

Antibacterial Effect of Extract from *Commiphora gileadensis*;
In Vitro Study

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Antibacterial Extract from *Commiphora gileadensis*; In Vitro Study

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Abstract

Objective: This in vitro study aims to investigate the antimicrobial effect of extracts from *Commiphora gileadensis* (*C. gileadensis*) on one and three weeks old oral anaerobic multispecies biofilms and to compare them to 2% chlorhexidine (CHX).

Material and Methods: Hydroxyapatite discs were coated with type I collagen and immersed in Brain Heart Infusion broth infused with an oral subgingival plaque obtained from two donors. The discs were then incubated under anaerobic conditions for one or three weeks. After biofilm growth, the discs were exposed to one or three minutes for the following solutions: 1 - *C. gileadensis* 1mg/ml water, 2 - *C. gileadensis* 0.1mg/ml water, 3 - *C. gileadensis* 1mg/ml 0.5% DMSO, 4 - *C. gileadensis* 0.1mg/ml 0.5% DMSO, 5 - water, 6 - 2%CHX and 7 - 0.5%DMSO. After the exposures, all discs were stained with a viability stain and scanned under a confocal laser scanning microscope. The percentage of dead bacteria was calculated using Imaris software. The data were submitted to Univariate analysis of variance and Tukey statistical tests ($P < 0.05$).

Results: *C. gileadensis* 1mg/ml water killed significantly more bacteria than all other groups ($P < 0.05$). 2%CHX and other groups of *C. gileadensis* killed bacteria without a significant difference among themselves ($P > 0.05$). In 0.5%DMSO and water groups only a few bacteria were killed.

Conclusion: *Commiphora gileadensis* extract 1mg/ml in water killed significantly more bacteria in oral anaerobic multispecies biofilm than 2% CHX.

Lay summary

Bacterial biofilms establish in the root canal system after the tooth becomes necrotic and infected. Root canal treatment heavily depends on the chemical irrigation procedures to clean and reach the root canal complexity. An optimal chemical irrigation solution is required to eliminate the biofilm bacteria proficiently. However, there is no such an ideal irrigation so far for root canal treatment. In this experiment a natural resource, *Commiphora gileadensis* extract, is used to investigate the effectiveness against oral multispecies anaerobic bacteria.

Preface

This thesis “Antibacterial Effect of Extract from *Commiphora gileadensis*; In Vitro Study” is an original, independent, and unpublished work by Khalid Abuljadayel performed under the supervision and direction of Dr. Markus Haapasalo and Dr. Ya Shen.

The tree extraction procedure was done by Biological Services Laboratory in the Department of Chemistry at the University of British Columbia. The writing and the lab experiments were done by Khalid Abuljadayel. Dr. Markus Haapasalo and Dr. Ya Shen contributed to the study design and thesis editing. Dr. Hakkinen has given advice as a member of the thesis committee. The relative contributions to this research project by Dr. Abuljadayel was 75%

Ethical approval was obtained from The University of British Columbia Research Ethics Board (application number H15-02793)

Table of Contents

Abstract.....	iii
Lay Summary.....	iv
Preface.....	v
Table of Contents.....	vi
List of Tables.....	viii
List of Figures.....	ix
List of Abbreviations.....	x
Acknowledgements.....	xi
Dedication.....	xii
Chapter 1: Literature Review.....	1
1.1 Endodontics infections	2
1.2 Endodontics bacteria.....	3
1.3 Biofilms.....	4
1.4 Eradication of endodontics biofilm.....	6
1.5 Irrigation in endodontics.....	7
1.5.1 Sodium hypochlorite.....	7
1.5.2 Ethylenediaminetetraacetic acid.....	8
1.5.3 Chlorhexidine.....	9
1.5.4 QMiX.....	11
1.5.5 Iodine potassium iodide.....	11
1.5.6 Hydrogen peroxide.....	12
1.6 Interappointment Medications.....	13
1.6.1 Calcium hydroxide.....	13
1.6.2 CHX gel.....	14

1.6.3	Combination of interappointment medications.....	15
1.6.4	Antibiotics.....	16
1.7	Alternative medicine.....	17
1.8	Plants in dentistry and endodontics.....	18
1.9	<i>Commiphora gileadensis</i>	19
Chapter 2:	Aim and Objectives.....	24
Chapter 3:	Materials and Methods.....	25
3.1	Pilot study.....	25
3.2	Plant preparation.....	25
3.3	Hydroxyapatite (HA) disc coating and biofilm preparation.....	27
3.4	Antibiofilm irrigating solutions and experimental procedure.....	28
3.5	Confocal laser scanning microscopy (CLSM).....	29
3.6	Image analysis.....	31
3.7	Statistical analysis.....	32
Chapter 4:	Results.....	33
4.1	Pilot study result.....	33
4.2	General findings.....	33
4.3	The effect of the agents on 1-week-old biofilm.....	35
4.4	The effect of the agents on 3-week-old biofilm.....	39
4.5	The effect of the exposure time.....	43
4.6	The effect of the age of the biofilm.....	43
Chapter 5:	Discussion.....	44
Chapter 6:	Conclusion.....	49
References	50

List of Tables

Table 1 Mean percentage of dead bacteria (\pm standard deviation) in one- and three-week-old biofilms after different exposure times; pilot results.....33

Table 2 Mean percentage of dead bacteria (\pm standard deviation) in one- and three-week-old biofilms after one and three minute exposure.....34

List of Figures

Figure 1. (A) <i>Commiphora gileadensis</i> shrub. (B) <i>Commiphora gileadensis</i> branch.....	23
Figure 2. <i>Commiphora gileadensis</i> methanol extract in a dry condition before being dissolved in distilled water or DSMO.....	26
Figure 3. <i>Commiphora gileadensis</i> extract, 10 mg/ml water.....	26
Figure 4. HA discs immersed in BHI-plaque suspension.....	27
Figure 5. One-week-old oral anaerobic multispecies biofilms on a HA disc after incubation in BHI broth under anaerobic conditions.....	29
Figure 6. Confocal laser scanning microscope (FV10i-LIV, Olympus, ON, Canada)	30
Figure 7. A general view of the biofilm was obtained by CLSM.....	30
Figure 8. (A) A view by Imaris 7.2 software (Bitplane Inc, St Paul, MN) of a 3D reconstruction of CLSM image of one of the areas of the biofilm that was selected, (B) calculating the green volume (live bacteria) (C) calculating the red volume (dead bacteria).....	31
Figure 9. Overall mean percentage (bar: S.D.) of dead bacteria in one and three weeks old biofilms after one and three minutes exposure to the examined agents	35
Figure 10. Mean percentage (and S.D.) of dead bacteria in one-week old biofilms after 1 min exposure to the indicated substances.....	36
Figure 11. Mean percentage (and S.D.) of dead bacteria in one-week old biofilms after 3 min exposure to the indicated substances.....	36
Figure 12. 3D reconstruction of CLSM images of one-week old biofilms exposed for 1min. The agents used and the percentage of dead bacteria volume are shown.....	37
Figure 13. 3D reconstruction of CLSM images of one-week old biofilms exposed for three minutes. The agents used, and the percentage of dead bacteria volume are shown.....	38
Figure 14. Mean percentage (and S.D.) of dead bacteria in three weeks old biofilms after one minute exposure to the indicated substances.....	40
Figure 15. Mean percentage (and S.D.) of dead bacteria in three weeks old biofilms after three minutes exposure to the indicated substances.....	40
Figure 16. 3D reconstruction of CLSM images of three-weeks old biofilms exposed for one minute. The solution agents used, and the percentage of dead bacteria volume are shown.....	41
Figure 17. 3D reconstruction of CLSM images of three-weeks old biofilms exposed for three minutes. The solution agents used, and the percentage of dead bacteria volume are shown.....	42

List of Abbreviations

3D: Three Dimensional
BHI: Brain Heart Infusion
C. gileadensis: *Commiphora gileadensis*
Ca[OH]₂: Calcium hydroxide
CFU: Colony-Forming Unit
CHX: Chlorhexidine
CLSM: Confocal Laser Scanning Microscopy
DAP: Double Antibiotics Paste
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
EDTA: Ethylenediaminetetraacetic acid
EPS: Extracellular Polymeric Substance
ESEM: Environmental Scanning Electron Microscope
H₂O₂: Hydrogen peroxide
HA: Hydroxyapatite
HCl: Hydrochloric acid
hDPCs: Human Dental Pulp Cells
IKI: Iodine potassium iodide
LD: Lethal Dose
LPS: Lipopolysaccharide
MIC: Minimum Inhibitory Concentration
MTAD: Mixture of Tetracycline, an Acid and a Detergent
MTT: Microculture Tetrazolium Assay
NaOCl: Sodium hypochlorite
OD: Optical Density
PCR: Polymerase Chain Reaction
PDL: Periodontal ligament
RNA: Ribonucleic acid
rRNA: Ribosomal Ribonucleic Acid
SEM: Scanning Electron Microscopes
TAP: Triple Antibiotics Paste
ZOE: Zinc oxide eugenol

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Dedication

I dedicate this thesis to my parents, Sana Edrees and Waleed Abuljadayel. I am very grateful for their unconditional and unlimited support and advice, and especially my father because he suggested examining the plant. Also, I dedicate this thesis to my grandfather Ramzy Edrees who passed away 8 years ago, as well as my grandmother Sabah Qabish for their encouragement and help during my journey of education. Lastly, I wish to thank the rest of my family, siblings and brothers-in-law, Dr.Layla, Abdulmaleek, Rabab, Ramzi, Ibrahim, Dr.Abdulraheem and Dr.Hani for their all supports.

Chapter 1: Literature Review

1.1 Endodontic infections

One of the main objectives of endodontic treatment of apical periodontitis is to eliminate biofilm and bacteria from the root canal system. Endodontic infections are the result of bacterial invasion through the dentin and pulp, and these infections are the leading cause of pulp necrosis. The primary source of bacteria is the oral plaque microflora (1–3). Later, the necrotic pulp becomes a habitat for facultatively anaerobic and anaerobic bacteria (4,5). The etiology of apical periodontitis is well established in many studies, such as in the study published by Kakehashi et al in 1965 (1). In that experiment, the exposed Fisher rat's dental pulps healed and formed dentinal bridges when the rats were germ-free. However, when the rats had normal microflora (were not germ-free), the pulp was not able to heal but became necrotic instead. Therefore, pulp injury without bacteria is not sufficient to develop apical periodontitis (1). Furthermore, a different study done on monkeys supported the proposal that bacteria are the reason for periapical periodontitis. The pulp chambers of the monkey were divided into two groups, those aseptically necrotized and those infected by the oral flora. After 6 to 7 months, the monkeys were sacrificed and the root canal microflora and presence of any periapical pathosis were examined. The results showed that aseptically necrotized teeth did not develop apical periodontitis, whereas in those teeth where the root canal was intentionally infected by oral flora did (6). Moreover, Sundqvist's well-known thesis from the 1970's stated that apical periodontitis is associated with the presence of bacteria in the necrotic root canal and that infected teeth have a mixture of bacteria that are predominantly obligate anaerobes (3).

Endotoxins are one of the virulence factors of bacteria (7). In one study, the pulps of cat teeth were infected with endotoxin from *Escherichia coli*, while the teeth pulp of control animals

were exposed to saline only. After 6 weeks, the cats were sacrificed, and the teeth and surrounding bone were processed for histological examination. The results showed that apical inflammation was more intense for the endotoxin group than for the saline control group. Also, radiographic examination showed that teeth injected with endotoxin had larger apical radiolucency than teeth injected with saline. Therefore, it was concluded that bacterial endotoxin may have a role in the production of periapical inflammation lesions (7). Kantz and Henry conducted a study to investigate the presence of bacteria in non-vital intact teeth, samples were collected, and direct microscopic counts were calculated. The results showed that 92% of the samples contained bacteria (8). Another study was done to examine the pulps of non-vital teeth necrotized by trauma. Thirty-two of the forty teeth had bacteria in the samples taken from the necrotic root canals (9). While healthy pulps have always been considered sterile, a recent study revealed interesting results; under a strict disinfection protocol, ten pulp samples were removed from different patients with healthy, virgin vital teeth that were to be extracted at a later date for orthodontic purposes. The pulps of these teeth were accessed employing extreme care to avoid contamination from the oral cavity and tooth surface. Several control samples were obtained to demonstrate that the deep dentinal cavity was free of bacteria and microbial DNA before pulpal perforation. After perforation into the pulp tissue, the pulp tissue of each tooth was examined for the presence of bacterial DNA by employing universal 16S ribosomal RNA polymerase chain interaction primers and MiSeq sequencing. It was found that bacterial genetic material was present in all pulp tissues sampled. This is the first, and only, study reporting the detection of bacterial DNA in vital, healthy pulp tissue of teeth with no detectable decay, restorations, or periodontal infection. The source of the DNA was not studied, but the authors speculated that it originated from the healthy pulp tissue (10).

1.2 Endodontic bacteria

Anaerobic bacteria are the most dominant bacteria in the infected necrotic root canal system due to a lack of oxygen and the type of nutrients present in necrotic root canals (4,11). The quantities and types of microbes isolated from root canal infections are diverse among the literature due to the different methodologies that have been used. In 1957, Macdonald et al using aerobic and anaerobic culture methods found seventy-one bacterial strains, of which only twenty-three were anaerobic bacteria. Alpha streptococci and anaerobic gram-positive cocci were the most dominant bacteria (12). Sundqvist et al found eighty-eight strains of bacteria associated with necrotic pulp, including members of the genera *Bacteroides*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus*, and *Campylobacteria*. Furthermore, Byström and Sundqvist studied the effect of 0.5%NaOCl irrigation on root canal microbes. Regardless of the irrigation effect, they found that around 88% of the bacteria were anaerobic (13). Additionally, 68% of the bacteria were strictly anaerobic when apical 5 mm of infected root canals with pulpal exposure and periapical lesion were cultured and examined (14). Haapasalo et al reported that symptomatic and sinus tract cases were associated with synergistic action of mixed anaerobic flora (3,15). Later, with the advancement of techniques such as molecular methods and utilization of scanning electron microscopes (SEM), discovery of new bacteria has been achieved (16). Molven et al examined the most apical 2 mm of the root canal with SEM. They found that 88% of the samples had bacteria. The canals predominately harboured rod-shaped bacteria and to a lesser extent other types of bacteria such as filaments, cocci, and spirochetes (17). In one study, 16S rDNA directed polymerase chain reaction (PCR) revealed the presence of *Actinomycetales* species, *Fusobacterium nucleatum*, *Streptococcus miller*, and for the first time *Tannerella forsythia* (formerly *Bacteroides forsythus*) (18). Studies with 16S rRNA gene clone library examination discovered that 40-55% of the bacterial taxa present in primary root canal infections have not been cultivated and identified (19,20). Cultural and molecular studies

estimated that there are around three hundred and ninety-one bacterial, four fungal, and one archaeal taxa in primary endodontic infections; these included different groups of gram-negative and gram-positive bacteria (21). Persistent interradicular infection refers to bacteria that exist in the primary infection and resist the root canal treatment (22). One hundred and three bacteria and six fungal taxa were identified by culture and molecular methods after chemo-mechanical preparation. Most species in this category were Firmicutes, Proteobacteria, and Actinobacteria (16). Although gram-negative bacteria may be found as persistent bacteria, gram-positive bacteria (such as streptococci, *Parvimonas micra*, *Actinomyces* species, *Propionibacterium* species, *Pseudoramibacter alactolyticus*, lactobacilli, *Enterococcus faecalis*, and *Olsenella uli* were the most dominant) (16,23–25). Secondary infections involve bacteria that re-enter after the root canal treatment. There is less bacterial diversity in secondary infections than in primary endodontic infections (26). In addition, studies that have been done with cultural and molecular methods identified one hundred and fifty-eight bacterial and three fungal taxa at the time of root canal re-treatment (16). Moreover, endodontically treated teeth are approximately nine times more likely to have *E. faecalis* than non-root canal filled teeth (27). Furthermore, *Candida albicans*, a fungus, can be present in the secondary infection at up to 18% (28).

1.3 Biofilms

When microorganisms are able to aggregate and form a community, these structures are known as biofilms. A biofilm can form and grow anywhere, for example on rotten fruit and vegetables, uncleaned places and items, and as dental plaque on tooth surfaces and in necrotic root canals within the human body (29). The first observation of a biofilm was done 300 years ago by a Dutch scientist Anthony van Leeuwenhoek, who referred to it as animalcules (30). A few centuries later, Henrici observed, that bacteria in water do not float freely; they combine

and grow submerged from the surface (31). However, with the current advance in technology and devices, we can study biofilms in more detail. For instance, with confocal scanning laser microscopy (CLSM) can evaluate the biofilm's development, structure, viability, and sensitivity to antimicrobial agents in three dimensions and without destruction (32). Biofilms can be defined as a group of microorganisms attached to a surface, embedded in a self-produced extracellular polymeric substance (EPS) and separated by water channels (33,34). Bacteria make up nearly one-tenth of the biofilm's volume, while the remainder is EPS (35,36). The majority of EPS is made up of polysaccharides, proteins, nucleic acids, and lipids that are produced by biofilm cells (33). EPS has many functions and is considered as fuel for the biofilm. It facilitates adhesion of the biofilm to a surface, provides stability for the biofilm, and plays a role in gaining nutrients and digestion. Furthermore, it allows biofilms to function as a unit in terms of genetic exchange, quorum sensing, and pathogenic synergism. It can also be a nutrient bank in case there is a nutrient shortage. Finally, EPS makes biofilms stronger and more resistant, protecting against host defense cells and penetration of antimicrobial agents (35,37). EPS has the ability to modify the response of the bacteria to antimicrobial agents through a "diffusion shield" and a "reaction neutralizer". Also, because of EPS' structure and charged ions, it can limit the penetration of antimicrobial agents (38).

Bacteria living within a biofilm are "stronger" than planktonic bacteria, possessing 10 to 1000 fold resistance to stressors (39). As biofilms create a habitat for multiple bacteria, it increases metabolic diversity, better protection, communication between the bacteria, and genetic exchange (40). The resistance of the biofilm is related to the biofilm's maturation, and it has been stated that biofilms may take 10 days to become mature (41). However, in an in vitro study, dental plaque biofilms were grown for up to 8 weeks and antibacterial agents were employed against these biofilms. Results indicated that the biofilm's resistance increased in the third week of maturation and resistance against the antibacterial solutions remained higher

for the rest of the maturation time (42). Biofilms resist antibacterial agents in several ways. The outer layers protect the deep layers through waste accumulation or an altered environment (pH, pO₂, etc.). In addition, antibacterial agents may get trapped and destroyed by enzymes in the biofilm matrix. Microbial growth rates vary inside the biofilm; therefore, non-growing bacteria are resistant to many antimicrobial agents, such cells are known as persister cells. Furthermore, a biofilm under stress can regulate the expression of stress-response genes, shock proteins, and multi-drug pumps (efflux pumps) (43).

1.4 Eradication of endodontic biofilms

Many of the biofilms in the root canal system cannot be removed by mechanical instrumentation alone as the instrumentation alone leaves large areas of the canal walls untouched (44,45). These biofilm are located in specific areas of root canal configurations such as isthmus, fins, accessory, and lateral canals which cannot be reached by the instruments and therefore not removed mechanically (44,45). Furthermore, bacteria can be present in the dentinal tubules of infected teeth deep into dentin (46,47). Therefore, mechanical instrumentation alone is insufficient to remove all the debris and biofilm from the root canal system. Consequently, irrigation is crucial to create mechanical washing effect, shear forces by rapid liquid flow in addition to chemical effects to clean the root canal system of microbes and debris (48). Chemical irrigation agents must diffuse through the EPS matrix before they can attack the bacteria. Interruption of the EPS is induced by shear stress on the biofilm by the irrigation. Additionally, chemical agents could in different ways change the properties of the EPS, and thereby influence the elimination of the biofilm (49,50). It has been suggested that the mean surface relative effective diffusion coefficient declines from the surface of the biofilm toward the base. The diffusion coefficient varies for biofilms of different ages and usually

decreases with older biofilm (51). Also, the base level regulates the attachment to the dentin surface and is usually the most difficult one to eradicate (52).

1.4 Irrigation in endodontics

An ideal endodontic irrigant should be a lubricant for the instrument, able to dissolve organic and inorganic tissue, eliminate bacteria, and disturb the biofilm structure. It should not be irritating to vital periapical tissue, toxic, carcinogenic, or alter dentin physical properties. Moreover, it should be inexpensive, practical, easy to handle, not cause tooth discoloration, or affect the sealing ability of the filling materials (47,48,53). As there is no single product with all the desired properties, many different irrigants, such as sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), chlorhexidine digluconate (CHX), and QMiX, are used during root canal treatments (54).

1.5.1 Sodium hypochlorite (NaOCl)

A diluted NaOCl (0.5%) solution was used by a chemist, Dakin, and a surgeon, Alix, to disinfect wounds during World War I (55). In 1919, Coolidge recommended its use in endodontics as a major irrigant due to its strong antibacterial and tissue dissolving abilities (56). Sodium hypochlorite solution is inexpensive, easy to obtain, and has a decent shelf life (57). Therefore, these criteria made it the best widely used irrigation solution in root canal therapy. However, it also has a couple of drawbacks (58). Sultzky-Goldberg et al reported that concentrations of 2.5% and 6% NaOCl with exposure times of 10 or 20 minutes significantly reduce microhardness of human root dentin, compared to a saline solution (59). Furthermore, NaOCl can negatively affect the dentin bonding of resin. The marginal adaptation and dentin bond strength of direct composite restoration may be reduced after dentin is exposed to NaOCl (60). It can also increase microleakage between the dentin margin and composite restoration

(61). Regarding cytotoxicity, it was found that a ratio as low as 1:1000 of 5.25% NaOCl in saline can cause complete hemolysis of red blood cells in vitro (62). Moreover, complications may occur during NaOCl irrigation. For instance, damage to a patient's or clinician's eyes results in instant pain, intense burning, erythema, and abundant watering. The outer layer of the cornea may be affected by the loss of epithelial cells during the accident (63). Additionally, when NaOCl escapes beyond the apical foramen, serious complications such as tissue necrosis, severe pain, ecchymosis, hematoma, hypersensitivity, swelling, secondary infections, and paresthesia can happen (64–67).

1.5.2 Ethylenediaminetetraacetic acid (EDTA)

EDTA, first described by Ferdinand Munz in 1935, is colorless, odorless, water soluble, and has the ability to dissolve limescale (68). In 1957, chelator agents such as EDTA were introduced into endodontics by Nygaard-Østby to negotiate narrow and calcified canals. A few years later, EDTA became popular among endodontists during the cleaning and shaping of root canals. Additionally, EDTA in paste form (RC-Prep) was introduced by Stewart et al to reduce friction during the use of rotary files (69). EDTA used after NaOCl can remove the inorganic part of the smear layer in less than one minute by binding to calcium. EDTA also has a lower surface tension than 1-5% NaOCl and becomes a weak antimicrobial agent by binding to the metal ions in the bacterial or fungal cell envelope (69,70). In clinical practice NaOCl has been by many used a second time after EDTA, in order to reduce the bacterial load after removal of the smear layer. However, EDTA exposure followed by NaOCl can lead to serious erosion of the canal wall dentin and cause weakening of the root (71,72). In addition, NaOCl and EDTA should not be combined (mixed) because of a loss of tissue-dissolving ability and chlorine (73).

1.5.3 Chlorhexidine (CHX)

CHX was developed by the British company Imperial Chemical Industries in 1951 during attempts to create anti-malarial agents (74). CHX is a synthetic cationic bis-guanide (75). CHX solutions of 0.1 – 0.2% are recommended for plaque control as a mouthwash and are considered bacteriostatic, whereas 2% solutions are bactericidal and advocated for chemical root canal irrigation (76). As an antibacterial agent, CHX enters the bacterial cell through interaction with phospholipids and lipopolysaccharides in the cell membrane (77). Furthermore, CHX has a substantivity feature, i.e. it is absorbed by oral tissue such as the tooth surface and released when it is at a low concentration in the surrounding area (78). Different results have been reported in the published literature regarding the period of the substantivity. White reported the antimicrobial substantivity of 2%CHX can last for 72 hours, while 0.12%CHX can only last for 6 to 24 hours (79). Khademi et al stated that a 5 minutes application of 2%CHX caused substantivity for 4 weeks, whereas Rosenthal found that 10-minute exposure of dentin to 2% CHX can produce substantivity for up to 12 weeks (80,81). A concentration of 0.2%CHX has been evaluated in the infected root canal. There was a significant decrease in microbial count after irrigation and instrumentation using 0.2%CHX compared with 0.9% sodium chloride (82). Gomes et al compared different concentrations of CHX and NaOCl for their effectiveness in killing planktonic *E. faecalis*. NaOCl solution in concentrations of 0.5%, 2.5%, and 4% killed *E. faecalis* in 30, 10, and 5 minutes, respectively. For 2%CHX gel, 1 minute was needed to kill *E. faecalis*. However, for 0.2%, 1%, and 2% CHX in liquid form, and 5.25%NaOCl solution, it took less than 30 seconds to kill *E. faecalis* (83). It should be noted, however, that the methods in this and several other studies from this time era were not reported in full detail, and it is possible that e.g. presence of organic matter from culture media may have interfered with the killing and resulted in longer killing times.

Shen et al studied the effect of different CHX products and exposure times on various ages of hydroxyapatite oral biofilm by using a CLSM (42). It was shown that biofilms increased in thickness over time. Furthermore, mature biofilms were more resistance to CHX products than fresh biofilms. Also, CHX plus (a commercial CHX product with surfactant to reduce surface tension; Vista Dental, Racine, WI, USA) was more effective than 2 CHX. Exposure times of 1 and 3 minutes with 2%CHX were tested on 1- and 3-week-old biofilms; the 1 and 3 minutes exposure times killed between 60% and 70% of the 1-week-old biofilm bacteria, this decreased to 20% - 30% for the 3-week-old biofilm (42). Clegg et al studied the effect of 2%CHX and different concentration as of NaOCl on apical root dentin biofilm. It was found that 3% and 6% NaOCl solutions were capable of physically removing (detaching) the biofilms, whereas 1%NaOCl was capable of only disturbing the biofilm. However, the biofilm seemed fairly intact after 2% CHX exposure (84). Dentin and other substances can have an inhibitory effect on several root canal irrigants. Haapasalo et al found that dentin powder can reduce the antimicrobial effect of 0.05% and 0.5% CHX acetate (85). Another study reported that bovine serum albumin can totally inhibit the antibacterial effect of 0.05%CHX, while hydroxylapatite has no effect on it (86). One of the main drawbacks of CHX is that it has no effect on tissue dissolution. Neither 2%CHX liquid or gel could dissolve bovine tissue pulp within 6 hours (87). Furthermore, when 10%CHX was compared with 1%NaOCl, 3% and 30% hydrogen peroxide, 10% peracetic acid, and 5% dichloroisocyanurate, the results showed that only NaOCl dissolved organic tissue (88).

CHX can improve the dentin bonding system. It has an inhibitory effect on endogenous metalloproteinases found within the dentin (89). This inhibitory mechanism can preserve the hybrid layer, which is beneficial for dentin bonding (90). Carrilho et al reported that application of 2% CHX onto acid etched dentin significantly improved bond strength even after 6 months (91). Regarding the cytotoxicity of CHX, Tatnall et al found that NaOCl, CHX, and hydrogen

peroxide completely killed human fibroblasts, basal keratinocytes, and transformed keratinocytes lines (92). Another finding from a different study found that fetal bovine serum, lecithin, albumin, and heat killed *E. coli* decreased the cytotoxicity of CHX (93). These outcomes imply that potential cytotoxicity is low in the periapical tissue during root canal treatment. However, if CHX comes into contact with conjunctiva it may cause permanent damage (94,95). Lastly, a potential allergy from CHX is rare, but may occur (96).

1.5.4 QMiX

QMiX was developed by Haapasalo et al in 2011 (54). It contains small amount of CHX, a surfactant and EDTA (54). Stojicic et al investigated the antibacterial efficiency of QMiX, 2%CHX, 17%EDTA, MTAD, and 1% and 2% NaOCl, along with the smear layer removal ability of QMiX and 17% EDTA. It was found that QMiX and 1% NaOCl killed all planktonic *E. faecalis* in 5 seconds, while 2% CHX and MTAD were unable to kill all bacteria with exposure times of 30 seconds. In addition, QMiX and 2%NaOCl killed more biofilm bacteria than 1%NaOCl and 2%CHX. Furthermore, QMiX and 17%EDTA exhibited equal proficiency at removing the smear layer (97). In another study, different versions of QMiX were compared to 17% EDTA and its ability to remove the smear layer. SEM results showed that the different versions of QMiX removed significantly more of the smear layer than 17%EDTA (98). Lastly, QMiX was compared with EDTA and CHX regarding the efficiency of AH26 sealer penetration into the dentinal tubules after the use of these solutions as final irrigants—no significant differences were detected (99).

1.5.5 Iodine potassium iodide

Iodine was discovered in seaweed in the nineteenth century by Bernard Courtois who tried to extract potassium chloride from seaweed. William Prout was the first physician to use

potassium iodide for medical purposes, as a goiter remedy in 1816 (100). In subsequent years, iodine has been employed in the medical field for treating many diseases and as a disinfectant agent (101,102). In 1976, Torneck advocated the use of iodine solution in endodontics because of its antimicrobial properties (103). Generally, iodine potassium iodide (IKI) has been used as a root canal disinfectant agent. It has satisfactory antimicrobial properties, a better odor, and lower toxicity than NaOCl. (104) IKI also has the ability to penetrate the dentinal tubules up to 1000 μm when used for 5 minutes (70). In one study, IKI was used as a final irrigant for a group of patients who had failed endodontically-treated teeth, while calcium hydroxide ($\text{Ca}[\text{OH}]_2$) was used in a different group. It was shown that IKI prevented bacterial re-growth more than $\text{Ca}[\text{OH}]_2$ (28). In addition, *E. faecalis* was successfully killed when IKI was combined with $\text{Ca}[\text{OH}]_2$ in an in vitro study (105). However, in a clinical trial, 2%IKI decreased bacterial load by only 15%, while 2.5%NaOCl reduced it by 90% (104). The disadvantages of IKI include its inability to dissolve tissue, and some patients may be allergic to iodine (48).

1.5.6 Hydrogen peroxide (H_2O_2)

In 1968, drinking water in several countries was disinfected with low concentrations of H_2O_2 (106). It is known to have antimicrobial properties, stemming from the release of hydroxyl radicals which can attack membrane lipids, DNA and other crucial cell components (107). Interestingly, it has been reported that a synergistic effect could occur with a combination of H_2O_2 and CHX against *E. faecalis* (107,108). In contrast, mixing NaOCl and H_2O_2 can result in loss of chlorine which is needed for bactericidal activity, because chlorine and sodium hydroxide neutralize each other (109). There is a shortage of data in the literature supporting the use of H_2O_2 in endodontics (48). In addition, there are a few case reports that indicate facial

emphysema may be a rare side effect of using H₂O₂, due to the oxygen released during its use (110).

1.6 Interappointment medications

When a non-surgical root canal treatment cannot be done in a single visit, intracanal medication will be used for further disinfection, to limit bacterial regrowth, and as a physical barrier (111). The ultimate intracanal medicament would be antimicrobial, biocompatible, easy to place inside the canal system, enters the dentinal tubules, simple to remove, and has no systemic effects (112).

1.6.1 Calcium hydroxide (Ca[OH]₂)

Hermann introduced Ca[OH]₂ into endodontics in 1920 (113). Although the use of calcium hydroxide is well documented in the literature, the efficacy of this material is controversial. (114). Ca[OH]₂ has a high pH value, around 12.5, most bacteria cannot survive in a highly alkaline environment. Additionally, its antibacterial activity is related to the release of hydroxyl ions which cause damage to the cytoplasmic membrane and DNA of the bacteria (115,116). Furthermore, Ca[OH]₂ is able to denature proteins and detoxify the lipopolysaccharides (LPS) of gram-negative bacteria (117,118). Shuping et al investigated one-week application of Ca[OH]₂. They found that the bacterial load was reduced by 61.9% after chemo-mechanical instrumentation and 92.5% after a 1-week placement of Ca[OH]₂ (119). In a different study, the outcome associated with root canal treatment was better when Ca[OH]₂ was applied for 15 or 30 days, rather than 7 days (120). However, Ca[OH]₂ has limited activity against *E. faecalis* and *C. albicans*. Gomes stated that a 30-day application of Ca[OH]₂ could not disinfect bovine root dentin harboring *E. faecalis* (121). Furthermore, when different *Candida* species were exposed to saturated aqueous Ca[OH]₂ solutions, it was found that *C. albicans* was the most

resistant among the *Candida* species tested. Most of the species did not survive past 20 minutes to 3 hours of incubation with Ca[OH]₂, while 6 hours were required to kill *C. albicans* (122). Other limitations of Ca[OH]₂ use include partial inactivation of its antimicrobial effect by dentin, difficulty to remove it completely from the root canal system, and its interference with several endodontics sealers (85,123).

1.6.2 CHX gel

CHX is not only used in solution, it can also be used in gel form—as an interappointment medicament or irrigant. CHX gel contains a carboxymethylcellulose gel base and chlorhexidine digluconate with a pH value of 5.5–7 (124). As a endodontic irrigant, it has similar antibacterial effectiveness against *E. faecalis* as solutions of 2%CHX and 5.25%NaOCl (125). However, in a different study, both CHX gel and liquid kept the colony forming units (CFUs) of *E. faecalis* low for 7 days, while 5.25%NaOCl did not (126). Furthermore, as an intracanal medication, CHX gel showed better antibacterial activity than Ca[OH]₂. Bovine dentin roots were infected with *E. faecalis* and were then exposed to Ca[OH]₂, CHX gel, or a combination of CHX and Ca[OH]₂. It was revealed that Ca[OH]₂ could not kill the bacteria and produce a negative culture for the whole period of the experiment; however, the CHX gel did (121). In an in vivo study, samples were taken before and after cleaning and shaping of the root canals, and were then medicated for 7 days with Ca[OH]₂, CHX gel, or a combination of the two. Results indicated there was a greater reduction of the bacteria after cleaning and shaping, than with just interappointment medication alone; there was also no difference among the medicated groups (127). Moreover, in another study, CHX gel showed less antifungal activity than Ca[OH]₂. The minimum inhibitory zone of Ca[OH]₂ against *C. albicans* was greater than that of the CHX gel (128). However, agar diffusion tests are presently regarded as unreliable in predicting the in vivo antimicrobial potential of endodontic irrigants and disinfecting agents.

1.6.3 Combinations of interappointment medications

Despite the problems with agar diffusion tests with endodontic materials, the test was widely used in earlier studies. The assessment of CHX mixed with Ca[OH]₂ was performed in both in vitro and in vivo studies. Basrani found that the zone of inhibition for the CHX gel or solution on their own and for Ca[OH]₂ mixed with CHX were similar in their effect against *E. faecalis* (129). Furthermore, Evans showed that the combination of Ca[OH]₂ and CHX killed more *E. faecalis* in dentinal tubules than Ca[OH]₂ alone, based on CFU data (130). However, in a different in vitro study, it was stated that mixing Ca[OH]₂ with either CHX or saline did not increase the zone of inhibition of Ca[OH]₂ against *E. faecalis* and *C. albicans*. Also, it was noticed that the zone of inhibition of CHX mixed with Ca[OH]₂ was smaller than with CHX alone (131). Presently, the results from these agar diffusion studies, however, are regarded to reflect mainly the diffusion properties of the different chemicals in the artificial agar environment while having little or no predictability for their effectiveness in vivo. The situation is quite different from the testing of systemically used antibiotics in agar diffusion tests, where inhibition zones on the agar plate of each antibiotic correlate with their known effective tissue concentrations in vivo in clinical use against specific bacteria. With endodontic materials, no such data is available.

The efficacy of mixing Ca[OH]₂ and CHX has been assessed in an in vivo study. It was revealed that there was no difference in the reduction of CFUs, after 1 week of medication, among the mixed samples, or when Ca[OH]₂ and CHX gel was administered on their own (127). The combination of calcium hydroxide and other antibacterial agents was also evaluated in different studies (105,132,133). IKI was added to Ca[OH]₂ to investigate the killing of *E. faecalis* by measuring the distance of disinfection within the bovine dentinal tubules. The killing of *E. faecalis* was enhanced when a combination of IKI and Ca[OH]₂ was used, rather than Ca[OH]₂ alone. Nevertheless, IKI alone had a longer depth of disinfection than Ca[OH]₂ or IKI mixed

with Ca[OH]₂ (105). Furthermore, using the CFU method, iodoform and silicone oil were added to Ca[OH]₂ to study human maxillary canines infected with *E. faecalis*. It was shown that the iodoform, silicone oil, and Ca[OH]₂ combination had the most dentinal tubule disinfection, followed by a combination of IKI and Ca[OH]₂ and then Ca[OH]₂ alone (133). Moreover, Ca[OH]₂, IKI, and a combination of ciprofloxacin, flagyl, and Ca[OH]₂ were examined against several bacteria through the minimum inhibitory concentration (MIC) technique. The medicament combination was able to kill all bacteria, whereas IKI was unable to kill *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and Ca[OH]₂ could not eliminate *P. aeruginosa* (132).

1.6.4 Antibiotics

Hoshino et al and Sato et al suggested disinfecting the root canal system with antibiotics, with the most common form being triple antibiotics paste (TAP) (134,135). TAP consists of ciprofloxacin, minocycline, and metronidazole (135). In one in vitro study, one-day-old multispecies oral anaerobic bacteria was exposed to TAP, Ca[OH]₂, and CHX for 7 days. The percentage of dead bacteria was the highest in the TAP group, followed by CHX and then Ca[OH]₂ (136). In a different experiment, MIC and agar well diffusion assay methods were used to assess TAP mixed with either saline or CHX, different antibiotics, and Ca[OH]₂ either mixed with saline or CHX. It was shown that TAPs mixed with either saline or CHX had similar inhibition zones, and they were larger than Ca[OH]₂ alone or mixed with CHX. In addition, TAP combined with saline had the highest MIC among all the other groups (137). Furthermore, a study was performed on dogs' teeth that had necrotic pulp and apical periodontitis. Samples were taken before and after the chemo-mechanical phase, and after 2 weeks of TAP medicament. There were significant decreases in CFUs in both phases — after the cleaning and shaping and after the period of medication with TAP (138). However, in a

different in vivo study, TAP was compared to a combination of Ca[OH]₂ and CHX used as an interappointment medicament for 3 weeks, and bacterial identification and counting were assessed with PCR (16S rRNA) and CFUs between dental appointments. The results revealed no difference in bacterial count between the groups after the medication period. There was also no relation between the identification of the bacteria and the intracanal medication that had been used (139). Disadvantages of TAP include the possibility of bacterial resistance arising and discoloration of teeth due to minocycline. Therefore, another version of the antibiotic paste was introduced, dual or double antibiotic paste (DAP), which contained only ciprofloxacin and metronidazole (140). It was found that DAP and TAP had similar effects against *E. faecalis* and *Porphyromonas gingivalis* biofilms. Thus, the authors suggested DAP can be a suitable substitute for TAP (141). However, Latham discovered that TAP killed significantly more bacteria than DAP and Ca[OH]₂ in an in vitro study (142). Finally, the higher concentration of TAP, DAP, or Augmentin antibiotic pastes, the lower the survival rates of human stem cells in the apical papilla (143).

1.7 Alternative medicine

Search for the curing power of plants occurred already in a prehistoric era. There is evidence that 60,000 years ago Neanderthals used plants such as hollyhock as a remedy, and this is still used around the world (144). Even the Abrahamic religions have mentioned many plants and herbs that are beneficial for human health. It is estimated that there are approximately half a million plant species on earth (145,146). Surprisingly, only 1–10% of these plants are being used by humans and animals, and only 1% have been phytochemically studied (144,147,148). In many parts of the world, traditional medicine has been used for thousands of years for various diseases. During the first century, Dioscorides wrote "De Materia Medica", which is a medicinal plant directory that became a model for modern pharmacopeias (147,149). In

developing countries, approximately 80% of people use traditional medicine for their health care (144). The bioactive compounds that have been extracted from numerous plants are sources for many chemical pharmaceutical drugs (150). Roughly 25% of these drugs contain one or more components from plants (145). Increasing resistance of microbes to drugs such as antibiotics and antiviral agents has led to an increasing interest in new drugs to combat this resistance, especially ones that are plant-based. Furthermore, plant-based medications tend to be less expensive and have fewer side effects than synthetic drugs (146).

1.8 Plants in dentistry and endodontics

More than 1400 years ago, prophet Mohammed recommended the use of a stem from the Meswak tree (*Salvadora persica* L) several times per day for teeth brushing (151,152). There are abundant studies that have examined the bioactivity of natural plants components against oral bacteria and inflammation (153). There are several daily-use dental products that have some plant extract components. For instance, the eugenol from zinc oxide eugenol (ZOE) is extracted from clove oil (*Syzygium aromaticum*) (154). Furthermore, the dominant endodontic obturation material Gutta-percha is extracted from *Palaquium gutta*, *Dichopsis gutta* and *Isonandra gutta* trees (155). In the endodontics literature, there are several studies that investigate plants to assess cleaning and disinfection, pulp and dentin repair, sealer cements, and storage media (156). The antibacterial property of *Glycyrrhiza glabra* was assessed against numerous bacteria including *E. faecalis*, *Streptococcus mutants*, and *Actinomyces viscosus*. MIC assays were used to compare its antibacterial properties with those of CHX. It was found that both materials gave similar results (157). Furthermore, *Salvadora persica* L extraction showed antibacterial effect against *Streptococcus salivarius*, *S. sanguis*, *Lactobacillus vulgaris*, *C. albicans*, and *E. faecalis* (158,159). Regarding pulp-dentine complex repair, it was found that Genipin, a substance extracted from gardenia fruit, stimulates odontogenic

differentiation of human dental pulp cells. Genipin increased alkaline phosphatase activity, odontogenic markers, and mineralized nodule formation (160). Calcium silicate sealer was modified by adding hinokitiol, which is a component derived from the wood of the *Cupressaceae* family. The modified hinokitiol not only exhibited suitable setting times and superior stimulation of the odontoblastic potential of human dental pulp cells (hDPCs), but also inhibited pro-inflammatory cytokines; it also exhibited improved cytocompatibility and antimicrobials activities (161). Aloe Vera, in different concentrations, was investigated as a storage media in supporting periodontal ligament (PDL) cell viability. Results indicate there was no significant difference between Aloe Vera and Dulbecco's Modified Eagle Medium (DMEM) when it came to maintaining PDL viability up to 9 hours after tooth extraction (162). Grapefruit, tangerine, lime, and lemon oils were compared to chloroform as solvents for gutta-percha; however, chloroform was the best gutta-percha solvent compared to the other plant-based solvents (163).

1.9 *Commiphora gileadensis*

The genus *Commiphora* has 190 plant species and comes from the *Burseraceae* family. They are allocated in southern Arabia (Saudi Arabia, Yemen, Oman), northeastern Africa (Sudan), and other parts of the world such as India and Pakistan (164,165). The *Commiphora* genus is composed of little trees or shrubs. It has held great economic significance since ancient times and it was also said that the plants have been used for many human diseases (166). Their aromatic resin (Myrrh) has been used for incense, food, and medicinal products (167). Furthermore, the plant species had been mentioned in the Bible several times. For instance, it was mention when God spoke to Moses "Take thou also unto thee principal spices, of pure myrrh five hundred shekels, and of sweet cinnamon half so much..." (Ex 30:22-33).

Commiphora gileadensis (*C. gileadensis*) is one such species in this plant's family (syn *Commiphora opobalsamum*); it is also known as balsam or Bisham (Fig 1). This mid-size scented shrub grows in dry and semi dry areas like the Red Sea region and other locations such as Oman, Palestine, and Yemen (168,169). *C. gileadensis* has antimicrobial activities that prove its usage in the local treatment of wound infection, according to Iluz. *E. coli* and *Bacillus cereus* were exposed to the plant's sap using the Kirby-Bauer method. The inhibition zone was 12 mm for *B. cereus* but *E. coli* was not affected (170). When *C. gileadensis* was extracted by 95% methanol and dissolved with 100% DMSO the antimicrobial activity was determined using MIC, it was found that *C. gileadensis* was greater than Ampicillin for a number of oral bacteria, such as *S. mutans*, *S. salivarius*, *Lactobacillus casei*, etc. However, *C. gileadensis* showed less diameter of zone inhibition by using the agar well diffusion method (171). In addition, the plant showed antimicrobial action against *S. aureus*, *P. aeruginosa*, *Klebsilla pneumoniae*, and *Candida* species using the modified agar well diffusion method, but did not have antimicrobial properties against *Staphylococcus haemolyticus* or *E. coli* (172). Furthermore, it has been observed that *C. gileadensis*, when dissolved in methanol/methylene chloride, showed some antibacterial action against *E. faecalis* and *P. aeruginosa*. When the extract was dissolved in water, inhibition zones were observed for *S. aureus*, *Bacillus*, and *P. aeruginosa* (173). Modern methods using polymicrobial biofilms and non-disruptive measuring of microbial killing by viability staining and confocal microscopy have not been used in the studies of the antimicrobial effects of *C. gileadensis* extracts.

It has been mentioned that monoterpenoid hydrocarbons and oxygenated monoterpenoids are the major components in the essential oils of this plant. Many studies have suggested that chemical compounds such as β -caryphyllene, α -pinene, sabinene, β -pinene, p-cymene, limonene, γ -terpinene, and terpinen exhibit antibacterial activities (174–178). Moreover, they can be found in *C. gileadensis* (179,180). However, two different studies showed *C. gileadensis*

had diverse compositions, suggesting a wide variation of the chemical structures within the population of this species (179,181). β -caryphyllene can be found in many plants such as *cannabis sativa*, clove, and rosemary (182–184). Yoo investigated β -caryphyllene's effect on *S. mutans* biofilm using CLSM and CFU techniques. The biofilm was exposed to 1.25% β -caryphyllene and 0.2% chlorhexidine for 10 minutes. It was revealed that both agents significantly killed and reduced bacterial colonies more than water which was used as a negative control (185). Sabinene and α -pinene are other chemical compounds and they can be found in holm oak, black pepper, and pine, as well as in *C. gileadensis* (186–188). An interesting study combined sabinene and α -pinene compounds and explored their effects on bacteria, fungi, and yeast. They found this combination created inhibition zones for *E. coli*, *P. aeruginosa*, *Corynebacterium* sp., *S. aureus*, *B. cereus*, *Alternaria* sp., *Aspergillus nidulans* and *niger*, and *C. albicans*. The inhibition zones were greater than those observed using antibiotics such as clindamycin, tetracycline, erythromycin, vancomycin, and ampicillin (189).

When the aqueous extract of this plant was intravenously injected into rats, they experienced a 20% drop in systemic atrial blood pressure and a 14% decrease in their heart rate (190). Moreover, oral administration of a *C. gileadensis* solution to rats significantly decreased anti-inflammatory reactions, as it has an antipyretic and diuretic effect (191). Al-Mahbashi et al showed that the plant has no toxicity by using the lethal dose (LD)₅₀ test. It was revealed that up to a 5000 mg/kg extract of *C. gileadensis*, extracted by methanol and dissolved in water, administered to albino mice was not toxic; however, there were reversible reductions in motor activities starting from 2500 mg/kg (172). Substances from the stem of this tree provided a selective apoptosis activator against tumor cell lines, such as mouse lymphoma cells and Epstein-Barr virus transformed human B lymphocytes, during experiments using MTT assays; it was also discovered that the apoptosis only occurred in tumor cells and not normal human

skin fibroblast (179). Moreover, the ethanolic extract of this plant showed apoptosis in transformed and immortalized human epidermal cell lines but did not affect normal dermal fibroblasts. They suggested that these cells did not divide after exposure to *C. gileadensis* extract solutions. Notably, apoptosis was happening during the S or G2 phases of the cell cycles, demonstrating that *C. gileadensis* toxicity targeted only mitotic cells and was cell cycle specific. Therefore, they proposed that apoptosis only happened to tumor cells because of their fast replication time and increase in mitotic stages (192). Furthermore, in a different study, two chemical compounds extracted from the plant exhibited moderate cytotoxicity against human prostate cancer cell lines (193). However, in a different study, the plant showed non-selective cytotoxicity for tumor cell lines and minimal toxicity toward normal monkey kidney fibroblast and pig kidney epithelia cells (194).



Figure 1. (A) *Commiphora gileadensis* shrub. (B) *Commiphora gileadensis* branch

Chapter 2: Aim and Objectives

The aim of this study was to investigate the in vitro antimicrobial effect of *C. gileadensis* extract in an oral multispecies biofilm model. The objectives were to:

1. Compare the antimicrobial effectiveness of *C. gileadensis* extract on 1- and 3-week-old oral biofilms.
2. Compare different concentrations and methods of extraction for *C. gileadensis* extract on 1- and 3-week-old oral biofilms.
3. Compare *C. gileadensis* extract to 2%CHX and water.

Study hypothesis

Null Hypothesis: *C. gileadensis* extract is equally effective against oral multispecies biofilms as 2%CHX.

Chapter 3: Materials and Methods

3.1 Pilot study

C. gileadensis extract in concentration 1mg/ml water was used in pilot experiments, against 1 week and 3 weeks old biofilms. The materials and methods were the same of the main experiment. 2%CHX and sterilized distilled water were used as control groups, and only one biofilm sample grown on sterilized, collagen coated hydroxyapatite (HA) disc was examined for each group in the pilot experiments.

3.2 Plant preparation

Dried branches of *C. gileadensis* were collected from the Makkah region in Saudi Arabia. The substances in the branches were extracted in the Biological Services Laboratory in the Department of Chemistry at the University of British Columbia. The tree branches were initially cut into small pieces by scissors and then a blender was used with methanol as the solvent. The methanol/stem mixture was soaked overnight. The suspension was centrifuged (Sorvall RC5-B) at 3900 rpm at room temperature for 10 min. The supernatant was dried using a Savant SpeedVac Concentrator, during the drying methanol evaporates and is no longer present in the extract. Aliquots of dry extract were suspended in distilled water and in solutions with different concentrations of Dimethyl sulfoxide (DMSO) (Fig 2). The following *C. gileadensis* extract solutions were prepared: 10 mg/ml water, 0.1 mg/ml water, 1 mg/ml 0.5%DMSO, and 0.1 mg/ml 0.5%DMSO (Fig 3).



Figure 2. *Commiphora gileadensis* methanol extract in a dry condition before being dissolved in distilled water or 0.5%DMSO.

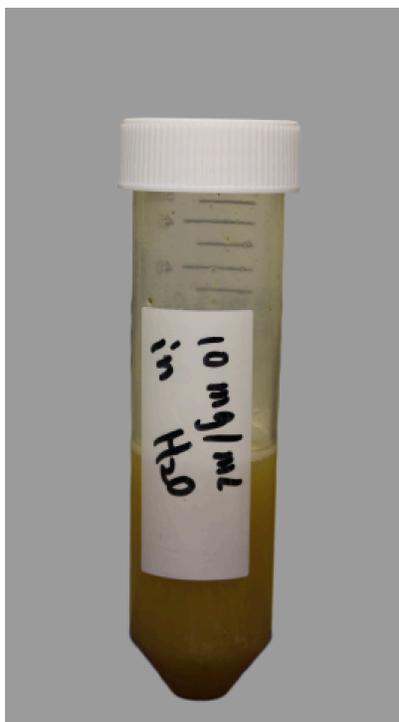


Figure 3. *Commiphora gileadensis* extract, 10 mg/ml water.

3.3 HA Disc coating and biofilm preparation

Sterilized HA discs (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, Williamsport, PA) were immersed in coating solution at 4°C for 24 h in a 24-well tissue culture plate. The coating solution contained sterilized distilled water, HCl (1 mol/L), and bovine dermal collagen type I. Subgingival and supragingival plaque was collected from two volunteers. (Human ethical study permission number: H15-02793). The plaques were mixed in sterilized brain heart infusion (BHI) broth with an optical density value between 0.08–0.12 (595 nm, 150 µL; polypropylene microtiter plates, Corning, NY). Then, the plaque in BHI was diluted 10 times in sterilized BHI. All discs were immersed in sterilized water for 1 min before being transferred into 2 ml of the plaque suspension in BHI (Fig4). The discs were placed in a 24-well tissue culture plate, sealed in a plastic bag containing an anaerobic conditioner (Thermo Scientific™ Oxoid™ AnaeroGen™ Compact Sachet, Hampshire, UK) and an oxygen level indicator (Oxoid™ Resazurin Anaerobic Indicator, Hampshire, UK). The discs were incubated at 37°C for 1 or 3 weeks. The BHI broth was refreshed once every week for the biofilm specimens that were incubated for 3 weeks.

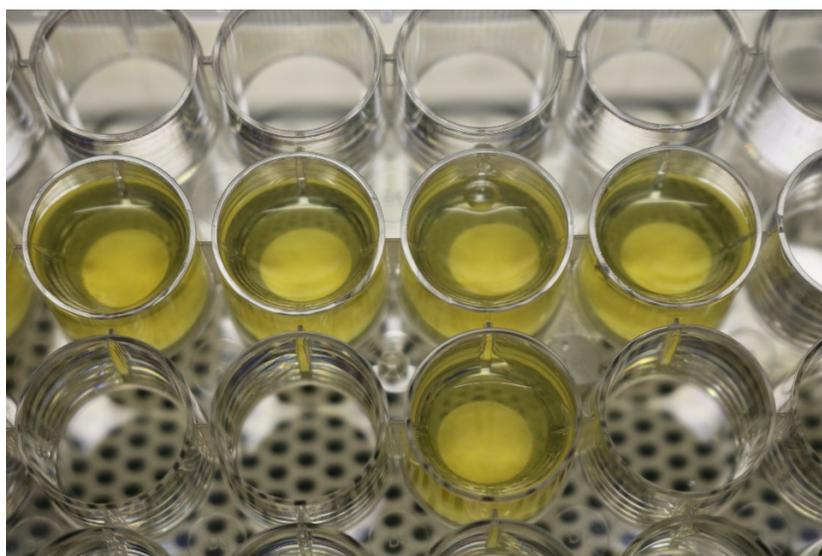


Figure 4. HA discs immersed in BHI-plaque suspension.

3.4 Antibiofilm solutions and experimental procedure

The biofilms were allowed to mature for 1 week or 3 weeks in BHI broth under anaerobic conditions (Fig 5). The samples were divided into seven groups according to the agents being used in biofilm exposure. In addition, each biofilm was exposed for 1 and 3 minutes to one of the agents studied. The groups were as follows:

- I. *C. gileadensis* extract - 1 mg/ml distilled water
- II. *C. gileadensis* extract - 0.1 mg/ml distilled water
- III. *C. gileadensis* extract - 1 mg/ml 0.5%DMSO
- IV. *C. gileadensis* extract - 0.1 mg/ml 0.5%DMSO
- V. 2%CHX (positive control)
- VI. 0.5%DMSO (positive control)
- VII. Sterilized distilled water (negative control)

Before the biofilm disc was exposed to the agents, the disc was immersed in sterilized distilled water for 1 minute to wash off the BHI broth. The selected agent (60 μ l) was gently applied to the biofilm using micropipettes, for either 1 or 3 minutes. The biofilm disc was once again placed in sterilized water for 1 minute to wash off the agent. A viability stain (50 μ l) was applied with micropipettes on the biofilm and the disc was placed in the dark for 5 minutes (LIVE/DEAD BacLight Bacterial Viability kit L-7012, Molecular probe, Eugene, OR, USA). The viability stain contained SYTO 9, propidium iodide, and phosphate buffered saline (PBS), and was prepared according to the manufacturer's instructions. Finally, the disc was immersed in phosphate buffered solution for 1 minute to remove the remnants of the dyes and to stabilize the biofilm.

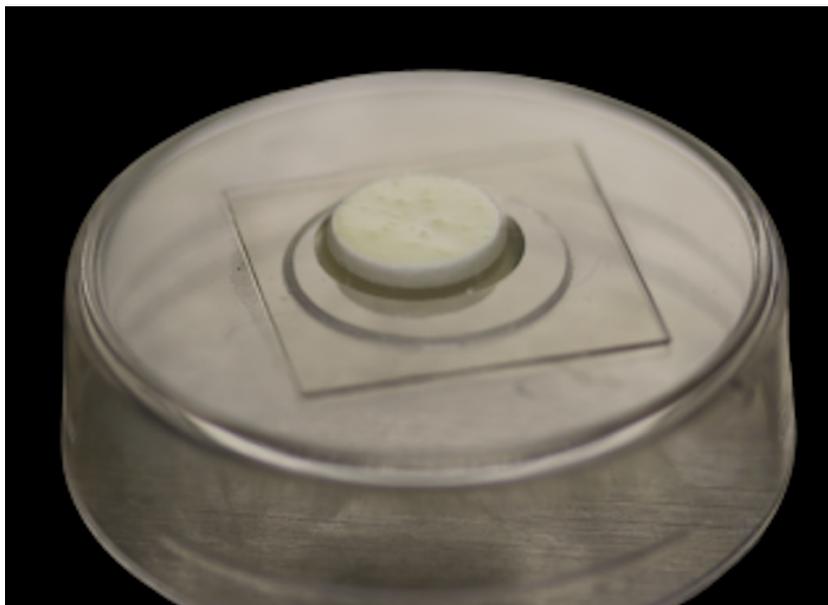


Figure 5. One-week-old oral anaerobic multispecies biofilms on a HA disc after incubation in BHI broth under anaerobic conditions.

3.5 Confocal laser scanning microscopy (CLSM)

The dyes mentioned above emitted fluorescence and were used for evaluation. The excitation/emission maxima were between 480/500 nm for the SYTO 9 stain and 490/635 nm for the propidium iodide stain. The treated biofilm disc was immediately placed in the CLSM chamber for scanning (FV10i-LIV, Olympus, ON, Canada) (Fig 6). The confocal microscope was attached to FV10i 2.1 software with a setting attuned to viability stain dyes. The machine automatically scanned the biofilm disc and a general view was obtained (Fig 7). The biofilm was observed under live scanning mode and five random areas were chosen. For standardization, the laser output was 3% for all the scanning areas. The photomultiplier (green & red) was adjusted to $50 \pm 10 \%$. The CLSM completed the scan for the five areas in 8 minutes (on average) for each disc.



Figure 6. Confocal laser scanning microscope (FV10i-LIV, Olympus, ON, Canada).

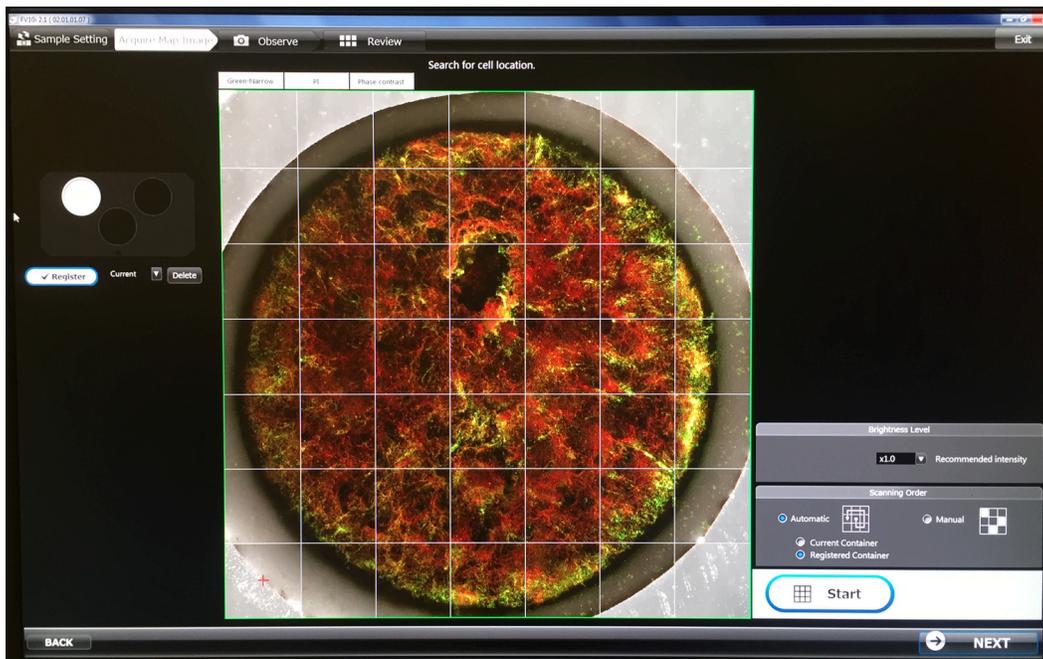


Figure 7. A general view of the biofilm was obtained by CLSM.

3.6 Image analysis

The scanned biofilm was evaluated for live and dead bacteria using Imaris 7.2 software (Bitplane Inc, St Paul, MN). The total volume of dead bacteria for each selected area was calculated as dead bacteria (red area) divided by the total volume of the biofilm (live and dead, or red and green) (Fig8).

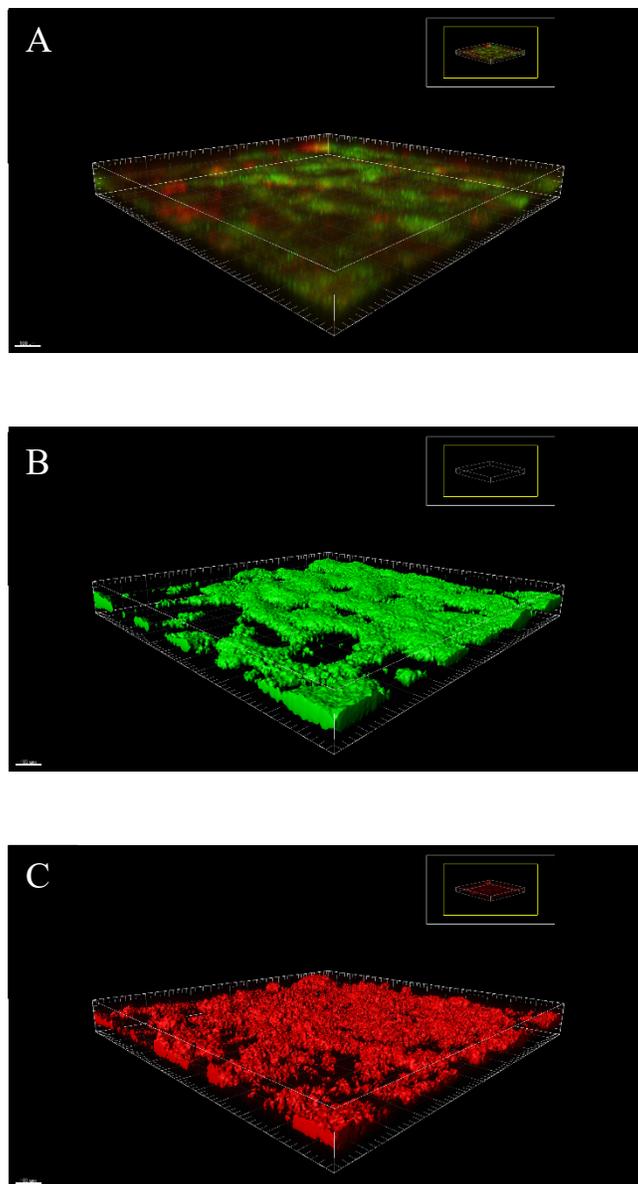


Figure 8. (A) A view by Imaris 7.2 software (Bitplane Inc, St Paul, MN) of a 3D reconstruction of CLSM image of one of the areas of the biofilm that was selected, (B) calculating the green volume (live bacteria), (C) calculating the red volume (dead bacteria).

3.7 Statistical analysis

Sample size was determined by using an online sample size calculator (<https://www.anzmtg.org/stats/PowerCalculator/PowerANOVA>). Using power = 0.90, effect size = 0.4, and number of groups/sub-groups = 28, it was revealed that 7 samples were needed for each group at a 0.05 significance level. Each HA disc provided 5 samples, resulting in 2 discs for each group. Univariate analysis of variance SPSS V.21 (SPSS INC. Chicago, IL) was used to assess the effect of the age of the biofilm and the exposure time. Furthermore, the post-hoc Tukey test SPSS V.21 (SPSS INC. Chicago, IL) was used for multiple comparisons among the agents.

Chapter 4: Results

4.1 Pilot study results

C. gileadensis extract at 1mg/ml in water killed significantly more bacteria in biofilm (46.1-61.1% \pm 5.1-10.4) than 2%CHX and water groups (P<0.05). Furthermore, 2%CHX significantly killed more bacteria than water (24.7-32.5% \pm 6.8-10.3) (P<0.05) (Table 1).

Group	1-week-old biofilm		3-week-old biofilm	
	1 min	3 min	1 min	3 min
<i>C. gileadensis</i> 1mg/ml water	46.10% \pm 8.71	46.20% \pm 5.11	61.10% \pm 10.39	47.31% \pm 9.41
2%CHX	N.D.	31.50% \pm 10.26	32.61% \pm 6.79	24.67% \pm 9.21
Water	2.70% \pm 1.35	N.D.	1.32% \pm 1.43	2.75% \pm 2.20

N.D. The sample was not done.

Table 1. Mean percentage of dead bacteria (\pm standard deviation) in one- and three-week-old biofilms after different exposure times; pilot results.

4.2 General findings

A total of 230 biofilm areas were scanned. Exposure of the biofilms to *C. gileadensis* extracts containing 1mg/ml water had the highest percentage of dead bacteria (42.13–46.67% \pm 4.5–8.0) among all the groups, regardless of the age of the biofilm and the time of exposure to the agents (1 or 3 mins), and the difference was statistically significant (P<0.05). Furthermore,

0.5%DMSO and sterilized distilled water used as controls killed fewer biofilm bacteria than any other groups (P<0.05) (Table 2 and Fig 9).

Group	1-week-old biofilm		3-week-old biofilm	
	1 min	3 min	1 min	3 min
I. <i>C. gileadensis</i> 1mg/ml water	46.67% ± 6.15	46.29% ± 4.58	43.25% ± 8.07	42.13% ± 6.72
II. <i>C. gileadensis</i> 0.1 mg/ml water	30.12% ± 8.93	38.53% ± 6.48	27.29% ± 11.66	28.32% ± 3.88
III. <i>C. gileadensis</i> 1mg/ml 0.5%DMSO	25.90% ±7.69	30.32% ±6.37	21.97% ±3.77	24.71% ±4.26
IV. <i>C. gileadensis</i> 0.1mg /ml 0.5%DMSO	29.126% ±6.91	30.69% ±8.18	10.53% ±3.24	22.51% ±3.68
V. 2%CHX	22.82% ± 8.33	28.90% ± 8.962	31.54% ± 7.94	26.68% ± 10.58
VI. 0.5%DMSO	12.12% ± 6.91	17.92% ± 8.76	9.95% ± 4.07	12.28% ± 4.17
VII. Water	2.4% ±1.76	4.5% ± 4.40	3.0% ± 2.33	2.75% ± 2.20

Table 2 Mean percentage of dead bacteria (± standard deviation) in one- and three-week-old biofilms after one and three minute exposure.

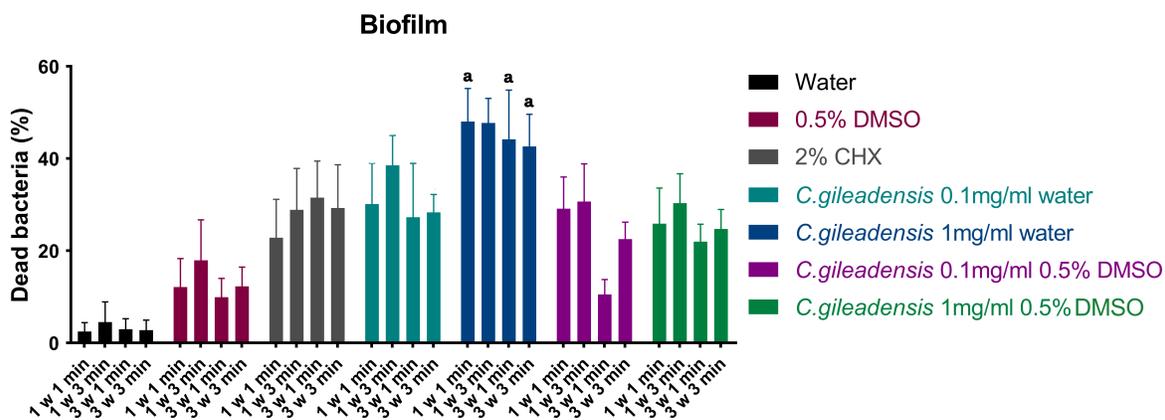


Figure 9. Overall mean percentage (bar: S.D.) of dead bacteria in one and three weeks old biofilms after one and three minutes exposure to the examined agents. Small superscript letters indicate statistically significant differences between the groups, $p < 0.05$ (Tuckey test).

4.3 The effect of the agents on 1-week-old biofilm

For 1-week-old biofilm and 1- or 3-minute exposure of the biofilms to the agents, the results indicated that all agents killed significantly more bacteria than water ($P < 0.05$).

After one minute of exposure, 2%CHX, *C. gileadensis* extract 0.1mg/ml water, *C. gileadensis* extract 0.1 mg/ml 0.5%DMSO, and *C. gileadensis* extract 1 mg/ml 0.5%DMSO did not show significant differences ($P > 0.05$). Exposure to water and 0.5%DMSO resulted in the lowest percentage of dead bacteria, with no significant difference between the two ($P > 0.05$) (Fig 12).

After three minutes of exposure *C. gileadensis* extract 1 mg/ml water killed significantly more bacteria than any other compound ($P < 0.05$), with the exception of the *C. gileadensis* extract 0.1 mg/ml water, where the difference was not significant ($P > 0.05$). Thus, the *C. gileadensis* extract 0.1 mg/ml water killed more bacteria than 2%CHX, *C. gileadensis* extract 1 mg/ml 0.5%DMSO, or *C. gileadensis* extract 0.1 mg/ml 0.5%DMSO solutions, however the difference was not significant ($P > 0.05$), as shown in (Fig 11).

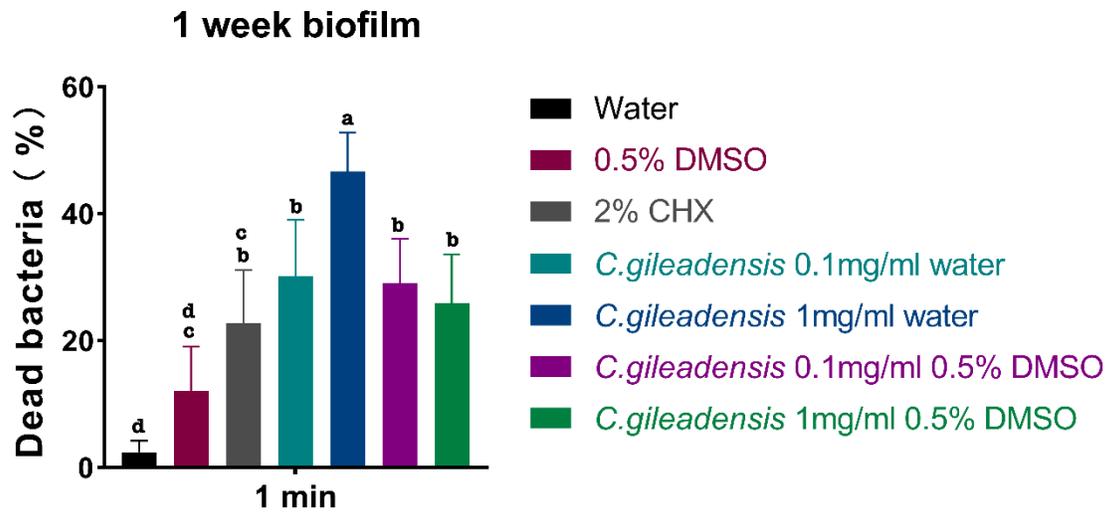


Figure 10. Mean percentage (and S.D.) of dead bacteria in one-week old biofilms after one minute exposure to the indicated substances. Small superscript letters indicate statistically significant differences between the groups, $p < 0.05$ (Tuckey test).

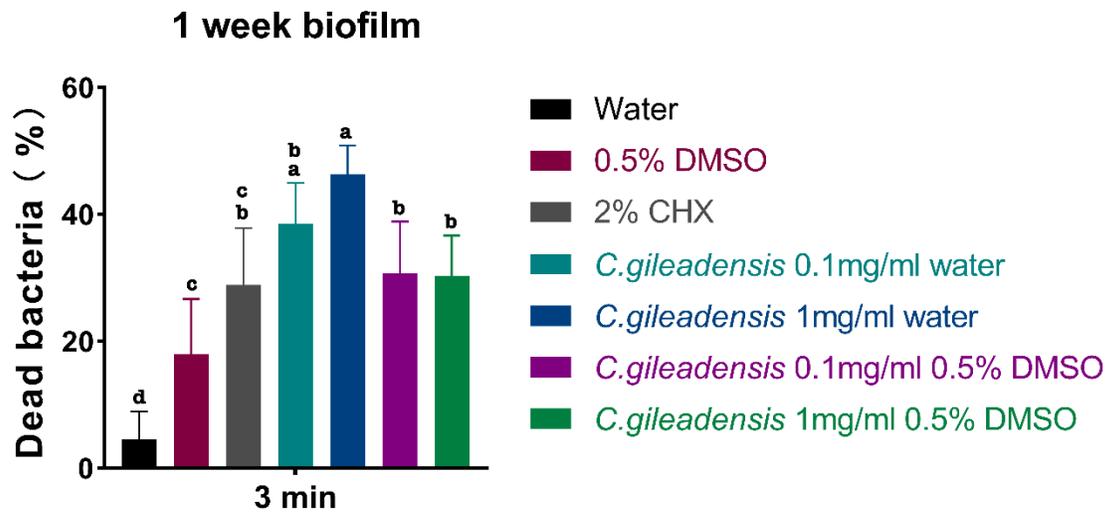
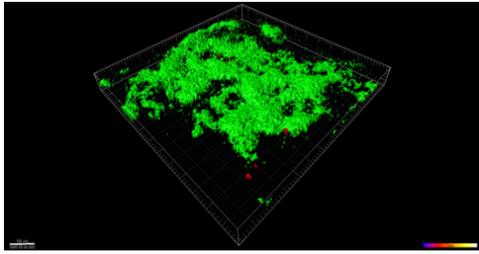
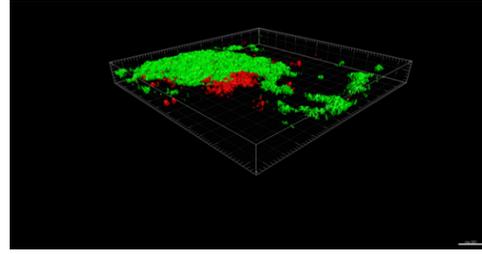


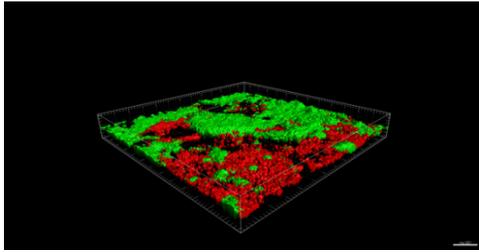
Figure 11. Mean percentage (and S.D.) of dead bacteria in one-week old biofilms after three minutes exposure to the indicated substances. Small superscript letters indicate statistically significant differences between the groups, $p < 0.05$ (Tuckey test).



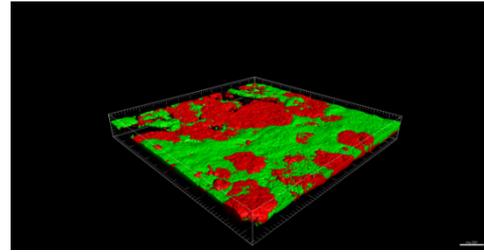
water 0.97%



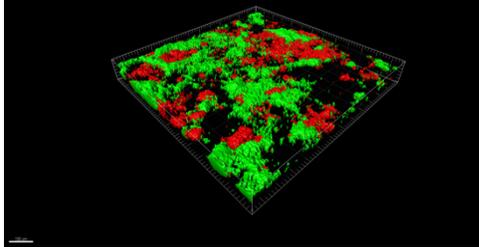
0.5% DMSO 7.86%



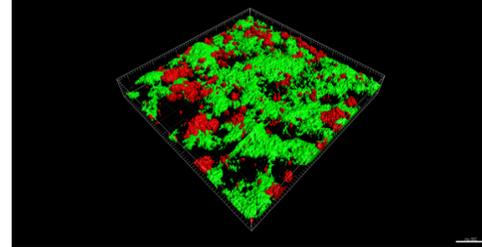
2% CHX 31.45%



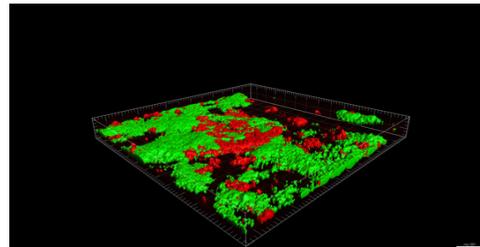
***C. gileadensis* 0.1mg/ml water 35.14%**



***C. gileadensis* 1mg/ml water 46.94%**

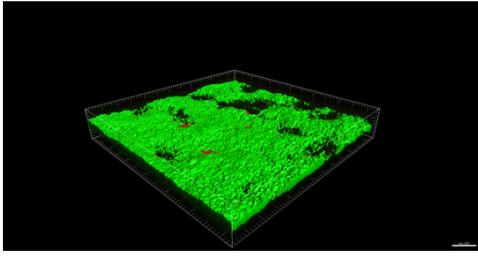


***C. gileadensis* 0.1mg/ml 0.5% DMSO 28.80%**

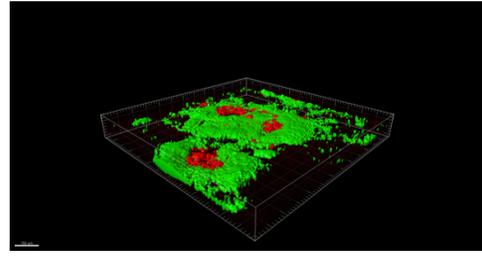


***C. gileadensis* 1mg/ml 0.5% DMSO 21.64%**

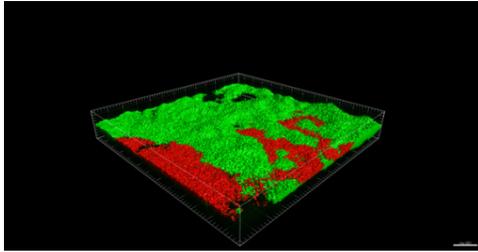
Figure 12. 3D reconstruction of CLSM images of one-week old biofilms exposed for one minute. The agents used, and the percentage of dead bacteria volume are shown.



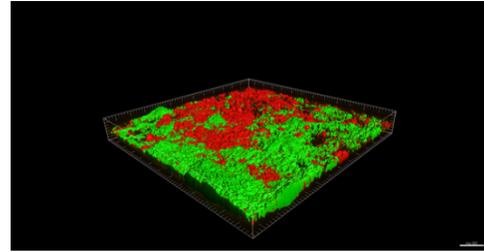
Water 6.57%



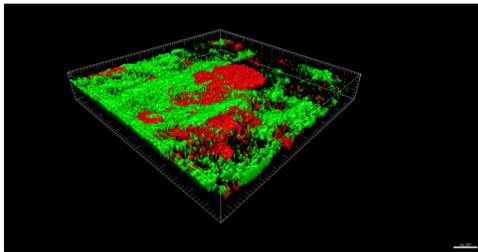
0.5% DMSO 12.81%



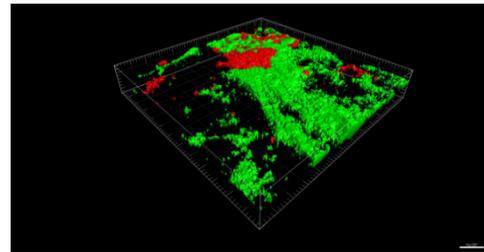
2% CHX 36.96%



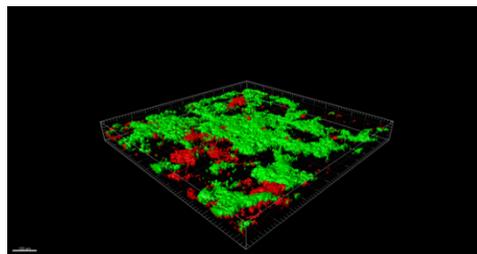
***C. gileadensis* 0.1mg/ml water 34.46**



***C. gileadensis* 1mg/ml water 48.29%**



***C. gileadensis* 0.1mg/ml 0.5% DMSO 36.13%**



***C. gileadensis* 1mg/ml 0.5% DMSO 31.19%**

Figure 13. 3D reconstruction of CLSM images of one-week old biofilms exposed for three minutes. The agents used, and the percentage of dead bacteria volume are shown.

4.4 The effect of the agents on 3-week-old biofilm

Regarding the 3-week-old biofilm and 1-minute exposure time, there was no significant difference between the killing effectiveness of water, 0.5%DMSO, and *C. gileadensis* - 0.1mg/ml 0.5%DMSO samples ($P>0.05$). Furthermore, 2%CHX performed better than the *C. gileadensis* 0.1mg/ml water solution, but the difference was not statistically significant ($P>0.05$). However, 2%CHX significantly killed more bacteria than water, 0.5%DMSO, *C. gileadensis* extract 0.1mg/ml 0.5%DMSO, and *C. gileadensis* extract 1mg/ml 0.5%DMSO ($P<0.05$) (Fig 14) (Fig 16). The results obtained for the 3-week-old biofilm after 3-minute exposure to the same substances showed that *C. gileadensis* extract 0.1mg/ml water samples killed significantly more bacteria than water and 0.5%DMSO ($P<0.05$). Also, the *C. gileadensis* extract 0.1/mg/ml water performed better than the *C. gileadensis* extract 1 mg/ml 0.5%DMSO, *C. gileadensis* extract 0.1 mg/ml 0.5%DMSO, and 2%CHX but without significant differences ($P>0.05$), as seen in (Fig 15) (Fig 17).

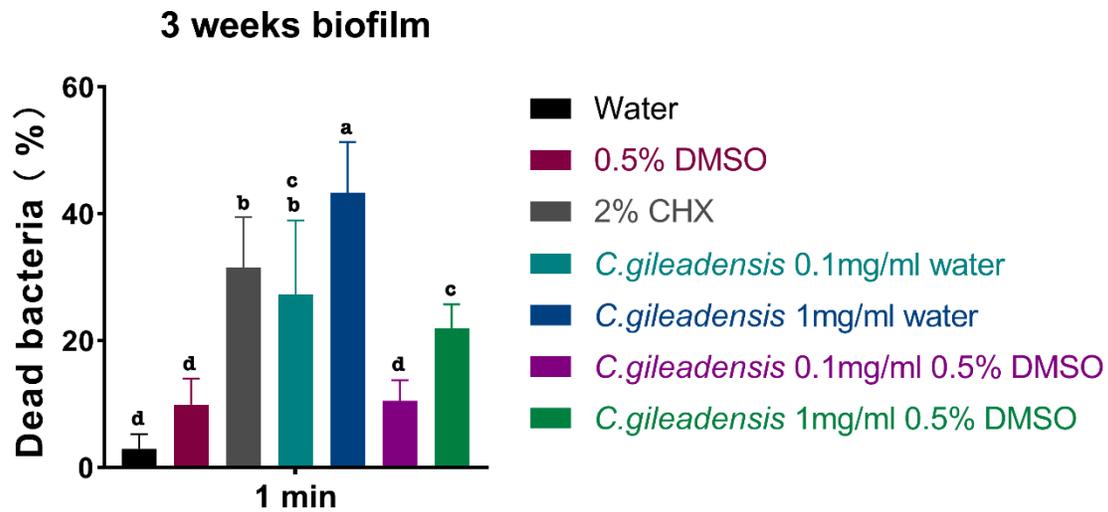


Figure 14. Mean percentage (and S.D.) of dead bacteria in three weeks old biofilms after one minute exposure to the indicated substances. Small superscript letters indicate statistically significant differences between the groups, $p < 0.05$ (Tuckey test).

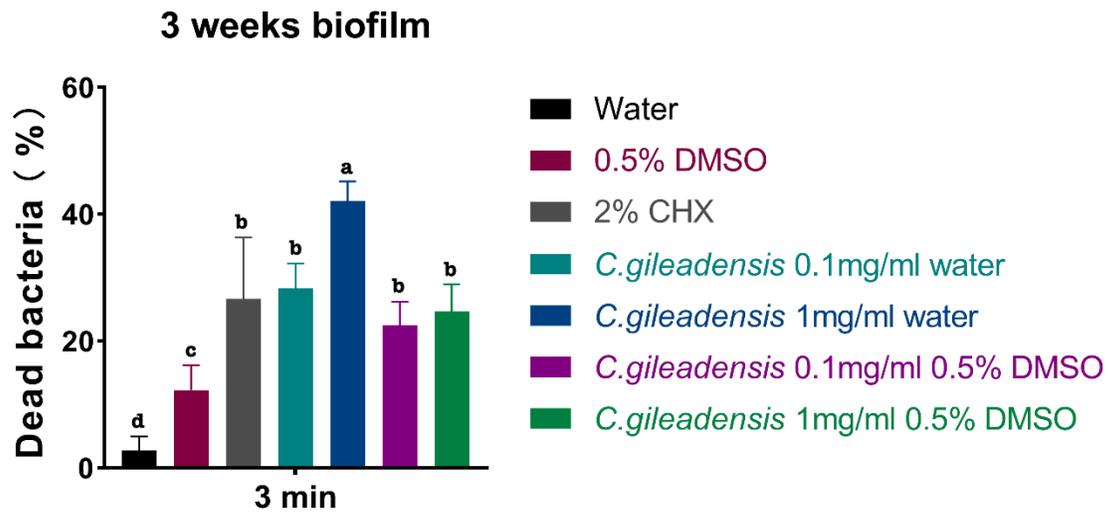
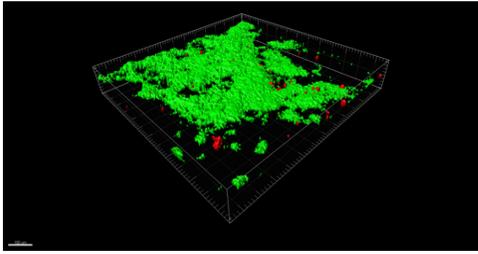
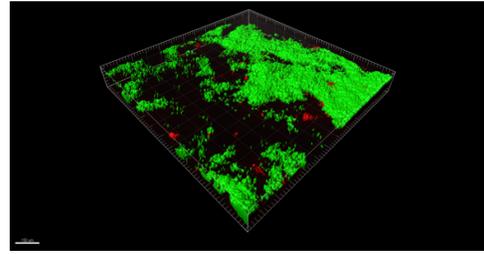


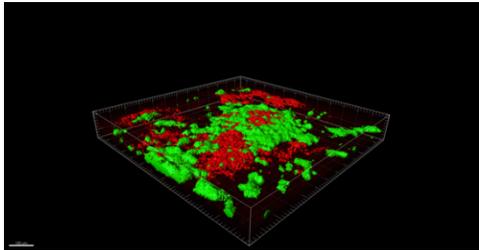
Figure 15. Mean percentage (and S.D.) of dead bacteria in three weeks old biofilms after three minute exposure to the indicated substances. Small superscript letters indicate statistically significant differences between the groups, $p < 0.05$ (Tuckey test).



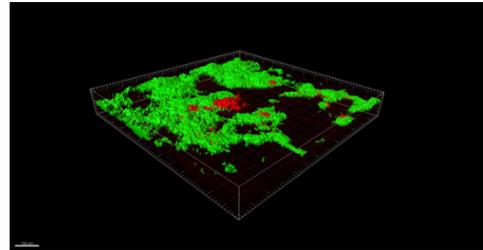
Water 2.30%



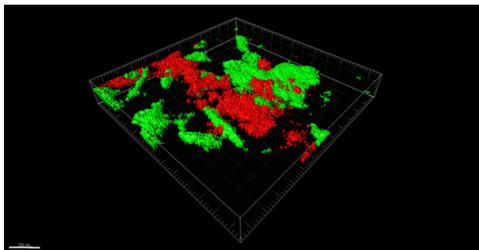
0.5% DMSO 5.91%



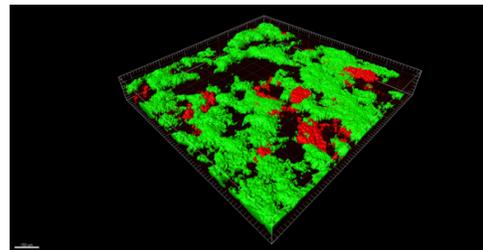
2% CHX 34.03%



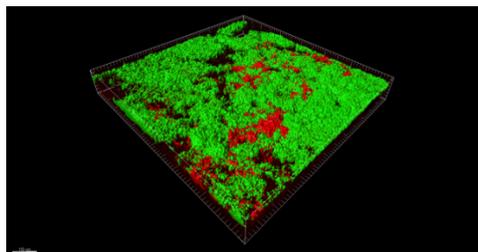
***C. gileadensis* 0.1mg/ml water 23.93%**



***C. gileadensis* 1mg/ml water 39.67%**

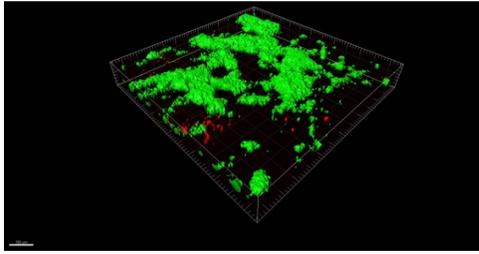


***C. gileadensis* 0.1mg/ml 0.5% DMSO 12.75%**

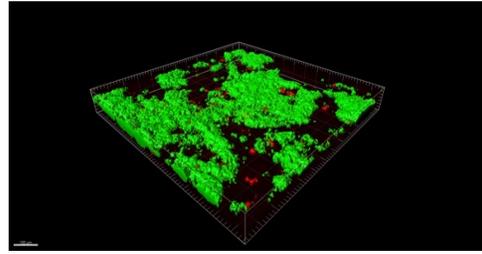


***C. gileadensis* 1mg/ml 0.5% DMSO 18.79%**

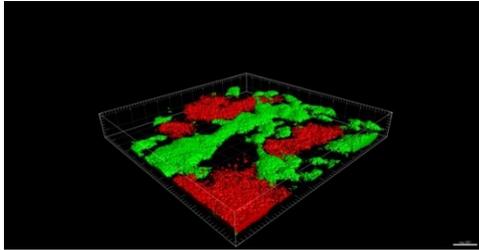
Figure 16. 3D reconstruction of CLSM images of three-weeks old biofilms exposed for one minute. The solution agents used, and the percentage of dead bacteria volume are shown.



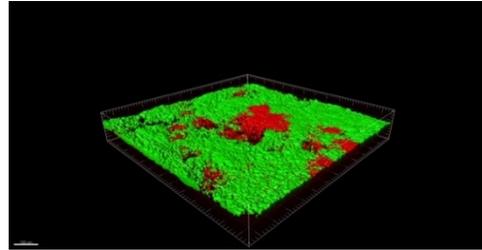
Water 2.40%



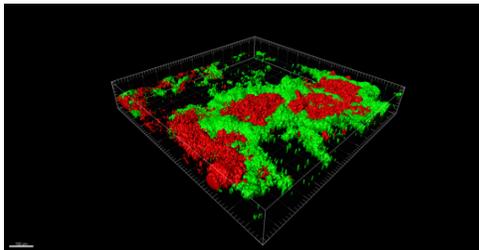
0.5% DMSO 10.82%



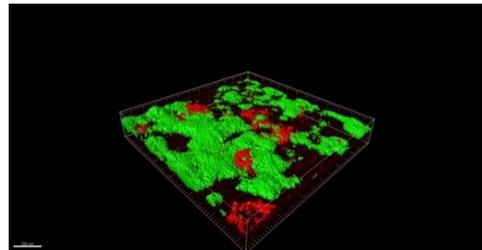
2% CHX 36.11%



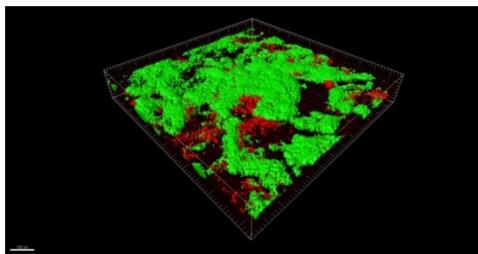
***C. gileadensis* 0.1mg/ml water 31.63%**



***C. gileadensis* 1mg/ml water 43.17%**



***C. gileadensis* 0.1mg/ml 0.5% DMSO 18.80%**



***C. gileadensis* 1mg/ml 0.5% DMSO 23.38%**

Figure 17. 3D reconstruction of CLSM images of three-weeks old biofilms exposed for three minutes. The solution agents used, and the percentage of dead bacteria volume are shown.

4.5 The effect of exposure time on killing of biofilm microbes

When taking the exposure time into consideration, regardless of the age of the biofilms, the *C. gileadensis* extract 0.1 mg/ml 0.5%DMSO performed better when the exposure time was 3 minutes instead of 1 minute ($P<0.05$). However, the exposure time did not play a significant role in other groups. Furthermore, when comparing 0.5%DMSO to water, the 0.5%DMSO samples had a significantly higher percentage of dead bacteria only when the exposure time was 3 minutes, regardless of the age of the biofilms ($P<0.05$).

4.6 The effect of the age of the biofilm

C. gileadensis - 0.1 mg/ml 0.5%DMSO and *C. gileadensis* -1 mg/ml water samples were the only solutions that killed more 1-week-old biofilm bacteria than 3-week-old biofilms bacteria ($P<0.05$).

Chapter 5: Discussion

Biofilms inhabiting the complex root canal systems contribute to the difficulty of achieving clean and bacteria free root canals by the endodontic treatment (195). Irrigation is a major component of root canal treatment. Without irrigation solutions, it is impossible to reach the regions of the root canal system inaccessible to endodontic instruments (files) (45). Searching for new techniques and materials, along with improving endodontic irrigation is one of the main parts of endodontic research and literature.

To the best of our knowledge, this is the first study examining the effectiveness of *C. gileadensis* extracts against anaerobic multispecies oral biofilms in vitro. The plant's branches were submitted to extraction protocols using different concentrations and solutions. We were interested to know if the extracts in obtainable concentrations would be able to kill significant numbers of biofilm bacteria. As expected, overall the 1 mg/mL water extract of *C. gileadensis* samples performed significantly better than the ten-fold diluted concentration (0.1 mg/mL water) of the extract. However, when the biofilm was 1 week old and the exposure time was 3 minutes, the difference between the two extract concentrations was not statistically significant, although killing by the stronger 1 mg/mL *C. gileadensis* extract was still slightly higher. Therefore, the extract solution, even at a low level of concentration may be still effective. Additionally, our study corroborates results from previous studies that showed *C. gileadensis* possesses antimicrobial effects (170–172). Nevertheless, we used a different technique to assess its effects, using a multispecies oral biofilm rather than a single species biofilm.

C. gileadensis extraction were dissolved with different solutions: water or 0.5%DMSO. We chose DMSO as the solvent because of its popularity among plant extraction studies and its ability to dissolve many kinds of chemical compounds (196). Thus, we were interested to discover if DMSO would dissolve the plant and release chemical compounds that were not

obtained by water extraction. On the other hand, DMSO has an antimicrobial effect which could be a confounding factor (197). Therefore, we selected the lowest possible concentration of DMSO for the extraction and added the same 0.5%DMSO as a control group. Surprisingly, the percentage of dead bacteria killed by extracts that were obtained with 0.5%DMSO was significantly less than those killed with water extract. A previous study that used an agar well diffusion method showed that when the plant was extracted with 100%DMSO it had a significantly larger antibacterial effect than when water was used (171). However, as mentioned earlier, agar diffusion studies are poorly suited for testing endodontic disinfecting agents and may not be reliable.

The sterilized distilled water and 2%CHX samples were also selected as control groups. Prior to exposure with the extract solution and scanning of the biofilm, we performed experiments with the control groups. This allowed us to confirm that the results with water and CHX are in line with previous results using the same method. For example, the percentage of dead bacteria in the water group (negative control) is usually less than 10% for 1-week-old biofilms and below 15% for the 3-week-old biofilms. Control experiments showed that the results were in good agreement with previous results when same methodology was employed (42). Importantly, our results on the effectiveness of 2%CHX against multispecies oral anaerobic bacteria also matched previously published studies. It has been previously reported that 2%CHX killed 20% and 30% of the bacteria in 3-week-old biofilms after 1 and 3 minute exposures, respectively (198–201).

The exposure times of one and three minutes were chosen because they are relevant in the clinic. Also, a study showed that bacterial killing by disinfecting solutions in infected dentin is time dependent. However, after the first 3 minutes of exposure, little additional killing was

achieved. NaOCl with high concentration was more efficient against *E. faecalis* biofilms than 2%CHX and 2%NaOCl. (198).

There are many different ways to assess the effectiveness of antimicrobial solutions on endodontic bacteria. For instance, MIC methods of planktonic cells in liquid medium can be used. However, it may be inappropriate to assess antibacterial agents using this method when bacteria are in biofilm form (202). One possible method to study the effect of antibacterial agents on biofilm is Scanning Electron Microscopy (SEM). This technique can show the complex structure of the microbial biofilm in high resolution. Nevertheless, multistep preparations steps are needed, such as fixation, dehydration, critical point drying and coating. Therefore, the original shape of the biofilm may be disturbed (203) and e.g. the EPS layer which is rich in water is invisible in conventional SEM specimens. Environmental Scanning Electron Microscopy (ESEM) is a substitute for SEM. With the ESEM technique, EPS can be preserved and the hydrated bacterial biofilm observed, contrary to SEM. (204) In our study, CLSM was used. This technique can reconstruct the thin scanned sections into a three-dimensional image. This technique can also preserve the status of the biofilm, with minimum disruptions, and provides scanning of the biofilm structure up to 200- μm 's thick (32,205). CLSM can also measure the width and allocation of cells in a biofilm and determine the pH value by using specific fluorescent dyes. Furthermore, dead and viable cells in a biofilm are detected through use of viability stain that emit green and red lights from the live and damaged (dead) those cells. This technique can also identify cells that cannot be detected in culturing techniques—for example, cells undergoing nutrition deprivation and species whose culture requirements are not yet properly understood. Thus, the true level of viability of the biofilm cells can be acquired using CLSM (206).

Although the multispecies biofilm was grown under anaerobic conditions, which corresponds well the ecology of the necrotic root canal. The surface (substrate) that was used was collagen coated commercial hydroxyapatite and not true dentin. Haapasalo et al have reported that dentin might have some inhibitory effect on some antimicrobial agents (207). Therefore, these antimicrobial solutions might not have identical effects when applied to dentin rather than HA discs. However, hydroxyapatite discs were used in this study for specific reasons; HA discs are synthetic and comparable (similar) in all samples, which helps in the standardization of the samples. In contrast, dentin is not uniform because it differs from tooth to tooth. Dentinal tubules have different orientations and densities, they are also variable within the same tooth (208). Some of the extracted teeth may have been previously exposed to a variety of chemicals, some of which could have interfered with the biofilm. Not having high level of standardization in the samples may influence the biofilm's growth and behavior; thus, playing a role in the response to the tested solutions.

There are many factors that influence the attachment of bacteria to a surface including environmental states such as fluid flow rate, pH, temperature, and nutrition accessibility. The types and growth phases of bacteria, along with the physical and chemical features of the substrate will also have an influence (209). Consequently, the bacterial species that were collected from supra- and sub-gingival bacteria are unknown. Although we incubated the multispecies bacteria in anaerobic condition and on collagen-coated hydroxyapatite discs in BHI medium, it is unknown if the bacteria will develop and grow exactly as they would in vivo. This is because of the difficulty in meeting all the criteria mentioned above.

This study only assesses the antibacterial solutions from a chemical perspective. We have very gently applied the solutions onto the biofilms, without adding any physical force to disturb the biofilm. On the other hand, in the clinic, we always add physical factors such as endodontic

files to interrupt the biofilm—except in cases where we use intra-appointment medications. Therefore, we need to interpret these results with caution and consider all the points that have been stated.

Although the results of this study are promising, and showed that *Commiphora gileadensis* extract performs significantly better than 2% chlorohexidine, there are still many questions left unanswered. For instance, how the fine particles of the plant solution will affect endodontic materials and what the outcome would be if the solutions interact with periapical tissues. Furthermore, it is important to determine what chemical compounds possess significant antimicrobial properties, the best methods to extract them, and whether it is best to use the extraction compound as an irrigant or as an interappointment medication. Therefore, further investigations should be done to assess the plant extract solution.

Chapter 6: Conclusion

Within the limitations of this study, 1 mg of *Commiphora gileadensis* dissolved in 1 ml of distilled water killed more of the 1- and 3-week-old biofilm bacteria than 2% chlorhexidine and other groups. Other different groups of the *Commiphora gileadensis* extracts killed bacterial biofilm equally well as the 2%CHX group. Lastly, the 0.5%DMSO alone killed significantly less bacteria than the other groups, but more than distilled water.

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