EFFICACY OF CHITOSAN PASTE AS INTRACANAL MEDICATION AGAINST *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* COMPARED TO CALCIUM HYDROXIDE

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ประสิทธิภาพของไคโตซานที่ใช้เป็นยาใส่ในคลองรากฟันเพื่อต้านเชื้อเอ็นเตอโรคอคคัส ฟีคัลลิส และแคนดิดาอัลบิแคนส์เปรียบเทียบกับแคลเซียมไฮดรอกไซด์

น.ส.พศิกา เทียนเงิน

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Thesis Title	EFFICACY OF CHITOSAN PASTE AS INTRACANAL MEDICATION AGAINST *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* COMPARED TO CALCIUM HYDROXIDE
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พศิกา เทียนเงิน : ประสิทธิภาพของไคโตซานที่ใช้เป็นยาใส่ในคลองรากฟันเพื่อต้านเชื้อเอ็นเตอโรคอคคัส ฟีคัลลิส และแคนดิดาอัลบิแคนส์เปรียบเทียบกับแคลเซียมไฮดรอกไซด์. (EFFICACY OF CHITOSAN PASTE AS INTRACANAL MEDICATION AGAINST *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* COMPARED TO CALCIUM HYDROXIDE) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.อัญชนา พานิชอัตรา, อ.ที่ปรึกษาร่วม : รศ. ทญ. ดร.อรนาฎ มาตังคสมบัติ

Enterococcus faecalis และ *Candida albicans* มักพบเป็นการติดเชื้อยืดเยื้อในการรักษาคลองรากฟัน ซึ่งการกำจัดเชื้อที่มีประสิทธิภาพจึงเป็นสิ่งที่สำคัญ ยาที่ใช้รักษาคลองรากฟันเป็นหนึ่งในขั้นตอนสำคัญ การศึกษานี้มีจุดประสงค์ที่จะทดสอบประสิทธิภาพของไคโตซานที่นำมาใช้เป็นยารักษาคลองรากฟันโดยเปรียบเทียบกับแคลเซียมไฮดรอกไซด์ โดยนำมาทดลองในฟันที่ถูกถอนทั้งหมด 68 ซี่ โดยแบ่งเป็นกลุ่ม *E. faecalis* ใช้ฟันจำนวน 36 ซี่ บ่มเชื้อในฟันเป็นเวลา 14 วัน และอีก 32 ซี่สำหรับกลุ่ม *C. albicans* ซึ่งใช้เวลาบ่มเชื้อในฟัน 48 ชั่วโมง เพื่อทำให้เกิดการสร้างไบโอฟิล์มในคลองรากฟัน หลังจากเกิดการสร้างไบโอฟิล์ม ฟันถูกนำมาแบ่งกลุ่มการทดลองเพื่อทดลองกับยาใส่ในคลองรากฟันโดยแบ่งกลุ่มการทดลองเป็น 6 กลุ่ม ได้แก่ กลุ่มที่ไม่มียาในคลองรากฟัน, กลุ่ม 20% โพลีเอทิลีนไกลคอล, กลุ่ม 20% โพรพิลีน ไกลคอล, กลุ่มไคโตซานผสมโพลีเอทิลีนไกลคอล, กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล และกลุ่มแคลเซียมไฮดรอกไซด์ ใส่ยาไว้ในคลองรากฟันเป็นเวลา 7 วัน เมื่อครบ 7 วัน เนื้อฟันในคลองรากฟันจะถูกเก็บตัวอย่าง โดยใช้เครื่องมือ Protaper next นำตัวอย่างเนื้อฟันที่เก็บได้มาทดสอบด้วยวิธีเจือจางตัวอย่างเชื้อเริ่มต้นและนำมาเพลท บนอาหารเลี้ยงเชื้อ Brain Heart infusion (BHI) agar สำหรับ *E. faecalis* และ Yeast Extract-Peptone-Dextrose (YPD) agar สำหรับ *C. albicans* เมื่อเชื้อเจริญขึ้นบนอาหารเลี้ยงเชื้อ นำมานับปริมาณเชื้อ เพื่อประเมินประสิทธิภาพยาในแต่ละชนิด การวิเคราะห์ประสิทธิภาพของยาประเมินจากร้อยละของเชื้อที่เหลืออยู่เมื่อเทียบกับกลุ่มที่ไม่ได้ใส่ยา โดยใช้ One-way ANOVA และ post-hoc Games-Howell test ผลการศึกษาพบว่าเชื้อ *E. faecalis,* กลุ่มไคโตซานผสมโพลีเอทิลีนไกลคอล, กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล มีประสิทธิภาพในการลดเชื้อลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ไม่มียาในคลองรากฟัน, กลุ่ม 20% โพลีเอทิลีนไกลคอล และกลุ่ม 20% โพรพิลีน ไกลคอล (P = 0.001, 0.003, 0.024, กลุ่มไคโตซานผสมโพลีเอทิลีนไกลคอล; P = 0.002, 0.003, 0.014, กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล) กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล มีประสิทธิภาพในการลดเชื้อมากกว่ากลุ่มแคลเซียมไฮดรอกไซด์ (P = 0.039) ผลการศึกษาในเชื้อ *C. albicans,* กลุ่มไคโตซานผสมโพลีเอทิลีนไกลคอล, กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล ไม่รวมกลุ่มแคลเซียมไฮดรอกไซด์ มีประสิทธิภาพในการกำจัดเชื้อมากกว่ากลุ่มที่ไม่มียาในคลองรากฟัน (P = 0.013, 0.005) สรุปไคโตซานที่ใช้เป็นยาใส่ในคลองรากฟันมีความสามารถในการกำจัดเชื้อ *E. faecalis* และ *C. albicans* เมื่อใส่ยาในคลองรากฟันเป็นเวลา 7 วัน กลุ่มไคโตซานผสมโพรพิลีน ไกลคอล มีประสิทธิภาพในการกำจัดเชื้อ *E. faecalis* มากกว่ากลุ่มแคลเซียมไฮดรอกไซด์ ไคโตซานจึงเหมาะที่จะพัฒนาเป็นยาทางเลือกสำหรับใส่ในคลองรากฟันที่ติดเชื้อยืดเยื้อ

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Pasika Thien-ngern : EFFICACY OF CHITOSAN PASTE AS INTRACANAL MEDICATION AGAINST *ENTEROCOCCUS FAECALIS* AND *CANDIDA ALBICANS* COMPARED TO CALCIUM HYDROXIDE. Advisor: Assoc. Prof. ANCHANA PANICHUTTRA, D.D.S., M.S., Ph.D. Co-advisor: Assoc. Prof. ORANART MATANGKASOMBUT, D.D.S. Ph.D.

Introduction: *Enterococcus faecalis* and *Candida albicans* are frequently found in persistent endodontic infection and are resistant to calcium hydroxide ($Ca(OH)_2$), a commonly used intracanal medication. Thus, an effective and safe antimicrobial medication against such refractory infection is necessary in endodontic retreatment, so we aimed to test the efficacy of chitosan paste against these microorganisms compared with $Ca(OH)_2$ in root canals of extracted human teeth. Methods: Thirty-six sterilized human root samples prepared from extracted premolars and upper maxillary incisors were infected with *E.faecalis* for 14 days, while 32 were infected with *C.albicans* for 48 hours, for mature biofilm formation. The samples were assigned to 6 groups of intracanal medications: no medication (negative control), 20% Polyethylene glycol (PEG), 20% Propylene glycol (PG), Chitosan+PEG, Chitosan+PG, and $Ca(OH)_2$. After 7 days, intracanal surface dentin was harvested using Protaper next, resuspended, serially diluted and spread on Brain-Heart-Infusion agar (for *E. faecalis)* and Yeast Extract-Peptone-Dextrose agar (for *C. albicans*) for colony count. Antimicrobial efficacy was determined as percentage of remaining colony forming unit (CFUs) relative to negative control and analyzed using One-way ANOVA and post-hoc Games-Howell test. The significance level was set at 0.05. Results: For *E. faecalis,* chitosan+PEG and chitosan+PG medication significantly reduced viable bacteria compared with negative control, PEG and PG (P = 0.001, 0.003, 0.024, respectively for chitosan+PEG; P = 0.002, 0.003, 0.014, respectively for chitosan+PG). Chitosan+PG also had significantly higher antibacterial activity than $Ca(OH)_2$ (P = 0.039). For *C.albicans,* chitosan+PEG and chitosan+PG, but not $Ca(OH)_2$, showed a significantly lower level of remaining CFUs compared with negative control (P = 0.013 and 0.005, respectively). Conclusion: Chitosan paste showed good efficacy as intracanal medication in reducing viable *E. faecalis* and *C. albicans* biofilm during 7 days in root canals. In particular, Chitosan+PG was significantly more effective against *E. faecalis* than $Ca(OH)_2$. Therefore, it could be developed as an effective alternative medication in endodontic retreatment.

Field of Study: Endodontics
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Student's Signature .....
Advisor's Signature .....
Co-advisor's Signature .....

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CHAPTER 1 INTRODUCTION

A major factor in endodontic failure in root canal treated teeth is persistent infection(1, 2). Thus, it is critical to have effective disinfection of the root canal system. Among the many steps of root canal treatment that help to eradicate microorganisms, intracanal medication is an important part that relies on the efficacy of antimicrobial agents(2). Calcium hydroxide has been used as a routine intracanal medication. However, certain microorganisms are still frequently detected in failed endodontic treated teeth, such as *Enterococcus faecalis* and *Candida albicans*(3).

E. faecalis is a Gram positive cocci that has a proton pump inhibitor mechanism to tolerate the alkalinity of $Ca(OH)_2$ medication. It can resist to a wide pH range, up to approximately pH 11.5, and could remain after root canal obturation(4). A previous study reported that $Ca(OH)_2$ has poor effectiveness against *E. faecalis*(5). *E. faecalis* can also suppress the activity of lymphocytes, invade dentinal tubules, form biofilm and attach to collagen in human serum, which can protect them from destruction(6). *C. albicans* is another microorganism that has been reported in persistent post-treatment apical periodontitis(7). An important virulence factor of *C. albicans* is the ability to switch between blastospore and hyphal form. The ability to grow as hyphal form make it able to invade host tissue and avoid phagocytosis from macrophages(8). Thigmotropism allow *C. albicans* to penetrate into deep dentinal tubules(9). *C. albicans* can form biofilm in 48 hours(10). It can survive in a wide range of pH, high alkaline environment and ecologically harsh conditions, which allow them to cause persistent infection(8).

Chitosan is a natural polysaccharide derived from deacetylation of chitin in crustacean shells. Chitosan has antimicrobial, antifungal properties and enhances wound healing(11). Chitosan can interact with microbial outer cellular components, the cytoplasm membrane and cytoplasmic constituents of microorganisms(12). Chitosan also has high biocompatibility and low toxicity; thus, it

could serve as a good alternative medication in endodontic treatment. Chitosan showed inhibitory effect on planktonic form and biofilm of *E. faecalis* and *C. albicans*(13, 14). Our group has previously showed that certain derivatives of chitosan are effective against *E. faecalis* and common oral *Candida* species, including *C. albicans*(15-17). In particular, we showed that 1700 kDa and 2100 kDa chitosan are effective against *E. faecalis,* but they require a long contact time (over 1 hour)(15). Thus, these chitosan derivatives are promising to be used as intracanal medication. In order to formulate chitosan into intracanal medication, Polyethylene glycol (PEG) and Propylene glycol (PG) can be used as vehicles to deliver intracanal medication through dentinal tubules and apical foramen as they showed better distribution of medication than using distilled water as a vehicle(18). The results from previous study showed that propylene glycol have antibacterial effect on *S. mutans, E. faecalis*, and *E. coli* by using broth dilution assay(19). Thus, they may also contribute to antimicrobial activity of the medication. The aim of this study is to develop chitosan paste as intracanal medication and test its antimicrobial activity against *E. faecalis* and *C. albicans* in comparison with $Ca(OH)_2$ in the root canals of extracted human teeth

Research questions

In the root canals of extracted human teeth, is chitosan paste as intracanal medication more effective in reducing the number of *E. faecalis* and *C. albicans* compared to $Ca(OH)_2$?

Research objectives

To test the antimicrobial activity against *E. faecalis* and *C. albicans* of chitosan paste compared to $Ca(OH)_2$ in the root canals of extracted human teeth.

CHAPTER II LITERATURE REVIEW

2.1 Cause of endodontic failure

Several studies found that persistence of microorganisms is the main cause of non-healed periapical tissue. Lin et al. studied in 236 cases of endodontic treatment failures, they found a correlation between the presence of bacterial infection in root canal and the persistence of periradicular rarefaction(2). The study exhibited 79% non healed periapical lesion teeth have remaining bacteria in root canal system. This studied implied that the microorganisms should be removed from the root canal before obturation(20). A systematic review studied showed that canals with negative culture before obturation have higher success rates than positive bacterial cultures canals(21). There are many cause of persisting of microorganisms such as untreated canal, anatomical variation (isthmus, fins, accessory canal), inappropriate mechanical debridement, inadequate filling of the root canal, improper coronal seal and persistence of bacterial infection(22).

2.2 Persistence microorganism

Bacteria in chronic infection may penetrate into dentinal tubules which are difficult to disinfect by mechanical and chemical procedures. Bacteria may remain in niches of root canal system and form biofilms(23). Gram positive bacteria commonly survive more than Gram negative bacteria in dehydration, lack of nutrients, changed ion strength, osmotic pressure and present of antimicrobial condition. Those conditions have been appreciated for *Enterococci, Streptococci, Lactobacilli, Actinomyces, Propioni-bacteria* and yeasts(23).

E. faecalis is commonly found in persistent infection canal. It is a non-spore forming fermentative facultative anaerobe, gram positive coccus. *E. faecalis* cell is ovoid shape with 0.5-1 μm in diameter. *E. faecalis* has a variety of forms, i.e. single, pairs, short chains but most common in elongated(24). Several studies by culture technique found that *E. faecalis* can be detected highly in

failed root canal treated teeth. Gomes et al. investigated microbial sampling of 19 previously treated teeth with persistent apical periodontitis. The results showed that *E. faecalis* is the most commonly isolated species, followed by *Streptococcus spp* and *Provetella*(25). Adib et al. found *Streptococcus* was the most prevalent at 35%, followed by *E. faecalis* at 12%(26).

Advanced microbiological culture technique was investigated the microorganisms associated with primary and secondary endodontic infection. The resulted showed that *E. faecalis* was the most dominant microorganism in secondary endodontic infection (36.6%), and *C. albicans* (20%)(3). Similarly, *E. faecalis* was reported the most prevalent species in failed endodontic treatment teeth(27). They was found as a small percentage of microorganisms in initial root canal treatment infection but higher percentage was observed in in the failed root canal teeth(28).

Another microorganism that has been reported in persistent post treatment apical periodontitis is *C. albicans*. Nair et al. collected 12 samples of asymptomatic persistence periapical lesion by apical surgery and detected yeast in 2 cases from 9 cases of perpendicular lesions refractory in failure treated teeth(1). Waltimo et al. reported fungi in 7% of 692 cases of persistent endodontic infections by culture technique. The result showed that *C. albicans* was the most common species isolated(29). Sundqvist et al removed gutta percha from previously root filled teeth with asymptomatic apical periodontitis and left empty canal to allow bacteria growth. After 7 days dentine debris was collected by hand files. They found *C. albicans* from 2 canals of 24 canals by advanced microbiological technique(30). Siqueira and Rocas exhibited 9% of *C. albicans* in 22 root filled teeth asymptomatic with persistence apical lesion by polymerase chain reaction technique(27). From previous studies this yeast play an important role in apical periodontitis case.

2.3 Calcium hydroxide intracanal medication

Calcium hydroxide has been used as common intracanal medication for clinical scenarios. They have several beneficial properties such as antimicrobial activity, antifungal activity, ability to dissolve tissue, inhibit radicular resorption, induction formation of hard tissue and anti-inflammatory effect(25).

Therapeutic effect of calcium hydroxide comes from the release of calcium ion and hydroxyl ion. Antibacterial effect highly depends on dissociation of the ions. The ions are released direct to the activation of enzymes of the cytoplasmic membrane of bacteria(31). The alkaline properties of calcium hydroxide are diffused through dentin(32). Direct contact of $Ca(OH)_2$ with microorganisms in vitro study was effective in 24 hours(33). In vitro study, the effective time for $Ca(OH)_2$ as intracanal medication is 7 days(34). Both *Candida* and enterococci have several properties that are necessary to survive in environment of failed treated teeth, including resistance to antimicrobial agents.

2.4 *E. faecalis* resistance to calcium hydroxide

Molander et al. reported that using calcium hydroxide as intracanal medication may be one reason for persistence of *E. faecalis* because it was more tolerant to high pH environment than other microorganisms. It can resist to wide pH range, up to around 11.5, and survive after root canal filling(4). The mechanism of high alkalinity tolerance of *E. faecalis* is the functioning of proton pump inhibitor. When environmental pH increases, *E. faecalis* can preserve intracellular pH by pumping protons across the cytoplasmic membrane. Evans et al. studied the proton pump mechanism of *E. faecalis* and discovered that carbonyl cyanide m -chlorphenylhydrazone (CCCP) can block normal function of proton pump in pH 11.1. The results showed that *E. faecalis* with CCCP are 20-fold cell decreased in compared to normal function cell(35). This ability makes *E. faecalis* adapt to various environments and survive when environment changes.

In biofilm form, *E. faecalis* can survive in environment with minimal nutrients and flourish with nutrient replacement(6, 27, 36). *E. faecalis* mature biofilm grown for 14 - 21 days is more difficult to eradicate by antimicrobial agents(37-39). Previous studies have shown that $Ca(OH)_2$ has poor effectiveness against *E. faecalis*. $Ca(OH)_2$ is not as effective as other substances by disc diffusion assay and could not inhibit *E. faecalis* by root canal direct contact test(5, 31).

2.5 *C. albicans* resistance to calcium hydroxide

An important virulence factor of *C. albicans* is the ability to switch between blastospore and hyphal formation. It can grow in any morphologic forms such as blastospores, germ tubes, true hyphae, pseudohyphae and chlamydospores depend on environment condition. *C. albicans* can form biofilm on difference surfaces, thus, *albicans* species can form biofilm more than other species such as *C. glabata*, *C. tropicalis* and *C.parapsilosis*. In biofilm form, It can be 100 fold more resistant to the antifungal fluconazole(8).

Initial period for *C. albicans* to adherence is 0-2 hour. Fungi begin to form microcolony after 2-4 hours. Yeast switching form budding to filamentous pseudo and true hyphal in 4-6 hour. Hyphal extensions will help yeast to interlink between micro-colony to form monolayer in 6-8 hour. Complex biofilm takes time on 3D architecture with spatial heterogeneity after 24-48 hour(10). *C. albicans* is able to survive in wide range of pH, high alkaline environment, ecologically harsh condition by switching of gene expression mechanism. Furthermore Ca^{2+} from calcium hydroxide solution is necessary for the growth and morphogenesis of *C. albicans*(8).

Waltimo et al. studied in 7 strains of *C. albicans* to 4 disinfection with iodide, chlorhexidine acetate, sodium hypochlorite, calcium hydroxide by filter paper test and found that $Ca(OH)_2$ had the weakest effect on *C. albicans* cells(40). The other study showed that $Ca(OH)_2$ used 6 hours to

eradicate *C. albicans*. They concluded that *Candida* species were highly resistant to calcium hydroxide solution(41).

2.6 Polyethylene glycol (PEG) and Propylene glycol (PG)

Polyethylene glycols are substance from ethylene oxide. The structure of compound is H-(O-CH_2- CH_2)$_n$ -OH. They are derived from petroleum and produced by the interaction between ethylene glycol or ethylene glycol oligomers. They have biocompatible and hydrophilic property that aid in penetration. It is considered to be safe by U.S. Food and Drug Administration and are used in medication as excipient in parenteral formulations(42).

Propylene glycol (CH_8O_2), or 1,2- dihydroxypropane or 1,2- propanediol, is an organic compound. The chemical formula is $C_3H_8O_2$ or $CH_3CHOHCH_2OH$. The substance consists of two alcohol functional groups. In the industry, propylene glycol can be produced from propylene oxide, but in laboratory it can be produced by fermentation method(43). It is clear, colorless, odorless and viscous. it dissolves in water, acetone and chloroform. Propylene glycol has been used in pharmaceuticals, preservation in solutions, parenteral nutrition, oral solution and topical medication(43). The difference between polyethylene glycol and propylene glycol is that polyethylene glycol is a polymer while propylene glycol is not(44).

Polyethylene glycol and Propylene glycol can deliver intracanal medication through dentinal tubule and apical foramen better than distilled water vehicle(18, 45). Cruz et al. exhibited that propylene glycol delivered dye through the root canal system more effectively than distilled water(18). According to Nalawade study PEG and PG have antibacterial effect to *S. mutans, E. faecalis*, and *E. coli* by using broth dilution assay(19).

2.7 Chitosan

Chitosan is a hydrophilic biopolymer gained from alkaline deacetylation of chitin extracted from shrimp shell. There are three major steps in the extraction procedure i.e.demineralization, deproteination and deacetylation. First, demineralization shrimp shells are treated with acid. Then the shrimp shell are washed up until its reach neutral pH and freeze at 80°C overnight for drying. Secondly, in deproteination, the samples are treated with NaOH followed by washing and drying. The last method is deacetylation; chitin are treated with strong alkali. The samples are washed until reaching neutral pH. After drying, the final product is chitosan(46).

Chitosan is a natural polysaccharide consist of copolymers of glucosamine and N-acetyl glucosamine which biocompatible, biodegradable, bioadhesion and lack cytoxicity(47). It has ability to form thin film that make chitosan useful to various applications in pharmaceutical industry. It is also non toxic and non-immunogenic(48). Chitosan is soluble in diluted acid. The properties of chitosan that related to dental treatment are antimicrobial activity, antifungal activity, and wound healing(11).

The antibacterial mechanisms of chitosan is the binding of cationic charged amino group to anionic components, such as N-acetyl muramic acid, sialic acid, and neuramic acid, on the bacterial cell surface. After chitosan interact with the negatively charged surface of outer cellular component, bacterial growth are suppressed because it impairs the exchanges with medium, chelating transition metal ions, and inhibiting protein synthesis(12). Varun et al. used chemical mechanism to extract chitosan from shrimp shell waste and investigated antimicrobial effect against four microbial i.e. *E. faecalis*, *Escherichia coli, Staphylococcus aureus* and *Enterobacter aerogenes* by agar well diffusion method. The results of zones of inhibitions was significantly more than control(46). Chitosan is also effective against common *Candida* and *C. albicans* on denture base acrylic resin. The results

showed that high molecular weight water soluble chitosan completely inhibited *C. albicans*(16).

2.8 Direct contact test & Colony forming unit assay

Direct contact test is an in vitro technique for measuring the effect of close contact between test bacteria and test material on the kinetic of bacteria growth(49).The colony forming unit (CFU) is a measure of viable clonogenic cell numbers in CFU/mL. This method shows the number of cell that remain viable. Viable cells must be able to proliferate and form small colonies on agar media after incubation period(50).

CHAPTER III MATERIALS AND METHODS

3.1 Root samples

This study was approved by the Ethics Review Committee for Research, Chulalongkorn University, Bangkok, Thailand (No.055/2020). Sixty eight intact premolars and upper maxillary incisors with straight root canal extracted for orthodontic and periodontitis reasons were collected. Teeth with caries, fractures, cracks or other defects detected by magnifying loupes were excluded. The tooth samples were prepared using method modified from Carpio-Perochena et al(51). All extracted teeth were stored in 0.1% thymol until prepared for the experiments. All soft-tissue remnants on the tooth surfaces were removed without harming the cementum. The crown and the coronal third of root were removed until the length of each root was 15 mm. The root canals were enlarged by Protaper next size X4 with 300 RPM speed and 2 gcm torque using a rotary handpiece. The samples were irrigated with 3 ml of 2.5 % sodium hypochlorite (NaOCl) followed by 1 ml of 17% ethylenediaminetetraacetic acid (EDTA) to remove organic and inorganic debris. Five ml of distilled water was used as an irrigation to remove the remaining of prior irrigants. Then, all the roots were autoclaved for 20 min at 121°C. The external surfaces were sealed with nail vanish. The root samples were divided into 2 groups, 36 teeth were inoculated with *E. faecalis* and 32 teeth were inoculated with *C. albicans.*

3.2 Preparation of microbial culture

E. faecalis (ATCC 29212) was incubated in brain heart infusion (BHI) broth (Himedia, Mumbai, India) at 37 C° until log phase. *C. albicans* (ATCC 90028) was incubated in Yeast extract – peptone-dextrose (YPD; Oxoid, UK and HiMedia, India) at 30 C° until log phase. The microbial suspension was adjusted to optical density of 0.5 for *E. faecalis* and 0.1 for *C. albicans* at 600 nm for inoculation.

3.3 Infection of the root samples

Log phase culture (30 μl) of *E. faecalis* or *C. albicans* was inoculated into the root canals. For *E. faecalis,* BHI media was replenished every 48 hours and the samples were incubated for 14 day for mature biofilm formation(37). For *C. albicans*, the root samples were incubated for 48 hours for mature biofilm formation(10). All procedures were carried out in a biosafety cabinet.

3.4 Preparation of intracanal medications

The chitosan powder (1700 KDa, Marine Bio Resources, Thailand) was dissolved in 1% acetic acid (Merck KGaA, Darmstadt, Germany) at 20 mg/ml. The chitosan pastes were prepared by mixing 1 ml of chitosan solution (20 mg/ml) with 1 ml of Polyethylene glycol (Krungthepchemi, Bangkok, Thailand) or propylene glycol (Krungthepchemi, Bangkok, Thailand), and 3 ml of distilled water. The final concentration of chitosan was 4 mg/ml in 20% PEG or PG. The pastes were sterilized by autoclave.

Calcium hydroxide intracanal medication was prepared by mixing 0.4 g of calcium hydroxide powder (Faculty of dentistry, Chulalongkorn university, Thailand) with 10 ml of distill water. The final concentration of calcium hydroxide was 40 mg/ml.

3.5 Antimicrobial assessment

After the specified incubation period, an aliquot of the media from each root sample was plated on solid media to check for purity and viability of the microbes. Root samples infected with *E. faecalis* and those with *C. albicans* were allocated to 6 groups and treated as follow: group I, no medication (negative control); group 2, 20% PEG; group 3, 20% PG; group 4, chitosan+PEG; Group 5, chitosan+PG; and group 6, $Ca(OH)_2$. Thirty μl of the assigned medication were applied into each root canal. The root samples were incubated at 37ºC for *E. faecalis* and 30ºC for *C. albicans* for 7

days. After 7 days, the canals were washed with 3 ml of sterile distilled water and Protaper next size X4 was used at 300 RPM speed and 2gcm torque to remove the medicament.

Dentin samples from the root canal surfaces were harvested by using Protaper next size X5 at 300 RPM speed and 2gcm torque and collected in 1 ml of phosphate buffer saline solution. Serial dilutions were performed and 100 μl of each dilutions were plated on BHI agar for *E. faecalis* and YPD agar for *C. albicans*. Plates were incubated for 24 hours at 37ºC for *E. faecalis* and 48 hours at 30ºC for *C. albicans*. Colonies were counted and calculated into percentage of remaining viable microorganisms relative to the negative control. All experiments were performed in duplicates and repeated 3 times (except for the PEG and PG groups for *C. albicans* where only one sample/group was available for two of experiments).

3.6 Statistical Analysis

Shapiro-Wilk was used to test for normality of the data. Welch's ANOVA was used to analyze the differences in percentage of remaining viable microorganisms after treatment among groups, followed by Games - Howell test to compare the data between experiment groups. Data were analyzed using IBM SPSS Statistics for Windows, Version 22.0 (IBM, Armonk, New York, USA). A *P*-value of < 0.05 was considered statistically significant.

CHAPTER IV RESULTS

After 7 days of treatment with various medications, the remaining viable microorganisms in the root canals were examined by plate count. The results are shown in Figure 1 for *E. faecalis* and Figure 2 for *C. albicans*. For *E. faecalis,* the average percentage of remaining bacteria in both chitosan+PEG (9.68± 8.6%) and chitosan+PG (3.02±2%) groups was significantly lower than that of the negative control (102.74±26%), PEG (85.7±25.3%) and PG (45.28±17.9%) groups. (*P*=0.001, 0.003, and 0.024, respectively for chitosan+PEG; *P*=0.002, 0.003, and 0.014, respectively for chitosan+PG). In addition, the PG group showed a significantly lower remaining *E. faecalis* than the negative control (*P*=0.015). $Ca(OH)_2$ (46.38±23.4%) also had a significantly lower level of remaining *E. faecalis* than the negative control group (*P*=0.034). Chitosan+PG could reduce the bacteria to a level that is significantly lower than the $Ca(OH)_2$ group (*P*=0.039) but this was not significantly different for the chitosan+PEG group (*P*=0.071).

For *C. albicans,* both chitosan+PEG (24.77± 20.5%) and chitosan+PG (7.57±14.6%) groups harbored significantly lower remaining viable *C. albicans* compared with the negative control (105.40±36.5%) (*P*=0.013 and 0.005, respectively). However, they were not significantly different from PEG (122.06±71.7%) and PG (95.36±64.8%) groups (*P*=0.292, and 0.441, respectively for chitosan+PEG; *P* = 0.206 and 0.293, respectively for chitosan+PG). In contrast, PEG, PG, and $Ca(OH)_2$ (41.19±38.1%) were not significantly different from the negative control (*P*=0.997, 1.00, and 0.106, respectively).

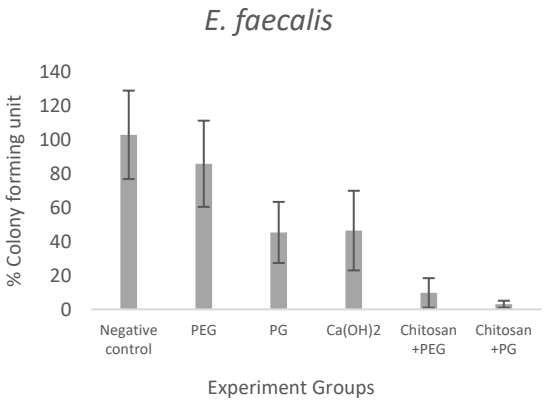


Fig 1

Figure 1: Percentage of remaining viable E. faecalis after treatment with intracanal medication relative to negative control.

Negative control group (102.74±26%), 20% Polyethylene glycol (PEG) group (85.7±25.3%), 20% Propylene glycol (PG) group (45.28±17.9%), calcium hydroxide ($Ca(OH)_2$) group (46.38±23.4%), chitosan+PEG group (9.68± 8.6%), chitosan+PG group (3.02±2%)

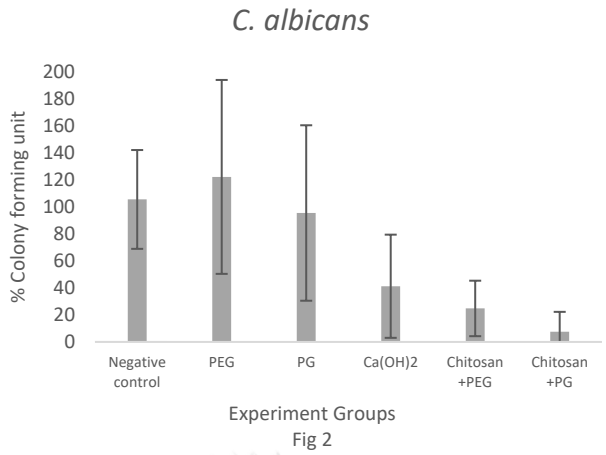


Figure 2: Percentage of remaining viable C. albicans after treatment with intracanal medication relative to negative control.

Negative control group (105.40±36.5%), 20% Polyethylene glycol (PEG) group (122.06±71.7%), 20% Propylene glycol (PG) group (95.36±64.8%), calcium hydroxide ($Ca(OH)_2$) group (41.19±38.1%), chitosan+PEG group (24.77± 20.5%) , chitosan+PG group (7.57±14.6%)

CHAPTER V DISCUSSION AND CONCLUSION

In this study, we showed that 1700 kDa chitosan (4 mg/ml) paste has good antimicrobial efficacy against *E. faecalis* and *C. albicans* biofilm in human root canals. The results suggest that chitosan paste could be developed into an alternative antimicrobial intracanal medication in cases with persistent infection.

Microbiological investigations found a complex community of bacteria and fungi in root canal treated teeth with persistent infection chronic apical periodontitis(1, 3). This suggests that previous endodontic treatment could not efficiently control these microorganisms. Mechanical instrumentation using larger size rotary instruments may help to remove bacteria and fungi in the root canals, but it cannot completely eradicate microbes in complex root canal structures and excessive instrumentation weakens root dentin(52-54). Thus, the use of effective antimicrobial intracanal medication can overcome the limitations of instrumentation to reduce microorganisms in complex anatomy of the root canals. $Ca(OH)_2$ has been used as routine intracanal medication, but it was not effective against *E. faecalis* and *C. albicans(35, 55-57).* Several studies have identified *E. faecalis* and *C. albicans* as important microorganisms using culture-dependent and molecular techniques(3, 27, 58). For example, a recent study used culture-dependent methods and showed that the most prevalent microorganisms in root canal treated teeth was *E. faecalis* (36.6%) and followed by *C. albicans* (20%)(3). Studies using PCR detected *E. faecalis* at a prevalence of up to 77%, and *C. albicans* up to 35%, of failed root-filled teeth(27, 58). Although *E. faecalis* was not the most abundant bacteria detected by metagenomic studies, it was observed at a greater frequency or proportion in secondary apical periodontitis than primary infection(59, 60). Thus, persistence of these microorganisms in the root canals are problematic for endodontic treatment and effective

antimicrobial agent against persisting *E. faecalis* and *C. albicans* in root canal system is clearly needed(5, 40, 57).

The antimicrobial efficacy of chitosan appears to be related to the type of microbes, type of chitosan, positive charge density, molecular weight, concentration, chelating capacity, and water solubility of chitosan(61). Our group has previously showed that 1700 kDa chitosan and 2100 kDa chitosan could effectively kill *E. faecalis* at an MBC of 2mg/ML(15). However, it needed a long contact time of over 10 min, so it may be more effective when applied as a root-canal medication rather than an irrigant. In addition, we also showed that these chitosan derivatives has a minimum fungicidal concentration against *C. albicans* at 4 mg/ml (17). Thus, in this study, we formulated 4 mg/mL of 1700 KDa chitosan in 0.2 % acetic acid into a paste for application as an antimicrobial intracanal medication. We used 20% PEG or PG to confer good flowability for easy handling and enhance penetration into dentinal tubules. In addition, PEG has hydrophilic property, so it can remove water, which is essential for microbial growth. However, it did not affect *E. faecalis* much when compared to the control group(19). Propylene glycol can inhibit the growth and proliferation of microbes and fungi because the structure of glycol (Methyl and propyl p – hydroxybenzoate) can interrupt surface tension of bacterial cell wall(62).

Our results showed that chitosan paste could eliminate more *E. faecalis* than the negative control, PEG, and PG groups. Chitosan+PG showed greater antibacterial effect against *E. faecalis* biofilm than PG, and also greater than $Ca(OH)_2$ groups. Although *E. faecalis* could resist to $Ca(OH)_2$ by a proton pump mechanism, our result showed that $Ca(OH)_2$ was better at reducing viable *E. faecalis* than no medication(63). However, other studies found that $Ca(OH)_2$ fail to eliminate *E. faecalis* biofilm(64, 65). Since PG could also reduce *E. faecalis* compared with negative control, the

combined effect of both chitosan and PG may explain why chitosan+PG is the most effective in this experiment.

Chitosan is positively charged and could interact with the negatively charged microbial cell membrane, while PG can help intracanal medication to penetrate deeper in dentinal tubules and it also has germicidal activity(19, 66). Similarly, chitosan-propolis nanoparticles was shown to be effective at eliminating *E. faecalis* biofilm after 7 days of medication(67). Chitosan has the ability to remove smear layer that contain organic and inorganic substances which may prevent the penetration of medication into dentinal tubules. Chitosan also has high chelating effect to eliminate dentin calcium ion(68). From an in vitro study, 0.2% chitosan as irrigant can remove more smear layer and cause less dentine erosion than 17% EDTA(69). Thus, chitosan is a promising alternative option for intracanal medication, either alone or in combination with other active ingredients, especially in secondary infection where persistent *E. faecalis* infection may play important roles.

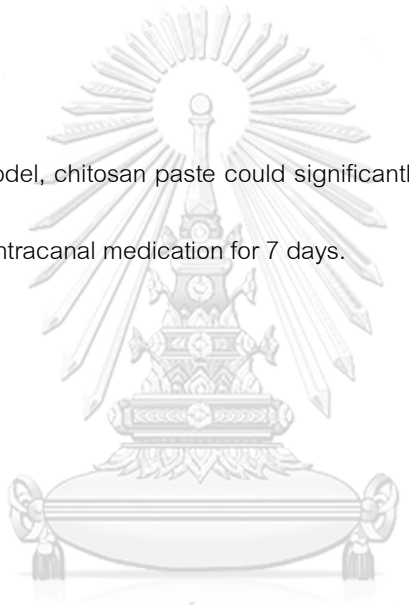
We also found that chitosan paste was effective against *C. albicans* biofilm as both chitosan groups had significantly less remaining viable *C. albicans* than the negative control. We could not detect significant differences among other groups, likely due to the wide variations in the CFU results. For *C. albicans*, $Ca(OH)_2$ was not significantly different from the negative control group. Our results suggest that $Ca(OH)_2$ is more effective against *E. faecalis* than *C. albicans.* This is concordant with a report by Ercan and colleagues(70).

Microorganisms in biofilm are more tolerant to antimicrobial agents, and this is the form that are found in the root canals(8, 38). Thus, we simulated such biofilm condition in this study using extracted human root specimens inoculated with *E. faecalis* and with *C. albicans.* We allowed sufficient time for them to form mature biofilm and penetrate in dentinal tubules, ie, 14 days for *E. faecalis* and 48 hours for *C. albicans,* according to previous reports(10, 38, 71). Our results showed

that chitosan paste could significantly reduce the number of viable microorganisms for both *E. faecalis* and *C. albicans* in this root canal biofilm model. Thus, it has a great advantage over antibiotics medication which are effective against only bacteria, but not fungi. Nevertheless, this biofilm model was of single species, unlike in vivo conditions where multiple species coexist in a community. Therefore, further studies on multi-species biofilm and clinical trials should be performed in order to develop chitosan paste for future clinical applications.

Conclusion

In this root canal biofilm model, chitosan paste could significantly reduce viability of *E. faecalis* and *C. albicans* when used as intracanal medication for 7 days.



REFERENCES

1. Nair PN, Sjogren U, Krey G, Kahnberg KE, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. J Endod. 1990;16(12):580-8.

2. Lin LM, Skribner JE, Gaengler P. Factors associated with endodontic treatment failures. J Endod. 1992;18(12):625-7.

3. Pourhajibagher M, Ghorbanzadeh R, Bahador A. Culture-dependent approaches to explore the prevalence of root canal pathogens from endodontic infections. Braz Oral Res. 2017;31:e108.

4. Molander A, Reit C, Dahlen G. The antimicrobial effect of calcium hydroxide in root canals pretreated with 5% iodine potassium iodide. Endod Dent Traumatol. 1999;15(5):205-9.

5. Basrani B, Tjaderhane L, Santos JM, Pascon E, Grad H, Lawrence HP, et al. Efficacy of chlorhexidine- and calcium hydroxide-containing medicaments against Enterococcus faecalis in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003;96(5):618-24.

6. Love RM. Enterococcus faecalis--a mechanism for its role in endodontic failure. Int Endod J. 2001;34(5):399-405.

7. Waltimo TM, Sen BH, Meurman JH, Orstavik D, Haapasalo MP. Yeasts in apical periodontitis. Crit Rev Oral Biol Med. 2003;14(2):128-37.

8. Siqueira JF, Jr., Sen BH. Fungi in endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97(5):632-41.

9. Chua EG, Parolia A, Ahlawat P, Pau A, Amalraj FD. Antifungal effectiveness of various intracanal medicaments against Candida albicans: an ex-vivo study. BMC Oral Health. 2014;14:53.

10. Tyagi SP, Sinha DJ, Garg P, Singh UP, Mishra CC, Nagpal R. Comparison of antimicrobial efficacy of propolis, Morinda citrifolia, Azadirachta indica (Neem) and 5% sodium hypochlorite on Candida albicans biofilm formed on tooth substrate: An in-vitro

study. J Conserv Dent. 2013;16(6):532-5.

11. Ballal N, Kundabala M, Bhat K, Acharya S, Ballal M, Kumar R, et al. Susceptibility of Candida albicans and Enterococcus faecalis to Chitosan, Chlorhexidine gluconate and their combination in vitro. Aust Endod J. 2009;35(1):29-33.

12. Raafat D, Sahl HG. Chitosan and its antimicrobial potential--a critical literature survey. Microb Biotechnol. 2009;2(2):186-201.

13. Pu Y, Liu A, Zheng Y, Ye B. In vitro damage of Candida albicans biofilms by chitosan. Exp Ther Med. 2014;8(3):929-34.

14. Wang N, Ji Y, Zhu Y, Wu X, Mei L, Zhang H, et al. Antibacterial effect of chitosan and its derivative on Enterococcus faecalis associated with endodontic infection. Exp Ther Med. 2020;19(6):3805-13.

15. Supotngarmkul A, Panichuttra A, Ratisoontorn C, Nawachinda M, Matangkasombut O. Antibacterial property of chitosan against E. faecalis standard strain and clinical isolates. Dent Mater J. 2020.

16. Namangkalakul W, Benjavongkulchai S, Pochana T, Promchai A, Satitviboon W, Howattanapanich S, et al. Activity of chitosan antifungal denture adhesive against common Candida species and Candida albicans adherence on denture base acrylic resin. J Prosthet Dent. 2020;123(1):181 e1- e7.

17. Srimaneepong V, Thanamee T, Wattanasirmkit K, Muangsawat S, Matangkasombut O. Efficacy of low-molecular weight chitosan against Candida albicans biofilm on polymethyl methacrylate resin. Aust Dent J. 2021.

18. Cruz EV, Kota K, Huque J, Iwaku M, Hoshino E. Penetration of propylene glycol into dentine. Int Endod J. 2002;35(4):330-6.

19. Nalawade TM, Bhat K, Sogi SH. Bactericidal activity of propylene glycol, glycerine, polyethylene glycol 400, and polyethylene glycol 1000 against selected microorganisms. J Int Soc Prev Community Dent. 2015;5(2):114-9.

20. Fabricius L, Dahlen G, Sundqvist G, Happonen RP, Moller AJ. Influence of residual bacteria on periapical tissue healing after chemomechanical treatment and root filling of experimentally infected monkey teeth. Eur J Oral Sci. 2006;114(4):278-85.

21. Ng YL, Mann V, Gulabivala K. Outcome of secondary root canal treatment: a systematic review of the literature. Int Endod J. 2008;41(12):1026-46.

22. Tabassum S, Khan FR. Failure of endodontic treatment: The usual suspects. Eur J Dent. 2016;10(1):144-7.

23. Fouad AF. Endodontic microbiology. Second edition. ed: Wiley; 2017.

24. Rocas IN, Siqueira JF, Jr., Santos KR. Association of Enterococcus faecalis with different forms of periradicular diseases. J Endod. 2004;30(5):315-20.

25. Gomes BP, Pinheiro ET, Gade-Neto CR, Sousa EL, Ferraz CC, Zaia AA, et al. Microbiological examination of infected dental root canals. Oral Microbiol Immunol. 2004;19(2):71-6.

26. Adib V, Spratt D, Ng YL, Gulabivala K. Cultivable microbial flora associated with persistent periapical disease and coronal leakage after root canal treatment: a preliminary study. Int Endod J. 2004;37(8):542-51.

27. Siqueira JF, Jr., Rocas IN. Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97(1):85-94.

28. Berman LH, Hargreaves KM, Rotstein I. Cohen's pathways of the pulp. Twelfth edition. ed: Elsevier; 2021.

29. Waltimo TM, Siren EK, Torkko HL, Olsen I, Haapasalo MP. Fungi in therapy-resistant apical periodontitis. Int Endod J. 1997;30(2):96-101.

30. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998;85(1):86-93.

31. Estrela C, Sydney GB, Bammann LL, Felippe Junior O. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Braz Dent J. 1995;6(2):85-90.

32. Oztan MD, Akman A, Dalat D. Intracanal placement of calcium hydroxide: a comparison of two different mixtures and carriers. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002;94(1):93-7.

33. Hargreaves KMB, Louis M. Cohen's pathways of the pulp. 7, editor2015.

34. Sjogren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J. 1991;24(3):119-25.

35. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J. 2002;35(3):221-8.

36. Stuart CH, Schwartz SA, Beeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod. 2006;32(2):93-8.

37. Taneja S, Kumar P, Malhotra K, Dhillon J. Antimicrobial effect of an oxazolidinone, lantibiotic and calcium hydroxide against Enterococcus faecalis biofilm: An in vitro study. Indian J Dent. 2015;6(4):190-4.

38. Guerreiro-Tanomaru JM, de Faria-Junior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod. 2013;39(3):346-50.

39. Bulacio Mde L, Galvan LR, Gaudioso C, Cangemi R, Erimbaue MI. Enterococcus Faecalis Biofilm. Formation and Development in Vitro Observed by Scanning Electron Microscopy. Acta Odontol Latinoam. 2015;28(3):210-4.

40. Waltimo TM, Orstavik D, Siren EK, Haapasalo MP. In vitro susceptibility of Candida albicans to four disinfectants and their combinations. Int Endod J. 1999;32(6):421-9.

41. Waltimo TM, Siren EK, Orstavik D, Haapasalo MP. Susceptibility of oral Candida species to calcium hydroxide in vitro. Int Endod J. 1999;32(2):94-8.

42. Gullapalli RP, Mazzitelli CL. Polyethylene glycols in oral and parenteral formulations--A critical review. Int J Pharm. 2015;496(2):219-39.

43. Propylene glycol: National Center for Biotechnology Information; 2022 [Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Propylene-glycol.

44. Madhu. What is the Difference Between Polyethylene Glycol and Propylene Glycol 2022 [Available from: https://www.differencebetween.com/what-is-the-difference-between-polyethylene-glycol-and-propylene-glycol/.

45. Teoh Y-Y, Athanassiadis B, Walsh LJJID. The influence of aqueous and PEG 400 solvent vehicles on hydroxyl ion release from calcium hydroxide medicaments.

2016;11:42-50.

46. Varun TK, Senani S, Jayapal N, Chikkerur J, Roy S, Tekulapally VB, et al. Extraction of chitosan and its oligomers from shrimp shell waste, their characterization and antimicrobial effect. Vet World. 2017;10(2):170-5.

47. Savitha A, SriRekha A, Vijay R, Ashwija, Champa C, Jaykumar T. An in vivo comparative evaluation of antimicrobial efficacy of chitosan, chlorhexidine gluconate gel and their combination as an intracanal medicament against Enterococcus faecalis in failed endodontic cases using real time polymerase chain reaction (qPCR). Saudi Dent J. 2019;31(3):360-6.

48. Ong TH, Chitra E, Ramamurthy S, Siddalingam RP, Yuen KH, Ambu SP, et al. Chitosan-propolis nanoparticle formulation demonstrates anti-bacterial activity against Enterococcus faecalis biofilms. PLoS One. 2017;12(3):e0174888.

49. Anumula L, Kumar S, Kumar VS, Sekhar C, Krishna M, Pathapati RM, et al. An Assessment of Antibacterial Activity of Four Endodontic Sealers on Enterococcus faecalis by a Direct Contact Test: An In Vitro Study. ISRN Dent. 2012;2012:989781.

50. Kumar R, Muralitharen S, Usman SM, Ramachandran RP, Sundararajan R, Madhivanan S, et al. Electrically-Enhanced Proliferation Control in Adult Human Mesenchymal Stem Cells. 2009.

51. Del Carpio-Perochena A, Kishen A, Felitti R, Bhagirath AY, Medapati MR, Lai C, et al. Antibacterial Properties of Chitosan Nanoparticles and Propolis Associated with Calcium Hydroxide against Single- and Multispecies Biofilms: An In Vitro and In Situ Study. J Endod. 2017;43(8):1332-6.

52. Zelic K, Vukicevic A, Jovicic G, Aleksandrovic S, Filipovic N, Djuric M. Mechanical weakening of devitalized teeth: three-dimensional Finite Element Analysis and prediction of tooth fracture. Int Endod J. 2015;48(9):850-63.

53. Usman N, Baumgartner JC, Marshall JG. Influence of instrument size on root canal debridement. J Endod. 2004;30(2):110-2.

54. Koprowicz A, Łęski M, Pawlicka H. Properties of Mechanical Endodontic Instruments and the Quality of the Simulated Canal Preparation. Dent Med Probl

2016;53(4):476–82.

55. Ballal V, Kundabala M, Acharya S, Ballal M. Antimicrobial action of calcium hydroxide, chlorhexidine and their combination on endodontic pathogens. Aust Dent J. 2007;52(2):118-21.

56. Turk BT, Sen BH, Ozturk T. In vitro antimicrobial activity of calcium hydroxide mixed with different vehicles against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2009;108(2):297-301.

57. Joy Sinha D, Garg P, Verma A, Malik V, Maccune ER, Vasudeva A. Dentinal Tubule Disinfection with Propolis & Two Extracts of Azadirachta indica Against Candida albicans Biofilm Formed on Tooth Substrate. Open Dent J. 2015;9:369-74.

58. Poptani B, Sharaff M, Archana G, Parekh V. Detection of Enterococcus faecalis and Candida albicans in previously root-filled teeth in a population of Gujarat with polymerase chain reaction. Contemp Clin Dent. 2013;4(1):62-6.

59. Bouillaguet S, Manoil D, Girard M, Louis J, Gaia N, Leo S, et al. Root Microbiota in Primary and Secondary Apical Periodontitis. Front Microbiol. 2018;9:2374.

60. Keskin C, Demiryurek EO, Onuk EE. Pyrosequencing Analysis of Cryogenically Ground Samples from Primary and Secondary/Persistent Endodontic Infections. J Endod. 2017;43(8):1309-16.

61. Sukmark T, Rachtanapun P, Rachtanapun CJA, Resources N. Antimicrobial activity of oligomer and polymer chitosan from different sources against foodborne pathogenic bacteria. Agriculture and Natural Resources. 2011;45(4):636-43.

62. Propylene Glycol: Hydra Technologies Ltd.; 2022 [Available from: https://hydratechglobal.net/technical/propylene-glycol/59/en.

63. Goy R, Britto D, Assis O. A review of the antimicrobial activity of chitosan. Polimeros: Ciencia e Tecnologia. 2009;19:241-7.

64. Zancan RF, Calefi PHS, Borges MMB, Lopes MRM, de Andrade FB, Vivan RR, et al. Antimicrobial activity of intracanal medications against both Enterococcus faecalis and Candida albicans biofilm. Microsc Res Tech. 2019;82(5):494-500.

65. Zancan RF, Vivan RR, Milanda Lopes MR, Weckwerth PH, de Andrade FB, Ponce

JB, et al. Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. J Endod. 2016;42(12):1822-8.

66. Olitzky I. Antimicrobial properties of a propylene glycol based topical therapeutic agent. J Pharm Sci. 1965;54(5):787-8.

67. Parolia A, Kumar H, Ramamurthy S, Davamani F, Pau A. Effectiveness of chitosan-propolis nanoparticle against Enterococcus faecalis biofilms in the root canal. BMC Oral Health. 2020;20(1):339.

68. Mathew SP, Pai VS, Usha G, Nadig RR. Comparative evaluation of smear layer removal by chitosan and ethylenediaminetetraacetic acid when used as irrigant and its effect on root dentine: An in vitro atomic force microscopic and energy-dispersive X-ray analysis. J Conserv Dent. 2017;20(4):245-50.

69. Silva PV, Guedes DF, Pecora JD, da Cruz-Filho AM. Time-dependent effects of chitosan on dentin structures. Braz Dent J. 2012;23(4):357-61.

70. Ercan E, Dalli M, Dulgergil CT. In vitro assessment of the effectiveness of chlorhexidine gel and calcium hydroxide paste with chlorhexidine against Enterococcus faecalis and Candida albicans. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006;102(2):e27-31.

71. Dezhurko-Korol VA, Novozhilova NE, Makeeva IM, Arkhipova AY, Moisenovich MM, Akhmadishina LV, et al. The influence of centrifugation and inoculation time on the number, distribution, and viability of intratubular bacteria and surface biofilm in deciduous and permanent bovine dentin. Arch Oral Biol. 2020;114:104716.

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