 6:6250 μg/ml; column 7: 12,500 μg/ml; column 8: 25,000 μg/ml; column 9: 50,000 μg/ml. Note in the test wells the similar density of staining in the lowest concentrations of propolis comparable to the positive control well (indicative of too low of a concentration to be inhibitory). As concentration reaches 1562.5 μg/ml turbidity decreases and becomes apparently consistent with extent of turbidity in relative negative control wells. The decrease in density due to bacterial biofilm inhibition is countered by an increase in density due to the density of the propolis (hence the need for spectrophotometer readings to compare each test group to a propolis concentration relative negative control group). Note in the control and test wells as concentration of propolis increased up to 6250 to 12,500 μg/ml the density of propolis staining in the wells increased and had an interesting (and consistent in all three trials) decrease in staining at 25,000 μg/ml and 50,000 μg/ml.

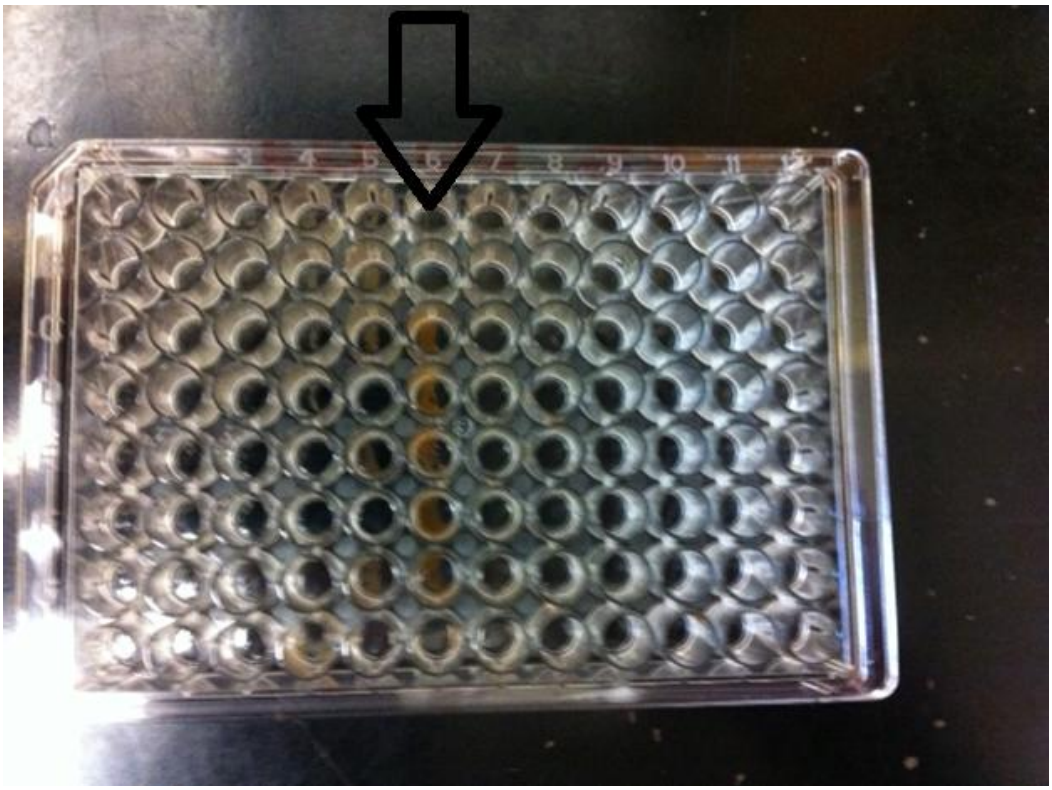

FIGURE 24. Note the visible precipitation of propolis in column 6 (6250 µg/ml; arrow) representative of the previously mentioned signs of the peak absorbance of propolis at 6250 to 12,500 µg/ml.

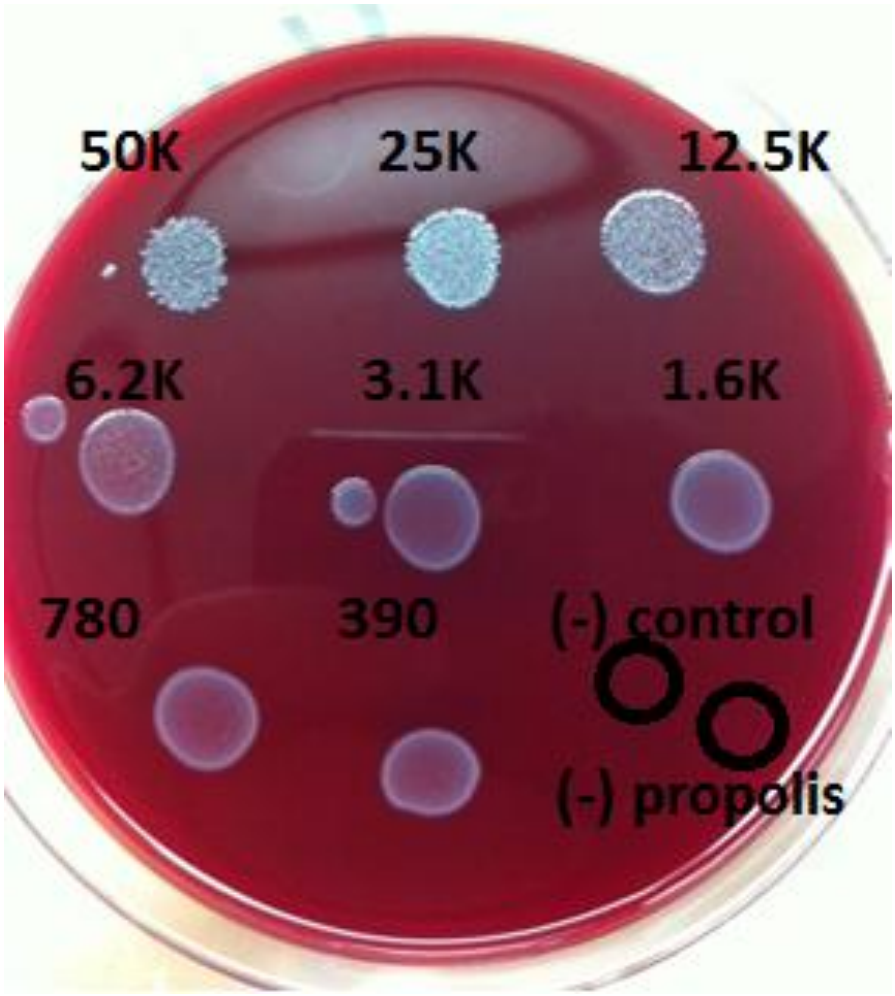

FIGURE 25. MBC blood agar plate shows growth of *F. nucleatum* at all tested concentrations (although appears to have a decreased ability to flourish at higher propolis concentrations) and confirmed no growth in the negative control of broth and negative control with propolis.

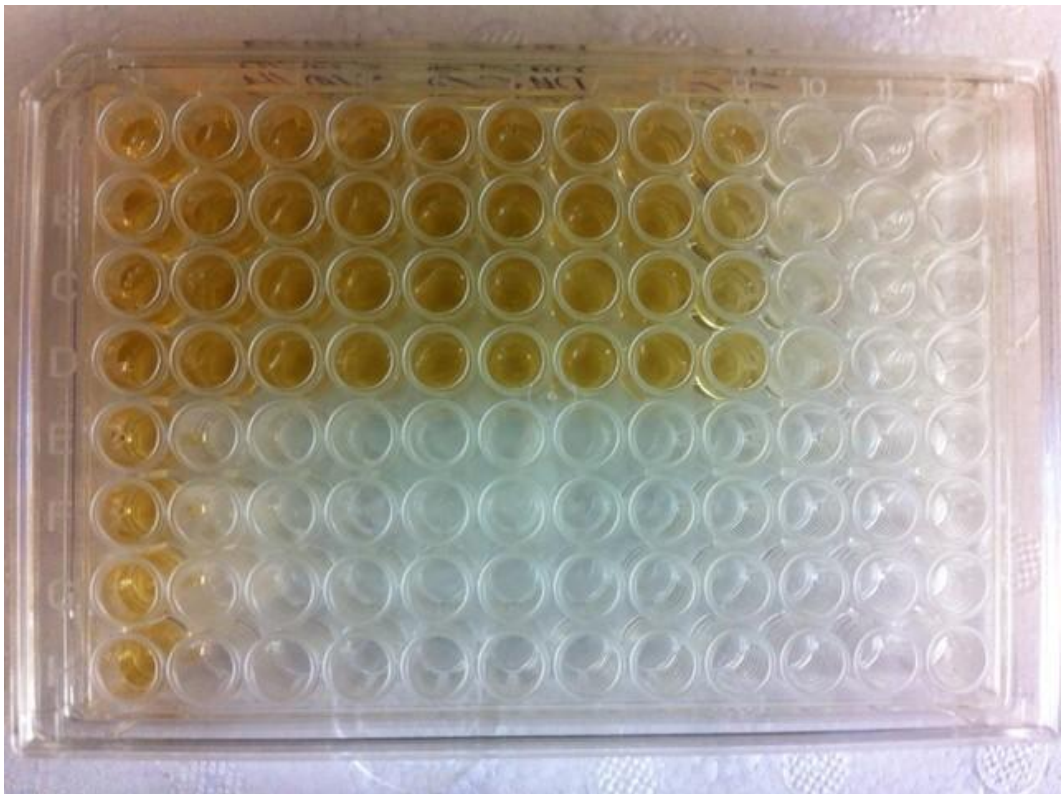

FIGURE 26. In order to verify the DMSO was not functioning as the antimicrobial in the wells, a control test of all concentrations of DMSO used was performed. Propolis was not included in this experiment. The DMSO concentrations by volume in each well were diluted with BHI:YE/vitamin K/hemin to achieve: 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% and 0.78%. Above is the view of the plate prior to incubation.

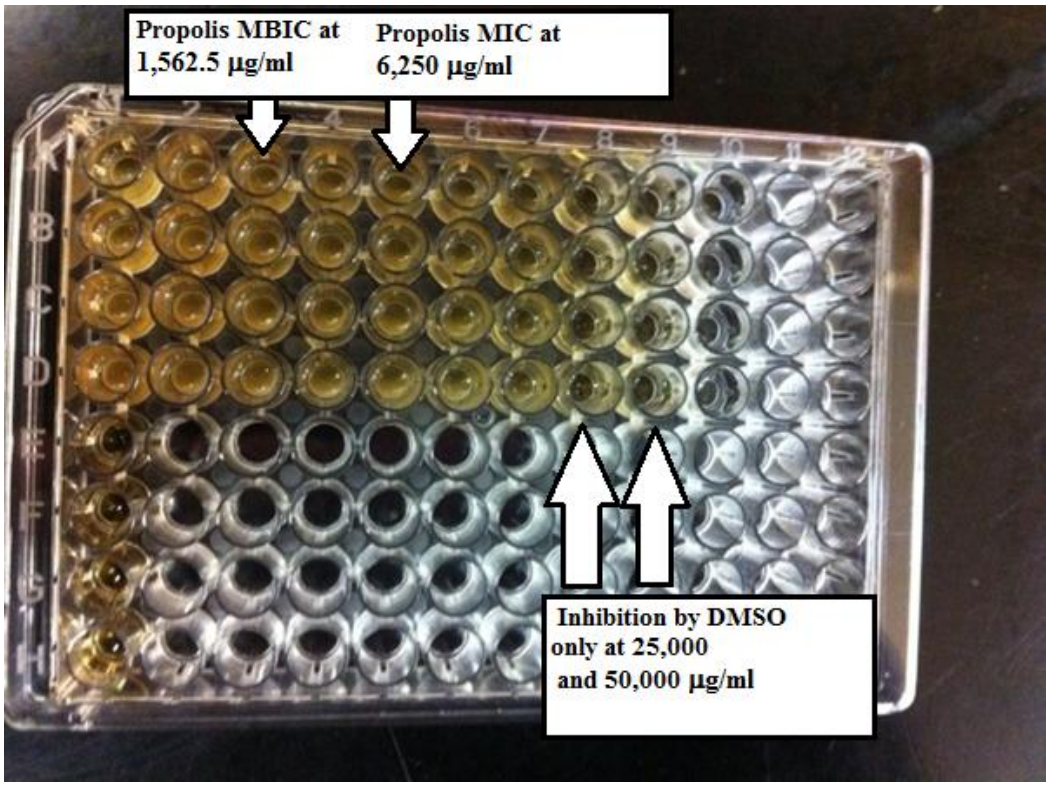

FIGURE 27. Above is a photo of the turbidity seen in the evaluation of the antimicrobial efficacy of DMSO. Propolis was not included in this experiment. Consistent visual turbidity indicative of bacterial growth was seen in quadruplicate of all test concentrations except at the highest two concentrations of 25-percent and 50-percent DMSO. The propolis MBIC and MIC concentrations are noted as a reference for comparison.

DISCUSSION

The first five experiments as well as experiment number eight could be considered a series of pilot studies and provide adjunctive information regarding a target concentration range of propolis/DMSO:BHI-YE, incubation time evaluation as well as determine the need to filter the propolis stock solution. It is noteworthy that during the initial experimentation without contamination of the initial stock solution, the MIC value appeared within a close estimated range to the final determined MIC concentration. This was interesting as it provides additional information of an estimated concentration at which propolis appears to be antimicrobial under these experimental conditions.

Using the information provided by the DMSO control study, the pilot study, and the triplicate experimental studies, it was confirmed that the propolis appears to be acting as an antimicrobial agent. If DMSO were acting as the antimicrobial, there would have been more inhibition of microbial growth in the pilot study at coinciding concentrations of DMSO to the final studies. Additionally, experiment eight which had an extended 96 hour incubation period provided similar data regarding the MIC/MBIC values coinciding the the propolis concentrations. Ultimately, the DMSO control study provided evidence that the DMSO did not have an antimicrobial effect at the concentrations at which the propolis inhibition of bacteria occurred.

In the DMSO control study, DMSO appeared to have an antimicrobial effect only at concentrations of 25-percent and 50-percent DMSO (see Figure 20 and 21), which in the triplicate experimental studies coincided with the 50,000 μg/ml and 25,000 μg/ml of propolis. Since the MIC and MBIC were determined to be at concentrations of 6250

μg/ml and 1562.5 μg/ml respectively, there is confirmed antimicrobial activity occurring prior to the DMSO has a profound effect. One could question whether DMSO may have a synergistic effect to increase the ability of the propolis to be a better antimicrobial. This is a valid question as Douwes et al. noted that DMSO may enhance the effect of other drugs.[152] Previous studies have used DMSO as a solvent for antimicrobial testing. Cuenca-Estrella used DMSO with stock solutions of antimicrobials starting at 100 times the concentration of that being evaluated within microtiter wells. They described that susceptibility testing standards recommend a series of dilutions from a stock solution to avoid dilution artifacts from precipitation of the test compound.[153] This description exemplifies that other researchers have also found precipitation of medicaments within microtiter wells as we noted at certain concentrations within our experimental results.

One of the interesting findings consistent in the three trials used (experiment six, seven, and nine), as well as seen in the additional unused trial (experiment eight) was the amount of precipitate noted at each diluted concentration of propolis (see Figure 18). It appeared consistent that the greatest amount of precipitation of propolis in the microtiter plate wells occurred at a concentration of 6250 to 12,500 μg/ml. Only speculation can be provided at this point why at the concentrations of 25,000 and 50,000 μg/ml of propolis adhered (formed precipitate along the well walls) less to the microtiter plate wells. One hypothesis could be that once the propolis concentration reaches a certain upper threshold, the viscosity of the solution prevents precipitate formation along the well walls. This may be due to an effect with the DMSO, as the highest two concentrations examined appear to show the most evident decrease in biofilm or propolis adherence to the wells. It appears that biofilm adheres to the wells best prior to the MIC/MBIC of

propolis, followed by propolis precipitation onto the wells past the MIC/MBIC, followed by less adherence of either at the highest concentrations of propolis/DMSO. There is potential that at the highest concentration of solvent, the propolis remains soluble and does not precipitate as easily.

Further description of this hypothesis is as follows: Propolis may be inhibiting aggregation of bacterial cells by inhibition of their outer membrane proteins or through quorum sensing, which may additionally be involved in adherence to other cells as well as the plate wells. At low concentrations, the propolis does not block enough outer membrane proteins or reduce quorum-sensing activity to disrupt coaggregation and biofilm adherence to the wells and there is not a high enough concentration of propolis particulate to leave a residue along the surface area of the wells; therefore, biofilm forms along the wells and not propolis particulate. At moderate concentrations, propolis inhibits the interactions of outer membrane proteins or inhibits quorum-sensing, thus it disrupts biofilm formation and bacteria adherence to the wells leaving more surface area of the wells for propolis particulate to precipitate onto. Therefore, there is less/no biofilm adhering to the surface area of the well walls and there is high enough concentration (with less concentrated solvent) of the propolis to leave particulate matter to precipitate onto the surface area of the wells. As the propolis concentration rises to higher concentrations, the propolis continues to inhibit biofilm adherence to the wells and there is a high enough concentrate of DMSO to keep the propolis soluble and remain suspended in the solution rather than precipitating onto the walls of the well.

Regarding previous publications of inhibitory concentrations of propolis, comparable methods to ours have been used. The use of the 96-well microtiter plate

method has been demonstrated in previous studies such as when Dziedzic et al. performed their MIC/MBC test of propolis on mutans streptococci and lactobacilli in 96-well microtiter plates using spectroscopic analysis at 600 nm.[112] Moncla et al. in 2012 found an MIC of 1600 μg/ml when tested against Enterococcus species. Using an agar dilution method, they tested human and animal isolates of Enterococci and found that human isolates of *E. faecium* and *E. faecalis* from refractory endodontic treatment cases were particularly susceptible to Brazilian propolis.[14] This MIC is a close approximation to our MIC value determined for *F. nucleatum* (6,250 μg/ml). Using a macrodilution method with an ethanol extract of propolis, Uzel et al. found the MIC of propolis of varying bacteria (staphylococcus, streptococcus, enterococcus, pseudomonas, salmonella) to be in a range of 2 to 32 μg/ml.[109] Using an agar dilution method, Koru et al. found that propolis samples were more effective against Gram positive anaerobic bacteria than Gram negatives when using an ethanol extract of propolis. They found MIC ranges from 4 to 512 μg/ml and MBC ranges from 8 to 512 μg/ml.[143] Dziedzic et al. as mentioned earlier, used a broth diffusion method and AlamarBlue assay and found the MIC for mutans streptococci to be 1100 mg/ml and the MBC to be 9010 mg/ml as well as the MIC for lactobacilli to be 700 mg/ml and the MBC to be 5910 mg/ml.[112] Arslan et al. used an ethanol extract of propolis in a macrobroth dilution method and found MIC and MBC antibacterial activity against *E. faecalis* and *C. albicans* in a concentration range from 2 to 2400 mg/ml.[15] Ghasem et al. found MIC values in weight per volume ratios for different propolis samples against *S. aureus* and *C. albicans* using an ethanol extract of propolis and a Petri dish bioassay method.[16] These studies and our own results reproduce data that consistently reveal that propolis may have a reasonably predictable MIC when

tested among a variety of microorganisms. Although the literature reveals that propolis has a reasonably predictable MIC range when tested among a variety of methods (agar dilution versus microtiter plate wells), using varying solvents (ethanol extract versus DMSO), and among a wide range of bacteria, the results from one method cannot be directly correlated or compared to that of another. Additionally, serial dilution methods have shown to provide more consistent results and microdilution methods have been utilized in a variety of studies to evaluate the antimicrobial effectiveness of propolis.[140]

Interestingly, several studies presented a MBC value for propolis including one for *F. nucleatum*.[143] In our study, we were unable to produce a MBC even when no suspicion of contamination was confirmed with negative control tests (see Figure 19). This is noteworthy because it does not agree with previous literature evaluating the antimicrobial properties of propolis. Several hypotheses will be presented of potential reasons why this finding may have occurred.

One hypothesis is that many other previous studies used an ethanol extract of propolis and this extraction process may free up more of the active antimicrobial components of propolis such as the flavonoids and flavonones. Macedo et al. stated, "Several herbal, animal and microbial extracts possess quorum-quenching activity but few active compounds and synthetic analogues are known."[137] It is possible that through the extraction process the compound availability is altered to enhance the antimicrobial effect. Similarly, the use of a commercially available formulation of propolis may exemplify variable antimicrobial properties.

Although there was no MBC, there appears to be a decreased ability of *F. nucleatum* to flourish at higher propolis concentrations. This was visually evaluated by

the less abundant growth seen on blood agar plates used to determine MBC at higher propolis concentrations. The discrepancy between previous studies and the current study may be due to the materials or methods involved in MBC examination. Several studies have described MBC as the ability to kill 50 percent of the bacteria. This may have allowed them to consider an MBC at a value that we did not consider as an MBC because our study was considering an MBC to be 100-percent MBC.

Another hypothesis to why there has been shown to be an MBC in previous studies and not in ours is that not all strains of *F. nucleatum* may respond the same to medicaments. It has been noted that "important concentrations are necessary to inhibit growth of some *F. nucleatum* strains. In the study by Joly et al., concentrations of 250 μg/ml are not sufficient to inhibit growth of some strains, but other strains are very sensitive."[123,154] Thus the use of strain ATCC 10953 in this study may provide variable results compared to other previous studies.

Another hypothesis to why propolis shows an MIC and MBIC with no MBC may be that propolis inhibits adherence to the microtiter plate well walls. This has been a proposed alternative method to fight biofilm infections; rather than destroy the microbes with antimicrobials, generate a functional surface to prevent the attachment of bacteria.[135]

Another hypothesis to why propolis shows an MIC and MBIC with no MBC is that propolis inhibits biofilm development or growth and inhibits the interaction or further replication of the bacteria. It may do so by interference with the outer membrane proteins or by interfering with quorum sensing. As previously described, propolis has shown anti-quorum sensing activity. Bulman et al. found propolis to contain compounds that suppress the quorum-sensing response. Our study may be exemplifying this anti-

quorum sensing effect.[107] By interfering with outer membrane proteins, such as those responsible for either producing the quorum-sensing molecule or the receptor that binds it, it may be reducing the aggregation of the bacteria and attach to the well walls or to each other. By interfering with quorum sensing, it may be disallowing bacteria to aggregate in a structural and functional manner that is necessary for them to thrive as a biofilm. Previous literature has stated that "several herbal, animal and microbial extracts possess quorum-quenching activity."[137] This could be one of the mechanisms by which the antimicrobial extracts of propolis function. Therefore, propolis may exhibit inhibitory effects within the experimental design, yet it may not be bactericidal under these experimental conditions.

One of the most interesting findings of this study was that the results indicate there is potentially significant interaction of propolis with biofilm. This was displayed by three main points within the study. First, the lower concentration needed to exhibit inhibitory effects on biofilm formation (1562.5 μg/ml) in comparison to the MIC of the total planktonic + biofilm (6250 μg/ml). This allows speculation that within the total MIC and the MBIC evaluation it appears that the biofilm inhibition is more likely what is being inhibited. Second, this indication is further exemplified by the lack of microbial inhibition seen in the planktonic evaluation. If propolis was acting on individual cells or cells in the planktonic form, one would expect to see a MIC in the planktonic evaluation which did not occur. Third, there was no MBC detected. If propolis inhibits biofilm formation or cell aggregation, it may not be cytotoxic to cells plated on growth media, which allows them to replicate. This may be evidence that the biofilm inhibitory effect is most profound on diminishing the ability for attachment to the well wall surface.

For the evaluation of biofilm under *in-vitro* experimental conditions, a variety of methods exist such as the microtiter plate method, tube test, radiolabeling, microscopy, and the Congo red agar plate test. The microtiter plate method is one of the most frequently used assays.[147] The quantification of microbial biofilm grown in 96-well microtiter plates has been described as an approach to studying biofilm and has been performed in previous studies.[138,141,147,149,150,155] It has been a published method of *F. nucleatum* biofilm evaluation in the oral microbiology literature.[144] It is understood that biofilm may adhere to non-biologic surfaces (such as the polystyrene of the microtiter plate) as one author defined biofilms as "matrix-enclosed microbial accretions (aggregations) that adhere to biological or non-biological surfaces."[135,136] The microtiter plate method was selected due to its ability to serve as a "rapid screening method sensitive enough to elucidate concentration-response relationships as well as differences between species responses to treatments."[156] Regarding the complexity of *F. nucleatum* biofilm growth *in vitro,* in one study biofilm forming ability was evaluated in 20 strains of bacteria common to apical periodontitis and *F. nucleatum* exhibited the strongest adherence to type-I collagen-coated polystyrene microplates[144] in methods that are similar to ours. Additionally, in a study published in the *Journal of Endodontics* testing a medicament's antibacterial effect on *F. nucleatum* biofilm, the authors used 24-well polystyrene TC plates. The authors of this study confirmed the attachment of bacteria on polystyrene, by using an extra set of biofilms grown and checked by light absorbance readings in a microplate spectrophotometer at 540-nm wavelength after staining with crystal violet.[145] Bachrach et al. used *F. nucleatum* in a microtiter plate coaggregation assay and found it to be sensitive and reproducible using 96-well microtiter plates.[146] In

our study we made no additional efforts to confirm the true attachment of bacteria or biofilm to the microtiter plates. The length of time it takes for biofilm formation to occur is for discussion. Previous studies have described inoculation of wells for a period of 2 days,[144] 16 hours,[146] or testing performed at time periods of 24 and 96 hours for biofilm,[145] or observed death of *F. nucleatum*.[143] We performed our experimental conditions under similar circumstances as previously published studies; therefore, we made no effort to confirm there was true biofilm formation in the sense of its proper definition. The previously mentioned publications appear to support the use of the microtiter plate method for the evaluation of the efficacy of propolis on the inhibition of *F. nucleatum* biofilm under circumstances similar to our study.

An additional question that was raised was whether DMSO could have had a contributory antimicrobial effect. DMSO at certain concentrations is known to be antimicrobial.[157] Previous studies of propolis have used DMSO, one which stated that concentrations below 5.0-percent DMSO have no antimicrobial contribution.[140,141] The DMSO trial performed to examine each individual concentration of DMSO used in the study confirmed bacterial growth at concentrations that showed inhibition when propolis was added. This confirmed that DMSO had no significant antimicrobial effect. Additionally, several initial pilot studies confirmed that DMSO had no significant antimicrobial effect. It showed that at very low concentrations of propolis (which were shown by our triplicate studies to not be high enough to be antimicrobial), even with higher concentrations of DMSO, there was no antimicrobial effect being seen by DMSO. Interestingly, DMSO has been described to have the ability to potentiate the effects of other drugs.[152] It is possible that although the DMSO does not appear to have an

antimicrobial effect, it may enhance the antimicrobial activity of propolis. This may be evident by comparison with a future similar study that is planned to take place at the same institution (Indiana University).

In the fourth and fifth experiments it was confirmed that the stock solution of propolis was contaminated (see Figure 13 to 16). No culture examination was performed to determine which microorganisms were present as the contaminants. Turbiditiy was seen in the broth used initially during experiment four. Experiment five confirmed using new broth and control wells that the stock solution of propolis was the source of contamination. Filter sterilization of the stock solution was performed prior to further experimentation, and control wells with each experiment confirmed there was no further contamination.

Future studies could be performed to examine a clinically applicable media that could be used as a carrier for intracanal medication, such as propylene glycol. Additionally, using the commercial formulation of propolis, there may be benefit to using higher concentrations to enable a slurry mix of the propolis powder rather than attempting to dissolve it. Furthermore, the use of propolis in nature is as a sealant for beehives. Consideration of testing propolis in a form that may allow it to “set” as it does in nature to create an antimicrobial seal may be of more benefit in endodontics as a sealer than its use as an intracanal medication.

SUMMARY AND CONCLUSIONS

The results indicate that the MIC of the total (biofilm+planktonic) appears to occur at a concentration of 6250 μg/ml. The MBIC was established at 1562.5 μg/ml. The planktonic results exhibited no significant difference in test and control wells. There was no MBC at any of the test concentrations. Propolis appears to inhibit bacterial growth and biofilm formation but does not appear to be bactericidal at any of the tested concentrations.

The results of this study indicate that propolis has an MIC and MBIC when tested *in vitro* against *F. nucleatum*, although it does not show an MBC. There appears to be a potentially significant interaction of propolis with biofilm as displayed by the lower concentration needed to exhibit inhibitory effects on biofilm formation. This information may contribute to the ability to develop a proper concentration of propolis to use *in vivo* when treating endodontic infections.

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ABSTRACT

EFFICACY OF PROPOLIS AGAINST *FUSOBACTERIUM NUCLEATUM* BIOFILM

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The primary goal of root canal treatment is to eliminate microbes from the root canal system, which is the cause of pulpal and periapical infections. Research shows that after a single visit of chemomechanical debridement microbes continue to remain within the canal system. An interappointment medication step has been advocated to maximize potential elimination of microbes within the root canal system. Previous studies have shown propolis to be antibacterial against common endodontic microbes. Studies have shown trends in different microbes being present in primary verus secondary endodontic infections. The majority of literature has focused on the efficacy of propolis against *Enterococcus faecalis,* a microbe commonly implicated in secondary endodontic

infections. The aim of this study was to demonstrate the efficacy of propolis against *Fusobacterium nucleatum*, a microbe commonly found in primary endodontic infections.

This study aims to demonstrate the efficacy of propolis against a bacterium of primary endodontic infections (*F. nucleatum*) as well as against microbial biofilm to further support its potential use as a novel intracanal medicament. Dilutions of propolis were added to cultures of *F. nucleatum* in microtiter plates in a range from 390 μg/ml to 50,000 μg/ml. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and the minimum biofilm inhibitory concentration (MBIC) were determined. The MIC was determined of the total solution (biofilm+planktonic), planktonic, and biofilm (MBIC) after a 48-hour incubation period. The MBIC was determined by fixing biofilm to the wells and using crystal violet staining with spectrophotometry. The MBC was examined by plating solution from each concentration test well and reading the plates after 48 hours of incubation.

The results show that the MIC of the total (biofilm+planktonic) appears to occur at a concentration of 6250 μg/ml. The MBIC appears to occur at the concentration of 1562.5 μg/ml. The planktonic results exhibit no significant difference in test and control wells. There was no MBC at any of the test concentrations. The propolis appears to inhibit bacterial growth and biofilm formation but does not appear to be bactericidal at any of the tested concentrations.

The results of this study indicate that propolis has an MIC and MBIC when tested *in vitro* against *F. nucleatum*, although it does not show an MBC. There appears to be potentially significant interaction of propolis with biofilm as displayed by the lower concentration needed to exibit inhibitory effects on biofilm formation. This information

may contribute to the ability to develop a proper concentration of propolis to use *in vivo* when treating endodontic infections.

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