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Rhamnus prinoides (gesho) extract Inhibits Streptococcus mutans and Candida albicans Polymicrobial Biofilm Formation

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RHAMNUS PRINOIDES (GESHO) EXTRACT INHIBITS *STREPTOCOCCUS MUTANS* AND *CANDIDA ALBICANS* POLYMICROBIAL BIOFILM FORMATION

by

RAGHDA FATHI

Under the Direction of Eric S. Gilbert, PhD

ABSTRACT

Polymicrobial biofilms contain multiple microbial species encased in an extracellular polymeric matrix. Synergistic interactions within polymicrobial biofilms contribute to elevated antibiotic resistance and chronic infections; furthermore, prevention and treatment is still an unresolved issue. The yeast *Candida albicans* and the Gram positive bacterium *Streptococcus mutans* are biofilm-forming oral pathogens that interact mutualistically, and were investigated in this work. Crystal violet-based biofilm formation assays were used to measure the effect of extracts from *Rhamnus prinoides* (gesho), an East African plant used in traditional medicine, on biofilm formation. The biomass of dual species biofilms was 70% greater than single-species biofilms, indicating a synergistic interaction. Treatment with gesho extracts reduced both single-species and

polymicrobial biofilm biomass by more than 90% relative to controls. Imaging by epifluorescence microscopy supported the findings of the biofilm formation assays. In conclusion, gesho exhibited significant potential for use as an anti-biofilm agent and warrants further investigation.

INDEX WORDS: Biofilms prevention, Gram Positive bacteria, Fungal biofilms, *Streptococcus mutans, Candida albicans*, Gesho

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DEDICATION

This thesis is dedicated to my parents Mr. Ayman Fathi and Dr. Dina El-Sawy who dedicated their lives to provide the best education for me and my sisters. And to my friends, whom without their support and love I couldn't have been able to standup through challenging time. A special thank you to my sisters I couldn't have done this without your encouragement.

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LIST OF ABBREVIATIONS

GSE: Gesho Stem Ethanol

GSW: Gesho Stem Water

GLE: Gesho Leaf Ethanol

GLW: Gesho Leaf Water

NT: No treatment

CA: *C.albicans*

SM: *S.mutans*

EPS: Extra polysaccharide matrix

BHI: Brain Heart Infusion

1 BACKGROUND AND SIGNIFICANCE

1.1 Biofilms

Biofilms are complex microbial communities (Stacy, McNally, Darch, Brown, & Whiteley, 2016). They are microbial cells encased in an extracellular polymeric matrix composed of proteins, carbohydrates and extracellular DNA. Biofilms are found to be different from planktonic cells in their different regulation of some of their genes as well as their slow growth rates. The matrix-stabilized environment within biofilms is beneficial for organisms to communicate with each other through quorum sensing as well as transfer of genetic material. Biofilms are most commonly found attached to surfaces to which they cannot easily detach, such as medical devices and pipes in water systems (Donlan, 2002). Biofilms can be beneficial or detrimental, depending on where they form and their inhabitants. For example, environmental biofilms are essential in the operation of wastewater treatment facilities. On the other hand, medical biofilms are the major cause of infections and persistent diseases (Bjarnsholt, 2013).

Biofilm communities are how bacteria survive in different environments (Høiby et al., 2011), their ability to overcome stressful conditions increased their persistence and made them a major cause of nosocomial infections, 50 % of nosocomial infections are related to indwelling devices such as catheters, dentures and heart valves (Roy, Tiwari, Donelli, & Tiwari, 2017). Biofilms comprised of pathogens can cause chronic infections. Cystic fibrosis pneumonia, device associated infections and chronic wound infections are the most common disease caused by biofilms that can lead to several deaths cases. The severity of infections caused by biofilms and their ability to be chronic is due to their high resistance to antibiotics and their ability to resist and evade the immune system (Bjarnsholt, 2013).

Biofilm treatment has been very difficult and the most effective method is removal of infected area such as the implant or the organ if possible, but in cases where that is not possible the main approaches are by combined antibiotic intake before the biofilm formation or by chronic intake of antibiotics in case the biofilm has already been formed (Bjarnsholt, 2013).

1.2 Polymicrobial Biofilms

Biofilms are often comprised of multiple species and are referred to as polymicrobial biofilms and can include both prokaryotic and eukaryotic microorganisms (Peters, Jabra-rizk, Costerton, & Shirtliff, 2012). The human body being complex, harboring the human microbiota including bacteria, fungi and archaea lead to the presence of polymicrobial biofilms that has become the more common feature of pathogenic biofilms. Polymicrobial biofilms display a complex environment that can be altered by various changes of host such as immunity. Therefore, the study of pathogenic biofilm should be expanded to have a more in-depth view of the polymicrobial biofilms (Nobile & Johnson, 2016).

Polymicrobial communities help biofilms become more antibiotic resistant through passive mechanisms; for example, where one organism uses the other's resistance capabilities to protect itself, a concept referred to as indirect pathogenicity (O'Connell et al., 2006) Moreover, members of polymicrobial biofilms enhance their quorum sensing communication as well as increase their metabolic products and the genetic pool where they will have access to wider variety and more diverse resources (Wolcott, Costerton, Raoult, & Cutler, 2013). It has been demonstrated that polymicrobial biofilms were not only formed from multiple bacterial species, but eukaryotic pathogens were involved as well. This

emphasizes the importance and necessity for increasing attention towards these biofilms and focus on their essential role in chronic infections (Harriott & Noverr, 2011)

1.3 Antibiotic resistance of biofilms

Several features of polymicrobial biofilms contribute to their enhanced antibiotic resistance. One important factor is the polymeric matrix surrounding the microorganisms, which acts as a protective shield against both the immune system of the host and antimicrobial medications. Prolonged treatment with various antibiotics can cause resistance through exposure to selective pressure , in addition it allows biofilm bacteria to adapt and acquire resistance through horizontal gene transfer (Fux, Costerton, Stewart, & Stoodley, 2005). Other mechanisms have been suggested for biofilm antimicrobial tolerance such as the phenotypic heterogenicity of cells within the biofilm which is directly related to unequal susceptibility to antimicrobial effects (Fux et al., 2005).

Stewart and Costerton (2001) hypothesized three mechanisms whereby biofilms are resistant to antibiotic treatments. Their first hypothesis was the inability of antibiotic molecules to diffuse through the biofilm matrix to deep layers of bacteria, they added that while some antibiotics can penetrate through the biofilm, the pace of penetration is highly limited when the antibiotics are deactivated by bacterial cells in the surface layers of the biofilm. On the other hand, the development of anaerobic layers with high pH differences between the biofilm layers due to oxygen consumption in surface layers and metabolic waste product accumulation can contribute to deactivation to antibiotic activity. The last hypothesis is the formation of a resistant spore like phenotype by a subpopulation of bacterial cells in the biofilm (Stewart & William Costerton, 2001). The significance of biofilms in chronic infections and antibiotic infections has lead to more research focus on prevention and treatment of biofilm communities.

1.4 Novel approaches for treating biofilms

Several methods have been under trials in effort to develop biofilm treatments. Among those methods are coating devices with antimicrobial agents to prevent the attachment of organisms to their surfaces and hinder their growth into biofilms. Other methods include quorum sensing inhibitors, bacteriophage therapy, oral drug combinations and new antimicrobial agents. Quorum sensing inhibitors proved to have significant antibiofilm effects but the need for further research on their safety as well as the debate on their inability to kill cells and only inhibiting their virulence has stood in the way of their emergence. Bacteriophage therapy concentrate on targeting bacteriophage to bacterial biofilms and thus removing the biofilm. Bacteriophage therapy is one of the recently studied mechanisms due to their safety and cost-effective production. Oral drug combination including quorum sensing inhibitors, enzymes, antifungals, herbs and antimicrobial agents have been investigated but the safety of such treatment is yet to be confirmed. Finally development of antimicrobial such as tigecycline have been under study for their biocidal effects on biofilm associated bacteria with promising effects (Savini et al., 2010).

1.5 Streptococcus mutans biofilms

Streptococcus mutans is a primary cause of dental carries, due to its ability to form biofilms in the oral cavity tissue. *S. mutans* produces adhesins that helps it to bind to the tooth surface as well as other proteins that help in its biofilm formation *S. mutans* biofilm formation mechanisms can be sucrose dependent or independent (Sug Joon Ahn, Ahn, Wen, Brady, & Burne, 2008). The ability of *S. mutans* to form robust biofilms lies in the secretion of an exoenzyme called glucosyltransferase. Through this exoenzyme, *S. mutans* utilizes sucrose

supplied from food to produce EPS. Moreover, *S. mutans* has the ability to overcome stressful and highly acidic environments, enabling it to survive and form biofilms leading to dental carries (He et al., 2017).

1.6 Candida albicans biofilms

Candida albicans represent the most common human fungal pathogen that can form biofilms. According to in vitro studies *Candida* forms biofilms in several stages. It starts with an early phase, where it goes through morphogenesis after adhering to a suitable surface. Formation of hyphae at this stage is essential for *Candida* to form biofilms. The second stage is the intermediate stage where hyphae continue their growth with the production of the extracellular matrix. Finally, the third stage is maturation where the yeast forms are present at the base with the hyphae at the surface of the biofilm and embedded in the polysaccharide matrix. *Candida* infection can be fatal, and studies are focused on their formation on abiotic and biotic surfaces, for example catheters and oral cavity (Harriott & Noverr, 2011).

Candida species were found to be the main pathogen causing infection for denture users leading to denture stomatitis. *Candida albicans* were found to be the most common among the *Candida* species. *C. albicans* can grow in different morphological forms as yeast or pseudohyphae or true hyphae. The elongated hyphae form has been observed to help the yeast penetrate into tissue by escaping from phagocytic cells. The ability of *C. albicans* to form biofilms through interaction with surfaces and formation of extracellular matrix is dependent on its ability to form hyphae (Pereira-Cenci et al., 2008).

1.7 *S. mutans* and *C. albicans* polymicrobial biofilms

Fungal and bacterial cooperation in biofilm formation synergizes biofilm activity and growth. They collaborate with each other to exchange metabolites or growth factors. For example, *S. mutans* metabolizes sucrose to glucose and fructose which can be a benefit for *C. albicans* (Kim et al., 2017).

Candida albicans or Candida species were found to be the main pathogen causing infection for denture users leading to denture stomatitis, bacteria were also found to be a factor in biofilm formation in dentures. Another example of fungal- bacterial association in biofilms is in the early-childhood caries ECC. *Streptococcus mutans* is a main bacterial pathogen for dental caries, especially in early-childhood caries (ECC). It was found that *S. mutans* is not solely present but *Candida albicans* were common in cases of highly infected plaque biofilms with *S. mutans* in children with ECC. *S. mutans* and *C. albicans* biofilms are enhanced by increase of exopolysaccarides by *C. albicans* and hence increasing the biomass. When animals were coinfected, biofilm virulence was synergized. In vitro studies shows that glucosyltransferase EPS derived was a main mediator in development of the dual specious biofilms and that *C. albicans* enhance the virulence genes expression in *S. mutans* (Falsetta et al., 2014).

1.8 *Rhamnus prinoides* (Gesho)

Rhamnus prinoidis is a plant that belongs family rhamnaceae. *R. prinoides* known as Gesho was found in Ethiopia and has been widely cultivated.

It was also found in Africa in the south countries like Kenya. The plant has an edible fruit and has been used for many medicinal treatments such as infectious diseases. It is known for its ethnomedicinal uses and its parts were used to treat nose, ear and throat infections in kenya while the leaves are used for tonsillitis in Ethiopia. In addition, gesho has been used in different case of

scabies, dandruff and hepatitis. Its decoction was used to treat stomach pain and was used in rheumatism and pneumonia as well. It's root extract was used for rheumatism and gonorrhea. Gesho has been studied for its antimicrobial activities and showed positive results that encouraged its study against diseases (Amabye, 2016; Molla, Nedi, Tadesse, Alemayehu, & Shibeshi, 2016)

2 RATIONALE

Rhamnus prinoides or gesho is a traditional plant used in East Africa for the treatment of a variety of infections. Gesho has been found to have a biocidal effect on planktonic cells of Gram- positive and Gram-negative bacteria, with the more potent effect on Gram positive species (Molla et al.2016). Our laboratory has found that gesho successfully prevented *Staphylococcus aureus* and *Bacillus subtilis* biofilm formation (unpublished data). Through preliminary research it was found that gesho has similar inhibitory effects on *Streptococcus mutans* biofilm formation. These findings led to the development of the research question that is the basis of this thesis: "what is gesho's effect on fungal and bacterial polymicrobial biofilms?". Polymicrobial biofilms of *S.mutans* and *Candida albicans* demonstrated enhanced biofilms with increased biomass compared to biofilms of single species (Falsetta et al .2014).The association of *C. albicans* with *S. mutans* in the human oral cavity biofilms and their co-presence that plays a role in dental carries and denture infections makes them an economically significant model for the study of gesho on polymicrobial biofilms.

2.1 Hypothesis

It is hypothesized that gesho extracts can prevent the formation of polymicrobial biofilms by *S. mutans* and *C. albicans*.

2.2 Aim

The aim of this study is to quantitatively test the effect of *Rhamnus prinoides* extracts on polymicrobial biofilms comprised of *C. albicans* and *S.mutans*. This will be achieved through testing the activity of *Rhamnus prinoides* toward the following:

1- *S. mutans* biofilms.

2- *C. albicans* biofilms.

3- Polymicrobial biofilms of *C. albicans* and *S.mutans* grown together.

The gesho extracts to be tested are gesho stem ethanol extract (GSE), gesho leaf ethanol extract (GSE), gesho stem water (GSW) and gesho leaf water (GLW). Each extract will be tested at several concentrations ranging from 0.25 mg/ml to 7 mg/ml.

3 METHODS

3.1 Biofilm formation assays:

3.1.1 S. mutans biofilms

Formation of in vitro biofilms was done in a 96- well microtiter plate, using 0.5% sucrose to assist *S. mutans* in biofilm formation (Kunze et al., 2010). Biofilms were grown overnight at 37 ºC aerobically on shaker (Sang Joon Ahn & Burne, 2007). The biofilm assay was done using BHI broth media for growth of *S. mutans*. *S. mutans* were cultured from -80 ^{0}C stocks in BHI broth and culture was grown overnight at 37 ^{0}C. The following day, the culture concentration was measured at OD 600 and adjusted to 0.01 to be used in biofilm assay. BHI- sucrose media

was used for biofilm assay, the BHI-sucrose was filter sterilized before adding *S. mutans* and proceeding with the assay. Gesho extracts were prepared at assigned concentrations and added to 96-well (100 µL per well) and the plate was set for overnight incubation at 37 ºC on shaker. The next day, the 96-well plates were washed and stained by crystal violet. Than using 95% ethanol, biofilms were de-stained to measure absorbance using MD plate reader at an optical density (OD) of 595 nm (O'Toole, 2011). The experiment was repeated at least three times independently.

3.1.2 C. albicans biofilms

C. albicans were grown on a 96-well plate according to Pierce et al (2015) with slight difference, using $1x10^7$ cells instead of $1x10^6$ cells by using hemocytometer cell counting and calculation. When using hemocytometer, *C. albicans* was stained by 0.1% v/v methylene blue. *C. albicans* was cultured on yeast peptone dextrose (YPD) agar plate overnight at 37 ºC form -80 stock. Then a loopful of colonies were cultured in 25 ml of YPD broth for 14-16 hours at 30ºC were *C. albicans* grows as budding yeast. Then *C. albicans* cells were centrifuged and washed twice with PBS and adjusted to $1x10^7$ cells using hemocytometer. For biofilm formation in 96-well plates, cells were added to RPMI buffered with 165mM morpholinepropanesulfonic acid (MOPS) (Pierce et al., 2015). The RPMI-MOPS were filter sterilized before *C. albicans* addition. Once *C. albicans* was added to RPMI-MOPS, gesho extracts were prepared at different concentration of 7,5,3,1,0.5 and 0.25 mg/ml to test its biofilm inhibition effect on *C. albicans* biofilms. Only GSE and GLE were tested with *C. albicans*. Biofilms were stained by crystal violet after washing the plates than measure absorbance by MD plate reader at OD 595 (O'Toole, 2011).

3.1.3 Dual species biofilm

The in-vitro growth of the polymicrobial biofilms were done similar to (de Oliveira et al, 2017) but with some changes. Biofilms of *S. mutans* and *C. albicans* will be grown using the same growth media used before for each. BHI/sucrose for *S. mutans* and RPMI-MOPS for *C. albicans* using equal volumes of each media (100 µL) with the same initial concentration used for each organism (0.01 OD for *S. mutans* and 1x 10^7 for *C. albicans*). Gesho ethanol extracts were tested at 3 mg/ml. The polymicrobial assay 96-well plate design included *S. mutans, C. albicans* and their dual species biofilms untreated compared to their treated counterparts at 3 mg/ml. The plates were washed and stained for reading as done previously. The biofilm biomass for the nontreated biofilms and the synergistic effect of adding the organisms together surpassed the MD plate reader reading limit, therefore a 1:10 dilution of the nontreated biofilms were done before reading and then multiplied by the dilution factor 10 before plotting the data into graphs.

3.2 Viability assays

These assays were done for each extraction after each biofilm assay, the aim is to test the effect of gesho on the planktonic cells in the suspensions of each well for each fraction. This is done as part of the effort for understanding the plants' mechanism for biofilm formation inhibition. Supernatant of cells from control nontreated cells as well as the 7,5 and 3 mg/ml were extracted from wells to be tested. Each fraction was diluted 1:10 dilutions from 10^{-1} to 10^{-7}. Using petri dishes divided into 8 quadrants for the dilutions, each quadrant inoculated with two drops of 10 µL each. Plates were incubated at 37ºC overnight. Dilutions showing the least number of colonies were counted and the data plotted on excel sheet to calculate log reductions in comparison to the control plate.

3.3 Polymicrobial Biofilms images

3.3.1 Flow cell biofilm growth system

To view and image the biofilms they were grown via flow cell system. The flow cell was done according to (Niu et al., 2013) but with few changes to fit our work. The main parts of the flow cell consist of the flow cell slide covered with glass cover slips which are glued using silicon, forming chambers to allow flow of media. The flow cell slide has two adjacent channels through which the media is pumped from the reservoir bottles and circulated for 24 hours at a rate of 0.9 ml/min at 37 ºC. The coverslips serve as surface for biofilm attachment. The entire system is pre-sterilized through autoclaving and bleaching. Two media reservoirs were prepared. First one for control with no gesho extracts was prepared with 25 ml of Sucrose/BHI broth with *S. mutans* at 0.01 OD as initial concentration, added to 25 ml of RPMI/ MOPs with *C. albicans* to reach a total of 50 ml. The second reservoir bottle was prepared like the first one with addition of GSE extract at a 3 mg/ml concentration. Each bottle was connected to a separate flow cell slide and they were incubated simultaneously for 24 hours at the same flow rate**.**

3.3.2 Epifluorescent microscope

C. albicans and *S. mutans* polymicrobial biofilms 2D images were taken using calcofluor white as a fluorescent dye for Candida and SYTO 9 for nucleic acid staining producing blue fluorescence under microscope for *C. albicans* and green for both *C. albicans* and *S. muatns*. The effect of gesho extracts tested on dual species biofilms will be examined by these images that will help primarily in viewing its effect on the biofilms in 2D dimensions.

3.3.3 Cell size of C. albicans and S. mutans by image analysis

AmScope 3.7 for digital camera (United states) software, was used to estimate the size of *S. mutans* and *C. albicans*. Epifluorescent or light microscopy images of biofilms collected as

described above were analyzed. Pixels were converted to µm using the following equation: [(Length in pixels) * (10000 µm/ cm)]/ [(resolution in pixels/cm) * (magnification)]. Representative images were selected and cell dimensions from a minimum of three images were evaluated for gesho-treated and non-treated biofilms.

3.4 Molecular analysis

3.4.1 RNA purification and extraction

The expression of the *S. mutans* glycosyltransferase genes (*gtfB, gtfC*) was measured using the protocol of Falsetta (Falsetta et al., 2014) with some modifications. Four biofilm cultures were prepared for RNA extraction: *S. mutans* cultivated alone with and without gesho extract, and dual species biofilms of *S. mutans* and *C. albicans* with and without gesho extract. RNA extraction and purification were done using a Direct-Zol ^{TM} RNA MiniPrep kit (Zymo research, USA). RNA was collected for both planktonic and biofilm cells. After DNase treatment, the RNA samples were measured for concentration and purity using a NanoDrop spectrophotometer (Thermo scientific, NanoDrop 2000), then stored at -80 °C. PCR reactions were performed on RNA samples to ensure the absence of DNA contamination. Genomic *S. mutans* DNA was extracted using a ZR Fungal/Bacterial DNA MicroPrep kit (Zymo research, USA). Genomic *S. mutans* DNA was used to confirm the primer annealing temperature in PCR reaction protocol, and to serve as a positive control in cDNA PCR gel electrophoresis. *S. mutans*16s rDNA primers were used in the PCR reaction to identify if any DNA was still present after DNase treatment and *S. mutans* genomic DNA was used as a positive control. While for annealing temperature confirmation and cDNA controls, the *gtfB, gtfC* primers were used for PCR reactions.

3.4.2 RT-qPCR

GtfB,C genes primers were ordered according to the primer sequence used by Klein (Klein et al., 2012). The Super Script III First-Strand synthesis system for RT-PCR (Invitrogen) was used to convert RNA samples to cDNA. PCR was used to confirm cDNA formation. Standards will be prepared by adding specific *gtfB, gtfC* primers to cDNA. SYBR green will be used for RT-qPCR. Samples concentrations will be measured using qPCR.

3.5 Statistical analysis

Non-parametric (Kuskal-Wallis Test and Median Test) analyses were performed. Cell size analysis were done using T-Test. Comparisons were done between control (non-treated) and gesho treated samples. Differences with a p-value < 0.05 were considered statistically significant and are noted with asterisk (*).

4 RESULTS

4.1 Effects of gesho extracts on *S. mutans* biofilm formation

Significant inhibition of biofilm formation for the GLE fraction with gradual increase in biofilm formation with the decrease in extract concentration. The most significant decrease in biofilms were at the 3, 5, and 7 mg/ml concentrations. The GLE 7mg/ml showed 90% biofilm reduction, 5mg/ml showed 80% biofilm reduction and 55 % reduction for 3mg/ml concentration. GSE assay resulted in similar effects as GLE with an average 90 % biofilm reduction for the 3, 5 and 7 mg/ml concentrations. While for the GSW 50 %, 25 % and 10 % reductions were shown for the 3, 5 and 7 mg/ml respectively. GLW had minor inhibitory effects with 10 % and 5 % reductions for the 5 and 7 mg/ml concentrations respectively. Each assay was repeated 3 times on independent occasions, results were consistent for each fraction concentrations and the average of the three assays were plotted as percent of control (Fig 1, Fig 2)

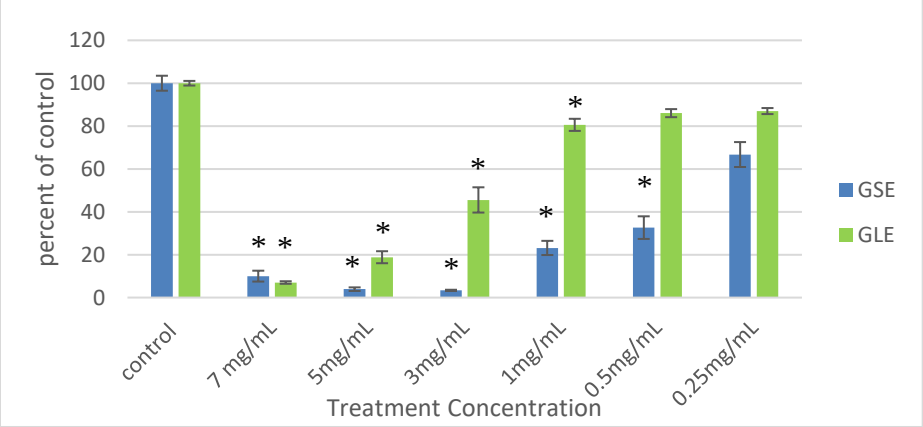

Figure 1 Percent of control average results of Gesho Ethanol leaf and stem extract effects on S. mutans, (*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control.

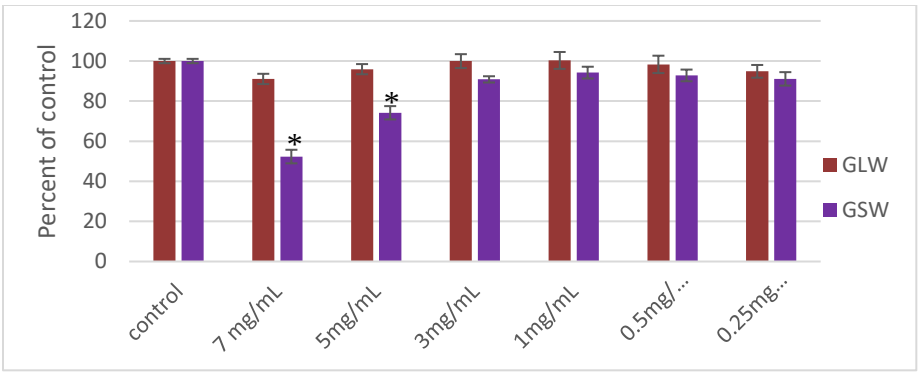

Figure 2 percent of control average of Gesho water leaf and stem extracts on S. mutans biofilms
(*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control

4.2 *S. mutans* viability assays

Viability assays for the *S. mutans* were carried on the 3,5 and 7 mg/ml concentrations since they represent the concentrations with the most significant effects. When compared to control; GLE showed average 2 log reductions for the 7mg/ml concentration, 2 log reductions for 5mg/ml and 1.5 log reduction for 3mg/ml. GSE showed an average 3 log reduction for the 7mg/ml compared to its control, 2.5 log reduction for 5mg/ml and 4 log reductions for 3mg/ml. GSW and GLW showed minor insignificant log reductions for viability assays with the GSW showing lower reduction than the GLW. Each assay was repeated three times and an average was plotted revealing the stated results (Figures 3 and 4).

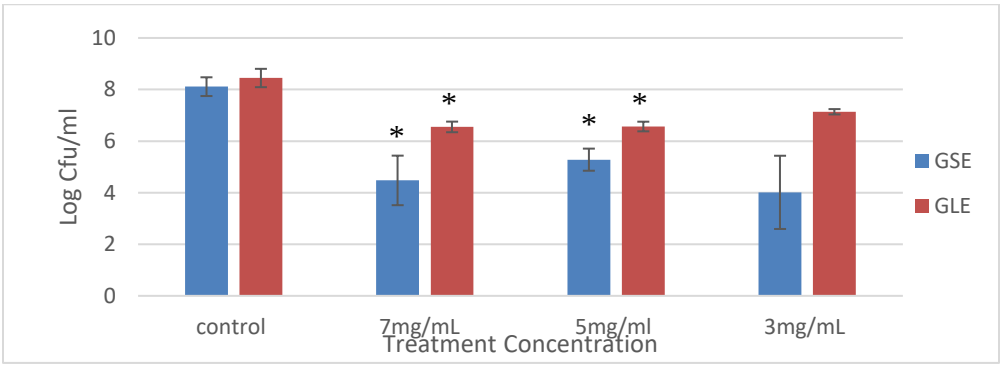

Figure 3 Viability assay showing log reductions of S. mutans with gesho ethanol extracts.
(*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control

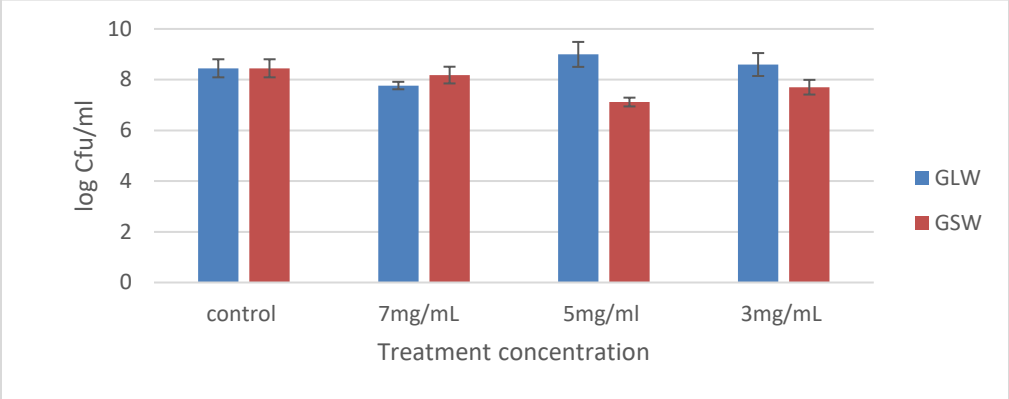

Figure 4 Viability assay showing log reductions of S. mutans with gesho water extracts

4.3 Effects of gesho ethanol extracts on *C. albicans* biofilm formation

Since the goal of our study is determination of gesho extract effects on polymicrobial biofilms we opted to work with the most effective extracts. From the *S. mutans* results gesho ethanol extracts had the significant effects. Hence, we chose to conduct the biofilm formation assay for *C.albicans* only on the ethanol extracts of gesho. Results showed significant inhibition of biofilm formation for the GSE fraction with more consistent results than GLE. The decrease in biofilms was similar through most of the tested concentrations 0.5,1,3,5 and 7 mg/ml. The GSE biofilm reduction ranged from 75-65% among those concentrations. GLE assay resulted in similar effects as GSE with an average 50% biofilm reduction for all concentrations, 0.25,0.5,1,3,5 and 7 mg/ml. Each assay was repeated 3 times on independent occasions and the average of the three assays were plotted as percent of control (Fig 5 and Fig 6).

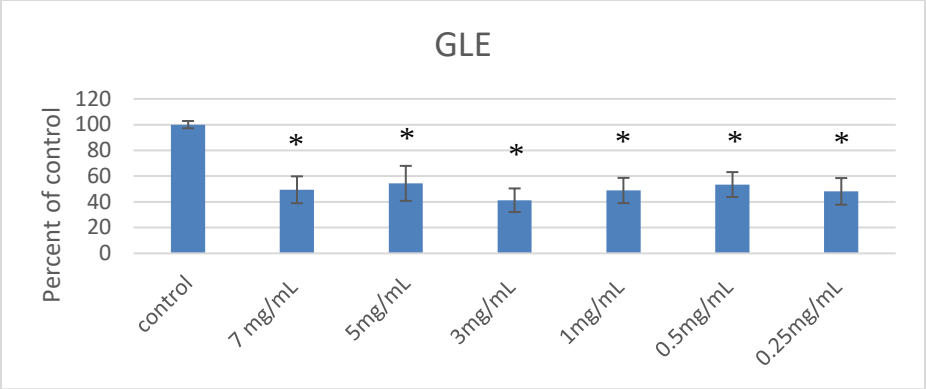

Figure 5 Gesho leaf ethanol extract effect on C. albicans biofilms, (*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control

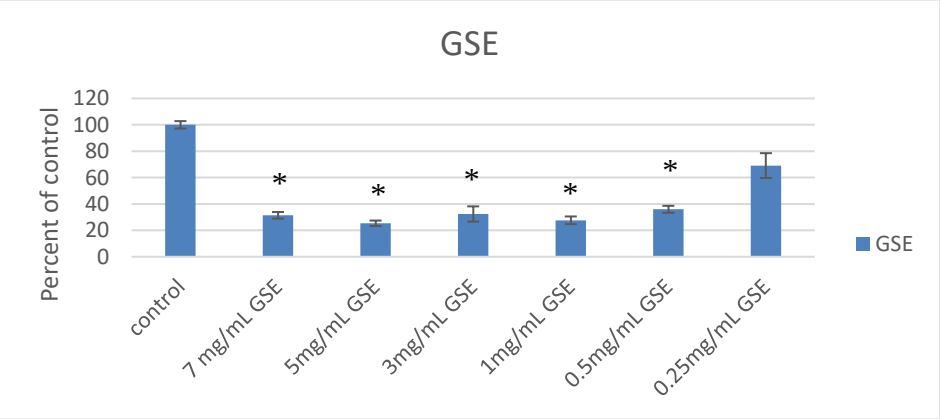

Figure 6 Gesho stem ethanol extract effect on C. albicans biofilm, (*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control

4.4 *C. albicans* Viability assays

Viability assays for *C. albicans* showed an increase to nonsignificant decrease in Log CfU/ml. GSE showed an increase of one log for 7mg/ml, while the 5 and 3 mg/ml concentration showed a decrease of less than one log. While GLE showed an increase of around one Log for 3, 5 and 7 mg/ml concentrations (Figure 7).

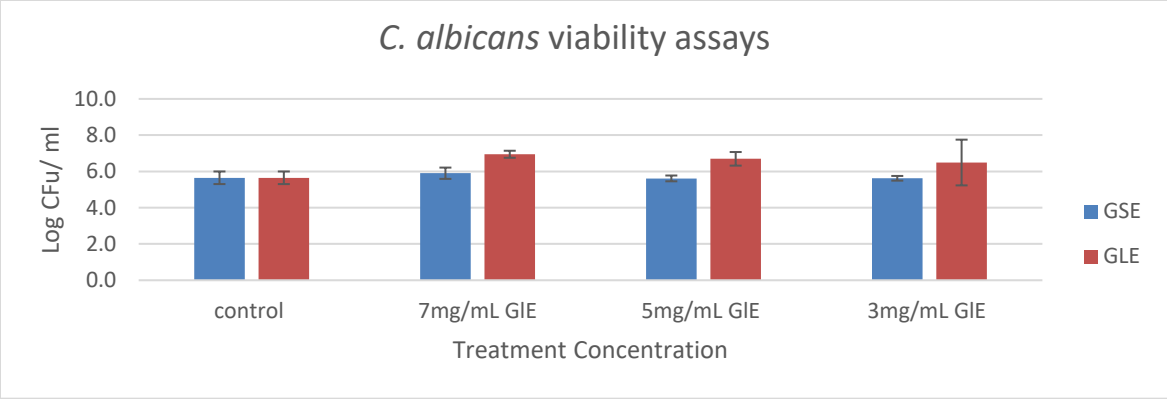

Figure 7 Viability assay showing C. albicans Log Cfu/ml increase.

4.5 Effects of GSE on *C. albicans* and *S. mutans* polymicrobial biofilms.

Gesho stem extract was chosen due to its more consistent results with *C. albicans.* GSE had similar inhibitory effects among most concentrations with *C. albicans* biofilms, while GSE 3 mg/ml was the lowest concentration with highest effect on *S. mutans* (Figure 8). Therefore, GSE 3 mg/ml was selected to proceed with performing gesho biofilm assays on polymicrobial biofilms. The design of this assay was performed to compare between polymicrobial biofilms of *C. albicans* and *S. mutans* and their monomicrobial biofilm biomass. Figure 9 shows the synergism that occurs in polymicrobial biofilms with more than double increase in biofilm biomass, almost 75%, compared to each organism alone. On the other hand, GSE treatment result showed significant decrease in biofilm formation when tested on the polymicrobial biofilm with even more inhibitory effects than each organism alone.

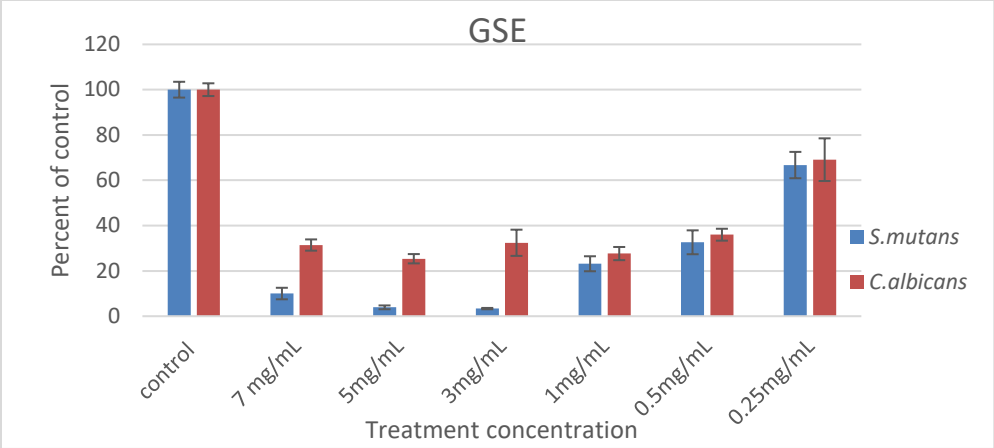

Figure 8 Effect of GSE on both S. mutans and C. albicans showing 3mg/ml with highest effect on S.mutans.

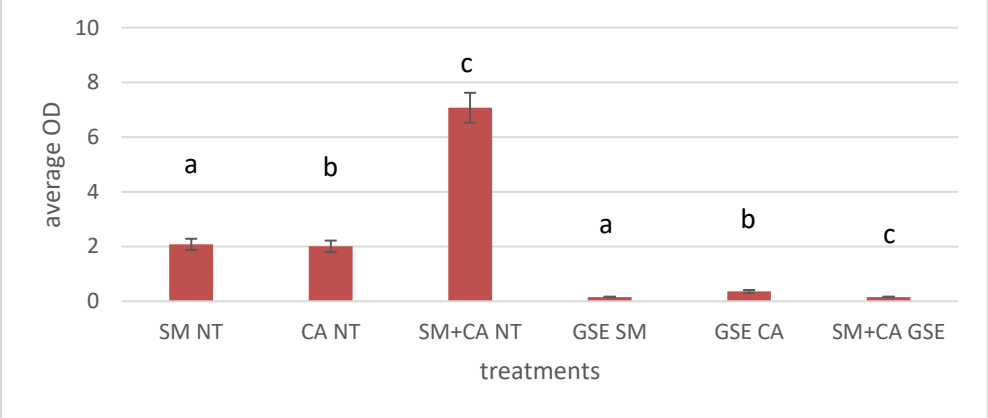

Figure 9 Effect of GSE 3mg/ml on S.mutans and C.albicans polymicrobial biofilms, each two similar letters represent a significant difference between each other, $p<0.05$

4.6 Epi-fluorescent microscope images

When comparing microscope images of *S. mutans- C. albicans* gesho treated dual species biofilm to untreated ones, the images indicated that gesho ethanol extracts significantly decreased the number of cells for both organisms without observing a prominent decrease in number for one over the other. *C. albicans* hyphae formation remains the same and is not affected. The EPS of the biofilms was extremely demolished, and we can only observe scattered

cells of *C. albicans* and *S. mutans* (Figure 10). The images for untreated biofilms show dense biofilms with thick layers of *C. albicans* and *S. mutans* (Figure 11).

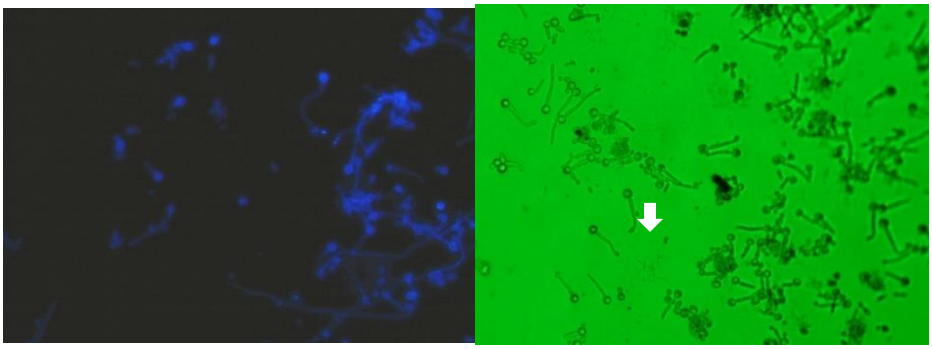

Figure 10 Images of treated Dual Species biofilms of C. albicans and S. mutans, white arrows pointes at S. mutans cells.

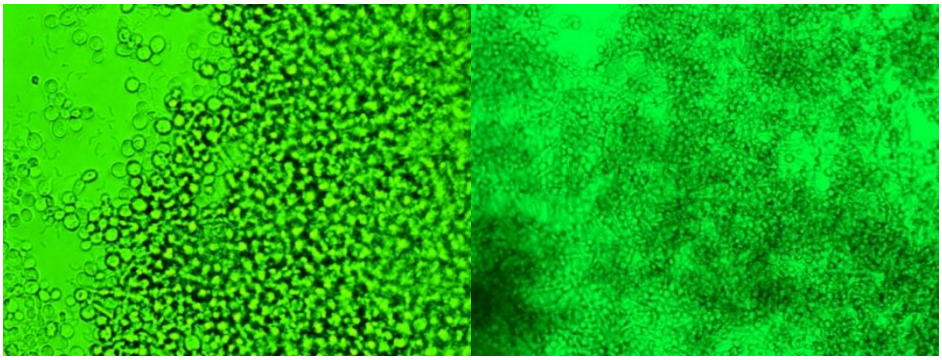

Figure 11 Images of untreated polymicrobial biofilms of C. albicans and S. mutans showing dense biofilms.

4.7 Cell size measurements results

For each image the size of *C. albicans* yeast cells, the *C. albicans* hyphae and *S. mutans* sizes were measured. Each image 3-4 measurement were taken, and an average of measurements was calculated and plotted into graph (Figure 12). We compared non-treated polymicrobial biofilms to GSE 3 mg/ml treated polymicrobial biofilms. We can see a difference in size measurements especially with *C. albicans* hyphae size, which can be an indication that gesho

might be affecting hyphae formation in *C. albicans*. And further investigation is required to confirm this observation.

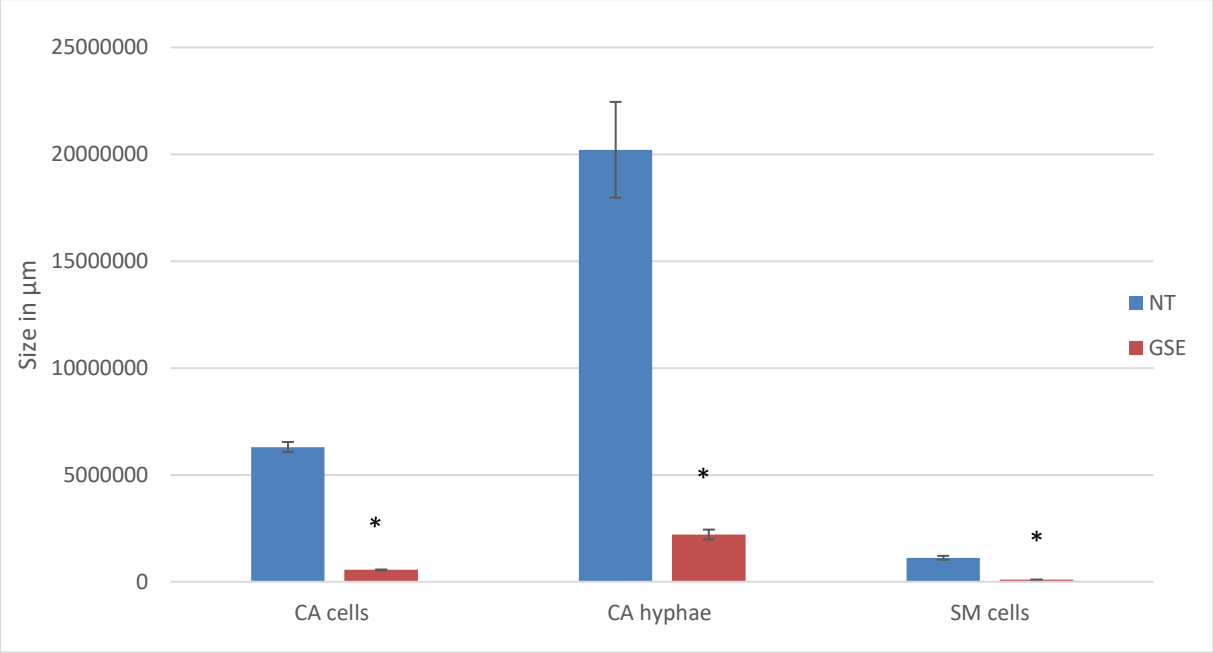

Figure 12 Size measurment of nontreated Vs. treated polymicroibial biofilms
, (*) indicates significant difference ($p < 0.05$) between treated samples and the untreated control

5 DISCUSSION

5.1 Gesho inhibits *S. mutans* and *C. albicans* polymicrobial biofilm formation

In previous studies, gesho leaf extracts demonstrated antibacterial activity specifically against Gram positive bacteria (Molla et al., 2016). In our laboratory research, gesho also successfully caused inhibitory effects on Gram positive bacterial biofilms (unpublished data). In this study, gesho inhibited *C. albicans* biofilms and polymicrobial biofilms comprised of both *C. albicans* and *S. mutans*. To test the hypothesis, we investigated the effect of gesho extracts on each organism alone. Through our results we confirm that gesho can inhibit the biofilm formation of each organism alone. GSE extracts were the most potent in inhibiting the biofilms of *S. mutans*, while for *C. albicans*, GSE and GLE had similar inhibitory effects, although they were more consistent with GSE. *C. albicans* biofilm formation inhibition percentages were similar for GSE at different concentrations, while for *S. mutans* GSE showed the highest inhibition with 3 mg/ml. Therefore, when testing polymicrobial biofilms we opted to work with GSE at 3 mg/ml concentration.

We compared the biomass of the untreated dual species biofilms to the biomass of *C. albican* and *S. mutans* single species biofilms. The results demonstrated a synergism that resulted in a 70 % biomass increase for the *C. albicans* and *S. mutans* dual species biofilm. These data confirm the previous study results by Falsetta (Falsetta et al., 2014). In their study, they presented a model which argues that *C. albicans* enhances the production of glycosyltransferase enzymes in *S. mutans*. Glycosyltransferase enzymes transfer sucrose to glucans, a major component of the EPS, strengthening the *S. mutans* component of the dual species biofilm and hence the overall biomass. Gesho stem extracts displayed significant inhibition of *C. albicans* and *S. mutans* dual species biofilm with GSE 3 mg/ml, even with the synergism gained with the

association of the two organisms together. Unlike previous studies (Amabye, 2016; Molla et al., 2016), our study started by testing the leaf and stem extracts separately. The stem extracts had a strong inhibitory effect. Moreover, we focused on using gesho as a natural remedy for polymicrobial biofilms formation. Few studies focused on natural treatments for polymicrobial biofilms, one similar study examined the antimicrobial effects of rosemary, a Mediterranean woody plant, for inhibiting polymicrobial biofilms (de Oliveira et al., 2017).

5.2 GSE extracts: potential mechanisms

S. mutans being a prokaryote and *C. albicans* a eukaryote, the mechanism by which gesho extracts inhibit biofilm formation for each organism is assumed to be different. Our viability assay results for *S. mutans* revealed significant log reductions compared to untreated controls, suggesting that gesho ethanol extracts possess biocidal effects, inhibiting biofilm formation. On the other hand, *C. albicans* viability assays showed an increase in *C. albicans* growth. We observed an increase or no notable change of CFU/ mL in the treated samples versus untreated samples. We hypothesize that gesho ethanol extracts do not kill *C. albicans* cells, but they can be quorum sensing inhibitors, inhibiting biofilm formation.

Images of *C. albicans* and *S. mutans* dual species biofilms assisted in exploring the mechanism by which gesho ethanol extract act. Based on our finding that GSE effected *S. mutans* biofilms by 90% compared to 70% for *C. albicans*, our initial hypothesis was that our images will display a lower *S. mutans* biofilms biomass compared to *C. albicans* biofilm biomass. Contradictory to our hypothesis, the images displayed a proportionate reduction in number for both organisms, with no significant reduction of one organism over the other. Our second hypothesis was related to the previous study model mentioned earlier (Falsetta et al., 2014). According to this model, gesho bactericidal effects *on S. mutans* can compromise its

ability to produce glucans for EPS formation and hence *C. albicans* biofilm formation is affected as well. Our images show a significant decrease in the EPS of the dual species biofilms, which indicates that gesho can be acting on the exopolysaccharide matrix formation. The mechanism of action by which gesho inhibits biofilms is still under question and requires further investigation.

5.3 Molecular analysis and future directions

5.3.1 S. mutans molecular analysis

Molecular analysis for genes involved in biofilm formation will enhance our knowledge on how gesho works. Each organism has its own genes involved in the biofilm construction process. In polymicrobial biofilms one organism can influence the gene expression of another. According to the Falsetta model (Falsetta et al., 2014), *C. albicans* increases the expression of glycosyltransferase genes (*gtfB, gtfC*), enhancing the EPS matrix formation. This model plus previous work by (Mattos-Graner, Napimoga, Duncan, Smith, & Fukushima, 2004) emphasize the essential role of the *gtf* genes in biofilm formation for *S. mutans*. Image analysis indicated a significant reduction in polysaccharide matrix formation in the treated polymicrobial biofilms. This observation is in agreement with our hypothesis that *gtf* expression is reduced by gesho extract. This hypothesis will be tested in future work. To this end, RNA extraction and preparation has been started in the laboratory; to date, cDNA has been synthesized.

5.3.2 C. albicans molecular analysis

Further insight into the mechanism of gesho can also be gained by studying other genes involved in *C. albicans* biofilm formation. Some studies are focused on such genes (Blankenship & Mitchell, 2006), which can be potential targets for our future studies, including *bcr1* and *efg1* (Falsetta et al., 2014). Through our biofilm assays, we confirm that gesho ethanol extracts have the ability to inhibit biofilm formation for the dual species biofilms of *C. albicans* and *S. mutans*

as well as their corresponding single species biofilms. Through our images, we know that both organism's ability to form biofilms is similarly compromised and the EPS biomass is significantly reduced. Molecular analysis to compare genetic expression with and without gesho will help understand the mechanism through which it works.

5.3.3 Applications

According to the National Center for Health Statistics, 37 % of children between ages 2-4 in the United States and 2.4 billion people around the world have dental carries. Dental disease can be difficult to treat and if stay untreated can cause further systemic complications as diabetes ,pneumonia and heart disease (Fernandes, Bhavsar, Sawarkar, & D'souza, 2018). Dental carries are caused by pathogenic bacteria forming oral biofilms. They have the ability to metabolize carbohydrates, producing acidic environment and building the EPS matrix that helps binding the bacteria to each other (Liu, Ren, Hwang, & Koo, 2018). While various methods have been studied for disruption or prevention of oral biofilms, antibiotic therapy was not one of the successful methods for biofilm treatment. The EPS matrix forms a barrier against penetration of antimicrobials, while decreasing antibiotic activity against oral biofilms (Liu et al., 2018).

Numerous novel methods have been investigated for oral biofilm treatment. Nanoparticles, phage therapy and photodynamic therapy are just to name a few. In spite of the variety of investigated new techniques, they still lack in vivo studies and their application and safety is still under question (Fernandes et al., 2018; Liu et al., 2018).

Natural products and plant extracts were proven to exhibit antimicrobial, anti-adhesive and anti-biofilm activities against oral pathogens (Karygianni et al., 2016). Plants represent a rich source of novel compounds and chemicals that can be used in pharmaceutical products. An estimated 500,000 species are present around the world with only 1% studied for their

phytochemical activity (Palombo, 2011). Herbs, in contrast to synthetic chemical compounds are a safe source of treatment. Many of which have been used traditionally for medicinal purposes, one of which is for oral health. Many herbal products are used as antimicrobials, anti-inflammatory and analgesics in dentistry (Kumar, Jalaluddin, Rout, Mohanty, & Dileep, 2013). The increased resistance to antibiotics as well as their adverse side effects in dentistry raised the need for other treatment options, natural products represented a promising and safe treatment alternative (Palombo, 2011). Gesho, an African plant, exhibited antimicrobial, anti-biofilm activity not only against Gram positive bacterial but also against two of the most common oral pathogens, *C. albicans* biofilms and *S. mutans* biofilms. Gesho represents an addition of a promising herbal treatment against the oral biofilms. It can be a source of natural anti-biofilm prevention source than can be used in many applications as mouthwashes or tooth pastes.

5.4 Conclusion

The enhanced resistance of biofilms to antimicrobials helps make them major source of chronic infection. The existence of polymicrobial biofilms increases their strength and their resistance. Gesho ethanol extracts were found to exhibit biofilm formation inhibitory effect. Its effect has been demonstrated on both *S. mutans* and *C. albicans* as well as their polymicrobial biofilms. The mechanism by which it prevents their biofilm formation is still not resolved, but some hypothesis include 1) it exhibits biocidal effects on *S. mutans*, 2) it has anti-quorum sensing effects on *C. albicans* or 3) it has the ability to inhibit formation of the EPS through different mechanisms.

In our study we show for the first time that gesho stem extracts possess inhibitory anti-biofilm effects. We also focus on polymicrobial biofilms that are formed of dual species of yeast and bacteria. And as part of our study we showed that gesho inhibits yeast biofilm formation as

well as Gram positive bacteria. Gesho's traditional use makes its future use in antibiofilm products applicable, for example toothpaste or mouthwash. Gesho anti-biofilm results make it a promising antibiofilm agent.

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