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RHAMNUS PRINOIDES PLANT EXTRACTS AND PURE COMPOUNDS INHIBIT
MICROBIAL GROWTH AND BIOFILM FORMATION

by

MARIYA M. CAMPBELL

Under the Direction of Eric Gilbert, PhD

ABSTRACT

The increased prevalence of antibiotic resistance threatens to render all of our current antibiotics ineffective in the fight against microbial infections. Biofilms, or microbial communities attached to biotic or abiotic surfaces, have enhanced antibiotic resistance and are associated with chronic infections including periodontitis, endocarditis and osteomyelitis. The “biofilm lifestyle” confers survival advantages against both physical and chemical threats, making biofilm eradication a major challenge. A need exists for anti-biofilm treatments that are “anti-pathogenic”, meaning they act against microbial virulence in a non-biocidal way, leading to reduced drug resistance. A potential source of anti-biofilm, anti-pathogenic agents is plants used in traditional medicine for

treating biofilm-associated conditions. My dissertation describes the anti-pathogenic, anti-biofilm activity of *Rhamnus prinoides* (gesho) extracts and specific chemicals derived from them.

Rhamnus prinoides, an evergreen shrub native to east Africa, is used in the fermented beverages te'j and tella and to treat a variety of illnesses including atopic dermatitis. Gesho has antibacterial and antiplasmodial activity but little is known about its effect against microbial biofilms. Preliminary work determined that gesho leaf ethanol extracts inhibited Gram positive bacterial biofilm formation up to 99 percent without inhibiting microbial growth, suggesting an anti-pathogenic mechanism of activity. Fractionation, chemical analysis and activity screens identified ethyl 4-ethoxybenzoic acid (EEB) as a novel gesho-derived compound with anti-pathogenic anti-biofilm activity. Structure-activity analysis of EEB-related compounds identified 4-ethoxybenzoic acid (4EB) as a more potent anti-pathogenic compound against *S. aureus* biofilms. 4EB inhibited 70 percent *S. aureus* biofilm formation with minimal impact on planktonic cell viability. 4EB decreased the fraction of hydrophobic *S. aureus* cells in culture, potentially reducing surface colonization. Additionally, treatments of existing biofilms with a combination of 4EB and vancomycin synergistically decreased the viability of biofilm dwelling cells up to 85 percent when compared to vancomycin alone. Work with gesho stem extracts measured more than 90 percent reduction of dual-species biofilms comprised of the oral pathogens *Streptococcus mutans* and *Candida albicans*. Reduced biofilm formation correlated with inhibition of extracellular polysaccharide production. Overall, gesho extracts and gesho-derived compounds have potential for use in topical and oral hygiene products, for wound treatments and other anti-biofilm applications.

INDEX WORDS: Biofilms, Anti-pathogenic, *Staphylococcus aureus*, Antimicrobial, *Rhamnus prinoides*, Phenolic compounds

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MICROBIAL GROWTH AND BIOFILM FORMATION

by

MARIYA M. CAMPBELL

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2020

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RHAMNUS PRINOIDES PLANT EXTRACTS AND PURE COMPOUNDS INHIBIT
MICROBIAL GROWTH AND BIOFILM FORMATION

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July 2020

DEDICATION

I dedicate this work to my wonderful parents Kendra and Lamont Powell. Thank you for all the sacrifices you have made to give me this wonderful life and for always supporting and encouraging me to follow my aspirations regardless of how challenging or eccentric they may be. I also dedicate this document to all the strong, empowered and driven women in my family.

You have made me who I am today and continue to shape who I will be in the future. Most importantly, thank you to God for blessing and guiding me all my life. I definitely would not be where I am today without the love and support of my creator.

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LIST OF ABBREVIATIONS

GSE: gesho stem ethanol extract

GLE: gesho leaf ethanol extract

GLW: gesho leaf water extract

GSE: gesho stem ethanol extract

4EB: 4-ethoxybenzoic acid

EEB: ethyl 4-ethoxybenzote

HMP: 4-hydroxy-4-methyl-2-pentanone

1 INTRODUCTION

1.1 Microbial biofilms

Biofilms are recalcitrant, complex, microbial communities that form on solid surfaces and can result in both acute and chronic illnesses. The biofilm lifestyle confers survival advantages against both physical and chemical threats that would easily eliminate their free-floating, planktonic counterparts; thus in nature, most microbes exist in biofilm communities. Biofilms form on a variety of surfaces including on water filters, inside industrial pipes, inside bioreactors and on animal tissues. In many cases, biofilms pose no threat to humans and often have beneficial applications however, when associated with human pathogens, they can be the root of chronic illnesses and facilitate the spread of antibiotic resistance.

1.1.1 The biofilm life cycle

The biofilm life cycle has four main stages: attachment, proliferation, maturation and detachment. Attachment occurs when free-floating, planktonic microorganisms in a liquid media interact with and anchor onto a solid substrate. Attachment can be reversible or irreversible with the later initiating the biofilm formation process. Both cellular characteristics (such as charge, hydrophobicity and appendage prevalence) and substrate features (including hydrophobicity, charge, composition, porosity and texture) play a role in attracting and anchoring organisms to a surface (1). Once attached, organisms will begin to proliferate, forming a thin film of cells on the substrate. As cellular proliferation continues and the biofilm matures, the direction of spread often switches from lateral to aerial, resulting in the formation of column or mushroom-like structures that protrude into the liquid media. These structures are made possible by the presence of a dense, cell-derived matrix of metabolites known as the extracellular polymeric substance (EPS) (2). The EPS is primarily composed of polysaccharides, proteins and extracellular DNA that work together

to provide structure as well as protection to the biofilm. As mass increases, biofilms becomes so dense that oxygen and vital nutrients are no longer able to effectively reach the older cells residing deep within the matrix (3, 4). The lack of available resources, coupled with the accumulation of both intra- and intercellular signaling molecules, leads the next stage in the biofilm life cycle, detachment (5). During detachment, parts of the biofilm become dislodged and travel to satellite locations where new biofilms can be established; this process is known as dispersal (5, 6). Biofilm dispersal exacerbates the infection and worsens both treatment efficacy and patient prognosis.

1.1.2 Biofilms and antibiotic resistance

Microbial biofilms exhibit a variety of characteristics that make them difficult to eradicate once formed. Low metabolic rates, persister cells, horizontal gene transfer and the extracellular matrix are all major factors that decrease the efficacy of both chemical and physical biofilm removal (7).

1.1.2.1 The matrix

The biofilm extracellular polymeric substance (EPS) is a complex matrix composed of polysaccharides, proteins and nucleic acids that work together to provide structure and protection to the biofilm and its inhabitants. The matrix is dense and sticky making penetration by antibiotics or immune cells challenging (2). Extracellular nucleic acids (primarily eDNA) are released into the matrix via cell lysis, active secretion or release from membrane vesicles. eDNA has been found to play a role in biofilm structure and antibiotic resistance through binding of cationic antibiotics (8–11).

Matrix proteins, another primary contributor to the biofilm structure, has been found to be important for the initial attachment of cells to surfaces and in cell accumulation (12, 13). Matrix proteins can be either secreted or anchored to the cell wall (CWA) (12, 14). CWA proteins have

been found to engage in both homo- and heterodimerization with surrounding microbial and host derived proteins, anchoring the microbial cells within the biofilm to each other and to host tissues (12, 14, 15). Interestingly, some biofilm associated proteins have been found to stimulate the production of other matrix components including extracellular DNA and polysaccharides, further enhancing biofilm formation (16).

Matrix polysaccharides make up a significant proportion of the biofilm matrix and perform similar functions as eDNA and matrix proteins. Matrix polysaccharide composition varies widely between microbial species and is greatly impacted by environmental conditions; despite this variability, most have been found to aid in cell aggregation, biofilm structural integrity and antibiotic resistance (17, 18). As in the case of biofilm proteins, biofilm carbohydrates have been found to be essential for proper assembly and maturation of biofilms with deficient mutants resulting in unusual biofilm morphologies or lacking the ability to form biofilms altogether (19). Additionally, matrix polysaccharides have been found to actively bind antimicrobial peptides (20) and prevent immune cell phagocytosis (18). The three major biofilm macromolecules (proteins, polysaccharides and nucleotide) together with less significant constituents (including lipids, bacteriophages and quorum sensing molecules) interact to form recalcitrant biofilms that can go on to establish resistant and chronic infections.

1.1.2.2 Decreased metabolism and persister cells

Cellular metabolism plays an important role in antibiotic efficacy with stationary-phase or metabolically attenuated cells exhibiting enhanced resistance to treatment when compared to their metabolically active counterparts (21–23). At any given time, biofilms contain cells at various stages in their life cycle. Cells at the biofilm perimeter are generally metabolically active while the majority of cells that reside at the base or in the interior of the biofilm column are in stationary

phase due to limited availability of resources such as nutrients and oxygen (3, 24, 25). The lack of metabolic activity exhibited by these senior biofilm-dwelling cells helps to protect them from biocidal and bacteriostatic antibiotics (22, 24). One method that has been found to reverse this form of resistance is to supplement antibiotic treatments with vital metabolites (22, 26). The metabolites stimulate microbial metabolism, restoring sensitivity to previously tolerant or resistant cells.

Unfortunately, resting or stationary phase cells are not the only population of biofilm-residing cells that exhibit inherent resistance to antibiotic treatment. Biofilms contain a subpopulation of antibiotic-tolerant, dormant cells known as persister cells that aid in biofilm longevity and infection persistence. Persister cells are genotypically identical but phenotypically distinct variants of microbial wildtype strains (27). They result from environmental stressors that trigger the expression of genes such as toxin-antitoxin modules that halt metabolism, translation and replication (28, 29). Persister cells can be found at every stage of growth as long as stressors such as nutrient and oxygen deprivation are present (30, 31). Despite the ubiquitous impact of the stressor, only a small subset of the total biofilm population (less than 1 percent) will go on to become persister cells (4, 27, 30, 31). The dormant nature of persister cells makes them highly tolerant to antibiotic treatments, requiring excessively high treatment concentrations to begin eliciting a biocidal response (27, 30, 32). This contributes to biofilm survival and chronic disease as standard antibiotic treatment dosages fail to eradicate these dormant cells allowing them go on to proliferate and reestablish infection once environmental conditions are favorable (30). This can lead to recurring infections and chronic illness (33, 34).

1.1.2.3 Heterogeneity and horizontal gene transfer

Horizontal gene transfer in biofilms can facilitate inter- and intraspecies spread of antibiotic resistance genes. In nature, biofilms are heterogeneous and contain cells from a variety of strains and species (35). The extracellular matrix surrounding biofilm cells positions disparate cells in close proximity to one another, facilitating swift, intercellular exchange of cellular components including DNA (36). Two forms of horizontal gene transfer occur in biofilms: transformation and conjugation (37, 38). Transformation is indirect and occurs when an organism appropriates extracellular DNA from the environment that is later incorporated into its own genome; conjugation, on the other hand, is the direct transfer of DNA between organisms through a pillus. The transfer of genetic information is especially important when it involves antibiotic resistance. Inter- and intraspecies transfer of antibiotic resistance genes has been observed in biofilms but is often limited to the actively metabolizing cells that reside on the biofilm perimeter (37, 38). This is thought to be due to the fact that both DNA uptake and pilli formation are energy expensive processes that can only be conducted by cells that are undergoing metabolism (39). Additionally, the ubiquitous spread of resistance genes throughout biofilms has been hypothesized to be hindered by the restrictive and structured nature of the biofilm matrix. Genetic information can only be exchanged between neighboring cells in close proximity, cells that are too distant are unable to interact halting further exchange. This limitation results in pockets or clusters of resistant communities within the matrix rather ubiquitous prevalence (39). Despite the localized occurrence of horizontal gene transfer in biofilms, it still plays an important role in the spread of antibiotic resistance and patient outcomes especially considering that multidrug resistant (MDR) microbial species have, in some cases, been found to produce more biofilm than their susceptible counterparts (40, 41).

1.1.3 Biofilm infections and chronic illnesses

Biofilm infections can result in debilitating, chronic illnesses as infections are recalcitrant to treatment and degrade host tissues overtime (42). Biofilms are commonly introduced into the body via contaminated abiotic materials such as joint replacements and catheters however, they can also form organically on biotic tissues as in the case of cystic fibrosis. Once introduced into the body, biofilm cells will bind to and invade host tissues to establish new biofilms inside the host. These infections begin as acute but can quickly become chronic due to the resistance mechanisms discussed previously (see section 1.1.2). Periodontitis, endocarditis, chronic rhinosinusitis and osteomyelitis are all serious illnesses that are attributed to chronic biofilm infections (43–46). Additionally, biofilms have been found in the wounds from chronically ill patients; these biofilms were not present in wounds from patients with acute infections (42). Wound colonization prevents healing and aids in the spread of infection to previously healthy tissues (47). Chronic biofilm infections such as this often require surgery or amputation to combat disease progression and when proper treatment is delayed, patients risk developing septicemia or death.

1.1.4 Societal impact of biofilm infections

Biofilm infections not only have negative health outcomes but also have serious societal and economic ramifications. In 2010, in the United States, it was estimated that over 14 million patients presented with biofilm infections each year resulting in approximately 350,000 deaths annually (43). The economic ramifications of treating and caring for these patients are considerable for both the patient and the country as a whole. Since the 1990's, the cost of treating biofilm related infections have steadily increased leaving patients with inflated medical bills totaling tens to hundreds of thousands of dollars (46). These exorbitant health care costs are further increased when infections exhibit active antibiotic resistance (antibiotic resistance genes are being

expressed) as additional hospitalization and care are required (46, 48–50). The average American citizen does not have sufficient income or financial reserves to immediately pay off such massive debts, leaving many struggling to pay their medical bills. In some cases, people are forced to sacrifice basic necessities such as food, housing and transportation in order to make healthcare payments (51). To make matters worse, severe infections often require the afflicted individual to stay home or check into a hospital in order to get well, this means increased absenteeism which may result in decreased income or termination of employment (46). The economic burden associated with biofilm infections does not solely fall on the patient but also impacts the nation as a whole as a significant population of the country are enrolled in Medicare or Medicaid. The United States has been estimated to spend over 94 billion USD on the treatment of biofilm infections each year with an additional estimate of 2-20 billion USD spent on the treatment of antibiotic resistant infections (43, 52, 53).

The costs of antibiotic resistance and biofilm infections are not strictly monetary, impacts on quality of life and mental health are also prevalent. When studying a cohort of individuals who developed post-surgery antibiotic resistant infections, Perencevich *et al.*, 2003 reported a significant decrease in overall mental and physical health in patients after surgery when compared to before surgery (50). A decrease in mental health was not observed in control patients and the reported impacts on physical health were less severe. This observation may have been due to a combination of financial woes, physical impairments and extensive care requirements (both in and outside the hospital) that come with the treatment of antibiotic resistant infections. Infections can also directly decrease a patient's quality of through inhibition of mobility or ability to perform basic day-to-day tasks. One example can be seen in patients with severe periodontitis, an oral biofilm infection that may result in irreversible tooth and bone loss. For the afflicted, the lack of

teeth and presence of oral pain make it difficult for many to eat, speak or properly conduct oral hygiene practices (ie. brushing and flossing) which negatively impacts their overall physical and psychological health (54–56). The debilitating epidemiological, financial and social implications of biofilms have inspired many researchers to investigate natural products as sources of anti-biofilm therapeutics.

1.2 Natural products in traditional medicine and anti-pathogenic phytotherapeutics

Humans have relied on homeopathic remedies to self-medicate for thousands of years. Prior to the development of modern medicine, people primarily used plant and animal products to alleviate symptoms and cure diseases. These treatments were commonly in the form of tonics, tinctures and creams whose recipes were passed down from generation to generation. Some of these treatments have since proven to be detrimental to human health while others are still widely used today (57, 58). Research into ethnopharmacologically relevant plant extracts and plant derived compounds have identified both anti-bacterial and anti-biofilm activities (59, 60). The anti-biofilm activity of many phytotherapeutics have been attributed to either biocidal or biostatic mechanisms; in time, this can prove problematic as selective pressure may lead to the development of resistance. In an effort to identify novel and effective anti-biofilm agents that do not potentiate antibiotic resistance, investigations into anti-pathogenic phytotherapeutics have increased (61–63).

Anti-pathogenic therapeutics are compounds that inhibit microbial virulence or pathogenesis while minimizing selective pressure thus delaying antibiotic resistance development (64, 65). Many plant extracts, essential oils and phytochemicals have been found to exhibit anti-pathogenic anti-biofilm activity including: *Rosa rugosa* (beach rose), *Tripterygium wilfordii* (thunder god vine), *Piper nigrum* (black pepper) and *Commiphora myrrha* (myrrh) (59, 61, 66). Targets of anti-pathogenic therapeutics include biofilm formation (61, 66, 67), quorum sensing signaling (59, 68),

hemolysis activity (61, 63) and motility (59, 66, 67). Despite the existence of many promising anti-pathogenic therapeutics, none are currently readily available to consumers.

1.2.1 Anti-pathogenic activity of benzoic compounds

Benzoic compounds are ubiquitous in plant tissues and are a major category of interest in anti-pathogenic antimicrobial research. Benzoic acid and its derivatives consist of a core benzene ring and a carboxylate substituent; many plant derived benzoic compounds such as epigallocatechin gallate, hamamelitannin, salicylic acid and ginkgolic acid possess additional substituents that aid in their antimicrobial activity. Benzoic compounds have been found to exhibit anti-pathogenic antimicrobial activity against a variety of microorganisms including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus mutans* and *Escherichia coli* through targeting extracellular polysaccharide production, exoprotease activity, fimbriae production, quorum sensing and motility (69, 70). The use of these compounds as antibiotic adjuvants has gained traction in an effort to restore sensitivity to resistant strains of microorganisms (71–73).

1.3 *Rhamnus prinoides* (gesho): more than just beer

Rhamnus prinoides, gesho, is a buckthorn plant that grows throughout East Africa. This plant has been used in the production of some of the oldest fermented beverages known to man but has also been used traditionally for the treatment of a variety of illnesses. Research into the therapeutic applications of gesho extracts have identified both antimicrobial and anti-plasmodial activities. Antimicrobial compounds such as quercetin, emodin and chrysophanol have been identified in gesho and may be responsible for its inhibitory effects (74). Despite the many studies that have been done using *R. prinoides*, many more investigations into gesho and gesho-derived compounds remain to be conducted.

1.3.1 What is gesho?

Rhamnus prinoides, a large evergreen shrub native to East Africa, is used in the production of two popular, traditional alcoholic beverages called tella and te'j (75). In beverage production, gesho is used as a bittering agent similar to hops in Western beer (76). The processes used for brewing tella and te'j consist of multiple stages and vary from person to person but several steps are conserved (77). The process begins with the smoking of clay pots for seasoning and sterilization (76–78). Gesho leaves or stems are then added to water in the pot and allowed to ferment for several days (76–78). When producing te'j, raw honey or sugar is added to the mixture while grains such as barley and malt are added when brewing tella (76, 77). Both drinks are allowed to ferment for 15-20 days or until the alcohol content reaches between 2-8% (76, 78). Despite the commercial production of te'j, the traditional origins of both tella and te'j have resulted in a variety of recipes as the instructions have been altered and passed down the generations (77).

1.3.2 Applications in traditional medicine

In addition to uses as a bittering agent in beverage production, gesho has also been used traditionally to treat a variety of illnesses. Aqueous tinctures containing gesho have been used for the treatment of arthritis, back pain, brucellosis, flu, common cold, indigestion, loss of appetite, pneumonia, fatigue, sexually transmitted diseases, stomach ache and ear nose and throat infections (79, 80). *R. prinoides* leaves have also been ground and mixed with butter to be used as an ointment for the treatment of eczema (81). In an attempt to validate or refute some of the acclaimed medicinal uses of gesho, researchers began investigating the plant for antibacterial and antiplasmodial activities. Researchers in Japan found that *R. prinoides* methanol extracts were effective at killing plasmodia both *in vitro* and *in vivo*, with decreased parasitemia and prolonged survival in mice treated with gesho extract compared to untreated control mice (82, 83). Gesho has

also been found to have biocidal activity against both Gram positive and Gram negative species of bacteria in planktonic culture (75, 84, 85). Despite the research that has been done on the antimicrobial and antiplasmodial activities of gesho, nothing is known about its effects on bacterial biofilms, this dissertation looks to help fill this gap in knowledge.

1.4 Hypothesis and objectives

The overall hypothesis of this work is *Rhamnus prinoides* (gesho) extracts and derived compounds exhibit anti-biofilm activity against Gram positive bacteria and yeast. To test this hypothesis, the following objectives were proposed:

1.4.1 Aim 1: Screen gesho extracts for anti-biofilm activity and identify active compounds

Four extracts were prepared from the leaves and stems of gesho and screened for anti-biofilm activity. 96-well microtiter plate, crystal violet assay were used to assess the effects of the extracts on biofilm formation. Resting cell viability counts were performed in order to determine if biofilm prevention was due to a biocidal, bacteriostatic or anti-pathogenic mechanism. Of the extracts tested, the leaf ethanol extract was selected for chemical analysis to identify individual compounds with anti-biofilm activity.

1.4.2 Aim 2: Evaluate the effects of gesho ethanol extracts on dual-species biofilms

Gesho ethanol extracts were screened for anti-biofilm activity against *Streptococcus mutans* and *Candida albicans* mono- and dual-species biofilms. 96-well microtiter plate, crystal violet assays were used to assess the effects on biofilm formation while resazurin assays were used to assess anti-microbial activity on biofilm dwelling cells. Growth curve experiments were conducted to characterize the anti-biofilm activity of the stem ethanol extract as biocidal,

bacteriostatic or anti-pathogenic. Finally, the effect of the stem ethanol extract on extracellular polysaccharide production was assessed to identify a potential mechanism for biofilm inhibition.

1.4.3 Aim 3: Identify benzoic compounds with anti-pathogenic anti-biofilm activity and characterize their phenotypic effects

Benzoic compounds with structural similarity to the gesho-derived ethyl 4-ethoxybenzoic acid were screened for anti-pathogenic anti-biofilm activity on *Staphylococcus aureus*. Three target compounds were identified and their effects on planktonic growth and antibiotic sensitization were evaluated. Phenotypic assays were used to characterize the effects of treatment on cell hydrophobicity, hemolysis activity, membrane permeability and EPS production.

2 *RHAMNUS PRINOIDES* (GESHO): A SOURCE OF DIVERSE ANTI-MICROBIAL ACTIVITY

2.1 Introduction

Microbial biofilms, or surface-attached communities of microorganisms, are a source of chronic infection. In contemporary medicine, concern over biofilms is frequently elicited by numerous conditions, including device-related infections and chronic wounds (42, 47, 86). A wide range of pathogenic bacteria are reported to establish biofilm infections (87, 88). These include *Staphylococcus aureus* and *Streptococcus mutans*, two opportunistic pathogens whose biofilms are responsible for diseases including endocarditis and tooth decay, respectively (45, 89). In traditional settings, biofilm-associated infections would most commonly be encountered in wounds, on the skin and in the mouth. The severity of biofilm infections in combination with the increased prevalence of antibiotic resistance, has led to an ethnopharmacological approach to finding novel anti-biofilm agents (40, 90).

Rhamnus prinoides (gesho) is a large evergreen shrub native to East Africa that is used as a bittering agent in the traditional East African fermented beverages tella and tej (77, 91). In addition to its culinary use, gesho has been used historically as a treatment for a variety of illnesses. Aqueous tinctures containing gesho have been used for the treatment of arthritis, back pain, brucellosis, flu, common cold, indigestion, loss of appetite, pneumonia, fatigue, sexually transmitted diseases, stomach ache and ear, nose and throat infections (79, 80). Notably, a mixture of ground *R. prinoides* leaves and butter has been used as an ointment for the treatment of atopic dermatitis (81), a skin condition occasionally associated with *Staphylococcus aureus* infections (92). Gesho contains a complex mixture of potentially therapeutic biocidal chemicals active against planktonic pathogens, notably geshoidin, quercetin, emodin, and various anthracene

derivatives (74, 85, 93). Based on this background information, we hypothesized that gesho could be a source of anti-biofilm compounds effective against *S. aureus* and other Gram positive bacteria. Additionally, we sought anti-biofilm compounds with low biocidal activity; i.e. that had anti-pathogenic properties (64, 65).

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

Four species of microorganisms were employed in this work: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 23059, *Pseudomonas aeruginosa* PA01 and *Streptococcus mutans*. *Streptococcus mutans* was a gift from Margaret Gould-Bartlett, Georgia State University. *Pseudomonas aeruginosa* PA01 was obtained from the laboratory of Jay Keasling, University of California, Berkeley. *S. aureus* was grown in Luria-Bertani (LB) broth (Becton Dickinson, USA). *B. subtilis* was cultivated in LB broth supplemented with 150 mM ammonium sulfate, 100 mM potassium phosphate, 34 mM sodium citrate, 1 mM MgSO₄ and 0.1% glucose (94). *S. mutans* was grown in Brain-Heart Infusion (BHI) medium (Becton, Dickinson, USA) supplemented with 0.5% sucrose. *P. aeruginosa* was cultivated in *Pseudomonas* basal mineral (PBM) medium containing 80 mM glucose (60).

2.2.2 Chemicals and reagents

The following reagents were purchased from Fisher Scientific (USA): crystal violet, potassium phosphate, magnesium sulfate, ammonium sulfate, hexanes, methanol, and sodium carbonate. Reagents purchased from Sigma Aldrich (USA) include: sucrose, butanol, Folin-Ciocalteu reagent, ninhydrin reagent, sodium bicarbonate, cupric sulfate. Sodium citrate, glucose and ethyl acetate were purchased from EM Science (USA), OmniPur (Germany) and Pharmco-Aaper (USA), respectively. 95% ethanol was purchased from Decon Labs (USA).

2.2.3 Extract preparation

Rhamnus prinoides stems and leaves were purchased from a local Ethiopian market (Buford, Georgia, USA). The authenticity of the stems and leaves was verified by two local experts on Ethiopian foods. 24 g of ground leaves or fractured stems were added to 150 ml of sterile water or 95% ethanol in a 250 ml flask (95, 96). Flasks were shaken in the absence of light at 200 rpm at room temperature for four days. The extraction liquid was then collected and particulate matter removed via centrifugation at 12,500 rpm at 4 °C for 5 min; clarified supernatants were recovered for further processing. Aqueous extracts were frozen at -80 °C and lyophilized (Virtis, USA) to remove all water. Ethanol extracts were air-dried under vacuum prior to lyophilization. Extracts were stored at -80 °C. The percent yield (w/w) of the leaf ethanol, leaf water, stem ethanol and stem water extracts were: 8, 13, 2 and 7%, respectively.

2.2.4 Log-phase cell antibacterial assay

Bacteria were inoculated in 20 ml of growth media at an initial optical density (OD₆₀₀) of 0.01 and incubated for 4 ± 1 h at 37 °C with shaking at 200 rpm until log phase (OD₆₀₀ ≈ 0.35) was reached. 1 ml of log-phase cells was transferred to 1.5 ml microfuge tubes and the growth medium was removed via centrifugation. Pelleted cells were washed twice with phosphate buffered saline (PBS) before resuspending the pellets in 1 ml of extract dissolved in PBS. Extract concentrations ranged from 2.5 – 10 mg/ml and were selected following a preliminary activity screen using *S. aureus* and then used for each of the other tested species. Treatments were incubated at 37 °C for 1 h and then serially diluted in PBS and radially plated on growth agar. Plate counts were used to assess biocidal activity.

2.2.5 Stationary-phase cell viability assay

To assess the viability of stationary phase planktonic cells, a growth agar-based assay was conducted. Spent media containing stationary phase planktonic cells were collected from microtiter plates or borosilicate glass culture tubes after the conclusion of each biofilm formation assay. The collected cells were serially diluted (1:10) in PBS and dilutions were radially plated in 10 μ l volumes on growth medium. Agar plates were incubated at 37 °C overnight to allow for colony formation and plate counts were used to assess viability.

2.2.6 *Staphylococcus aureus*, *Streptococcus mutans* and *Pseudomonas aeruginosa* biofilm formation assay

Staphylococcus aureus, *Streptococcus mutans* and *Pseudomonas aeruginosa* biofilms were grown in LB broth, BHI medium containing sucrose and PBM-glucose, respectively. Biofilm formation was assessed using a polystyrene 96 well microtiter plate crystal violet assay (97). Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh media and combined with gesho extract to obtain a final concentration of 7 mg/ml. Treatments were then serially diluted to final extract concentrations of 5, 3, 1, 0.5 and 0.25 mg/ml. These concentrations were selected after preliminary screens of the four extracts using *S. aureus* and were then used for each of the additional species that were examined. No vehicles were added to solubilize the treatments. Negative controls consisted of cells diluted to an initial OD₆₀₀ of 0.01 in growth media without plant extract. 100 μ l of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C for 24 h (98, 99). After 24 h, planktonic cells and spent growth media were removed from each well and the plate was washed 3 times in sterile water. Biofilms were then stained with 150 μ l of 0.1% crystal violet for 15 min at 200 rpm. Excess dye was removed via washing in sterile water and the plate was allowed to air dry. Crystal violet was solubilized in 150

µl of 95% ethanol and absorbance measurements at 595 nm were taken using an MD SPECTRAMax plate reader (Molecular Devices Corporation, USA).

2.2.7 Bacillus subtilis biofilm formation assay

Bacillus subtilis biofilms were grown in LB broth supplemented with 150 mM ammonium sulfate, 100 mM potassium phosphate, 34 mM sodium citrate, 1 mM MgSO₄ and 0.1% glucose (94). Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh supplemented LB media and combined with gesho extract to obtain a final concentration of 7 mg/ml. Treatments were then serially diluted to final concentrations of 5, 3, 1, 0.5 and 0.25 mg/ml. No vehicles were added to solubilize the treatments. Negative controls consisted of cells diluted to an initial OD₆₀₀ of 0.01 in growth media without plant extract. 1 ml of treated and untreated samples were added to sterile 10 ml borosilicate glass culture tubes and statically incubated at 30 °C for 48 h to allow for pellicle formation. After 2 days, planktonic cells and spent growth media were removed from below the pellicle that formed at the air to liquid interface. Pellicles were vortexed and pipetted for resuspension in 1 ml PBS then serially diluted (1:10) in PBS and radially plated on LB agar plates. Agar plates were incubated at 37 °C overnight to allow for colony formation and plate counts were used to quantify the number of biofilm cells. *Bacillus subtilis* biofilms were cultivated in glass test tubes due to a lack of robust biofilm formation in polystyrene microtiter plates.

2.2.8 Pure compounds on Staphylococcus aureus and Pseudomonas aeruginosa biofilm formation assay

Staphylococcus aureus and *Pseudomonas aeruginosa* biofilms were grown in LB broth and PBM-glucose, respectively. Biofilm formation was assessed using a polystyrene 96 well microtiter plate crystal violet assay (97). Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh media and combined with pure compounds to obtain a final concentration of 6.2%

(v/v). Treatments were then serially diluted to final concentrations of 3.1, 1.7, 0.8 and 0.4% (v/v). DMSO was added to solubilize the treatments. Negative controls consisted of cells diluted to an initial OD₆₀₀ of 0.01 in growth media without treatment. 100 µl of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C for 24 h. After 24 h, planktonic cells and spent growth media were removed from each well and the plate was washed 3 times in sterile water. Biofilms were then stained with 150 µl of 0.1% crystal violet for 15 min at 200 rpm. Excess dye was removed via washing in sterile water and the plate was allowed to air dry. Crystal violet was solubilized in 150 µl of 95% ethanol and absorbance measurements at 595 nm were taken using an MD SPECTRAMax plate reader (Molecular Devices Corporation, USA).

2.2.9 Liquid to liquid extraction

The fractionation strategy employed in this work followed the approach described by Quave et al., 2012 (100). Liquid to liquid extraction was conducted to separate the various components of the gesho leaf ethanol extract. A 1:1 ratio of water and hexanes was added to a separatory funnel. 500 mg of gesho leaf ethanol extract were solubilized in 10 ml of 95% ethanol was added to the separatory funnel. Solvents were thoroughly mixed via inversion and then allowed to separate before fraction collection in 250 ml Erlenmeyer flasks. Aqueous fractions were reintroduced to the separatory funnel and an additional 10 ml hexanes were added. Solvents were mixed via inversion and allowed to separate for fraction collection. A final hexane separation was conducted using an additional 10 ml of hexane. Subsequent extractions were performed with water-saturated butanol and ethyl acetate following the aforementioned method. Subsequently all fractions were dried under air to remove solvents, re-suspended in water and lyophilized.

2.2.10 Low Pressure Liquid Chromatography (LPLC)

LPLC was performed using Bondapack C18/ Corasil as the stationary phase. 10 mg and 7 mg of the butanol and ethyl acetate residual compounds, respectively, were dissolved in 1 ml of ethanol and applied to the column. Gradient separations were performed by changing mobile phase at 10% intervals from 100% methanol to 100% water. Eluted fractions were collected in polystyrene culture tubes at 5 ml intervals and stored at 4 °C.

2.2.11 Chemical tests

Chemical tests were used to help identify the chemical components present in *R. prinoides* ethanol extracts. Ninhydrin (101), Folin-Ciocalteu (102), Baeyer (103), Jones Oxidation (104), carboxylic acid (105) and Biuret tests (106) were conducted according to standard protocols with the following modifications: Ninhydrin test: instead of spraying chromatography paper with the ninhydrin reagent, 10 µl drops of ninhydrin reagent were applied directly to spots of dried sample. Folin-Ciocalteu test: volumes were modified to allow this assay to be performed in a microtiter plate rather than a cuvette; 18 µl of sample, 36 µl of Folin-Ciocalteu reagent and 145 µl of 20% sodium carbonate were used. Baeyer test: 1 drop of each sample was dissolved in 500 µl of acetone. Jones Oxidation: 5 drops of acetone and chromate solution were used to dissolve 4 drops of each sample. Carboxylic acid test: 1 ml of 100 mg/ml sodium bicarbonate solution was added to 3 drops of each sample. Biuret test: a 25 mg/ml cupric sulfate solution was used for analysis.

2.2.12 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent Technology 7890A gas chromatograph equipped with an Agilent Technology 5977A mass spectrometer. A sample volume of 5 µL was manually injected into an Agilent (30 m, 0.530 mm) HP-5MS column. The oven temperature was held at 60 °C for 5 min, then was raised to 100 °C at

25 °C/min. The temperature was stabilized at 100 °C for 1 min then raised to 200 °C at 25 °C/min and held at 200 °C for 1 min. Temperature was raised to 250 °C at a rate of 5 °C/min then held at the final baking temperature of 250 °C for 7 min. Agilent MassHunter software was used for mass spectrometry data acquisition and analysis.

2.2.13 Fourier Transform Infrared Spectroscopy (FTIR)

A Varian UMA 600 FTIR microscope equipped with He-Ne laser and MCT detector was used for analysis. 3 µL of select LPLC fractions were vacuum desiccated onto zinc solenoid windows. Data were collected in the 3750–925 cm⁻¹ region at a 4 cm⁻¹ spectral resolution.

2.2.14 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 software. Non-parametric (Kuskal-Wallis Test and Median Test) or parametric (ANOVA followed by Tukey HSD test or t-test) analyses of variance were conducted based on the characteristics of the data. Comparisons were conducted between the extract treated samples and the untreated control. Differences with a p-value < 0.05 were considered statistically significant and are designated with an asterisk (*).

2.3 Results and Discussion

2.3.1 Gesho ethanol extracts have biocidal activity against log phase planktonic cells

Ethanol and aqueous extracts of gesho were tested for their ability to kill log-phase, planktonic cells of *Staphylococcus aureus*, *Streptococcus mutans* and *Bacillus subtilis*. Ethanol extract treatments resulted in a 2 to 10 log reduction in colony forming units (CFU) per ml, whereas aqueous extract treatments resulted in a 1 to 3 log reduction in CFU per ml (Figure 1). Both stem and leaf ethanol extracts exhibited significant biocidal activity against all three species tested. The aqueous extracts exhibited greater variability; in particular, aqueous stem extract was active against *S. aureus* and *B. subtilis* but not *S. mutans*. The strong biocidal activity of the ethanol

extracts and lower activity of the aqueous extracts were consistent with the findings of Molla et al., 2016 who reported MICs for gesho extracts from 2 to 8 mg/mL; but greater than Amabye, 2015 at 0.2 to 0.4 mg/mL and less than Berhanu, 2014 who reported active concentrations ranging from 97.5 to 780 mg/mL (75, 84, 85).

2.3.2 Gesho ethanol extracts influence the growth of stationary phase cells

Gesho ethanol extracts exhibited biocidal or bacteriostatic activity against stationary phase cells. The number of viable cells present in the inocula, indicated by a dotted red line, were quantified to allow for comparisons of growth relative to starting conditions (Figure 2). Treatments of *B. subtilis* or *S. mutans* with stem ethanol extract resulted in a decrease in biomass when compared to the untreated inoculum, signifying biocidal activity. Stem ethanol treatment of *S. aureus* and leaf ethanol treatments of *S. mutans* and *B. subtilis* exhibited a bacteriostatic effect, showing little change in cell number compared to the inocula. Leaf ethanol extract impaired *S. aureus* growth in comparison to growth of the untreated control, possibly due to slight bactericidal activity or toxicity of the treatment. The variability of these findings suggests that the gesho ethanol extracts contain a mixture of chemicals that impact Gram positive bacteria in a species specific manner. Overall, these findings indicated that the stem ethanol extract was a good source of biocidal, anti-biofilm compounds, whereas the leaf ethanol extract was a good source of non-biocidal antibacterial (i.e. anti-pathogenic) compounds. Due to the superior inhibition of biofilm formation caused by the ethanol extracts, we elected not to assess the activity of the aqueous extracts on stationary phase cells (Figure 3).

2.3.3 Gesho ethanol extracts prevent Gram positive bacterial biofilm formation

Gesho ethanol extracts strongly inhibited Gram positive bacterial biofilm formation. Both ethanol stem and leaf extracts significantly inhibited *S. aureus*, *S. mutans* and *B. subtilis* biofilm

formation up to 99% relative to untreated control biofilms (Figure 3). The extent of inhibition was species dependent with *S. aureus* showing less susceptibility to treatment than *B. subtilis* and *S. mutans*. Aqueous extracts inhibited *S. aureus* and *S. mutans* biofilms but increased *B. subtilis* biofilm formation. *B. subtilis* biofilm formation may have been stimulated by the presence of polysaccharides (107) or peptides with pheromone-like activity (108) in the extract; these hypotheses have not yet been tested. As noted above, the effects of aqueous extracts were more variable in activity, resulting in inhibition of *S. aureus* and *S. mutans* but not *B. subtilis* biofilms. Stem extracts generally resulted in greater biofilm inhibition than their leaf counterparts, suggesting a difference in chemical composition between the two tissues. All extracts were ineffective at disrupting existing biofilms or inhibiting Gram negative bacterial biofilm formation (Figure 4).

2.3.4 Gesho-derived small molecules inhibit Gram positive biofilm formation

Further work on the leaf ethanol extract was pursued because the data in Figures 2 and 3 indicated that compounds contained within prevented biofilm formation with minimal bactericidal activity. Liquid to liquid fractionation was used to separate the extract on the basis of polarity, followed by chemical tests, Fourier Transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC-MS) (Figure 5).

Low pressure liquid chromatography of the butanol and ethyl acetate liquid to liquid fractions indicated that the most effective compounds were present in the more polar liquid phases (Figure 6). Colorimetric chemical tests indicated the presence of polyphenolic and alcoholic compounds (Table 1). FTIR supported these findings, showing a strong hydroxyl peak and several alkyl or alkene peaks (Figure 7).

GC-MS analysis found numerous peaks within the butanol fraction (Figure 8); compound identities were determined using the NIST11 chemical library. Compounds resulting in a certainty score greater than 85% were selected for further analysis. Activity screens identified two compounds with anti-biofilm activity: ethyl 4-ethoxybenzoate (CAS# 23676-09-7) and 4-hydroxy-4-methyl pentanone (CAS# 123-42-2) (Figure 8). Ethyl 4-ethoxybenzoate (EEB) and 4-hydroxy-4-methyl pentanone (HMP) are naturally occurring compounds that have been previously been extracted from plants (109–112). 4-hydroxy-4-methyl pentanone, also known as diacetone alcohol (DAA), is commonly used as an industrial solvent; however, HMP was not used as a solvent in this study, suggesting that it occurs naturally in gesho. Benzoic acid and 2-pentanone, compounds that are structurally similar to EEB and HMP respectively, were evaluated for anti-biofilm activity at 0.8% and 3%. Neither benzoic acid nor 2-pentanone had a statistically significant effect on biofilm formation. These data support the concept that the observed anti-biofilm activity of EEB and HMP was caused by their chemical structures rather than by non-specific effects.

A major peak in the butanol fraction was identified as dimethyl sulfoxide (DMSO). DMSO is commonly used in laboratories as a solvent, but, in this study, it was a component of the GC-MS output (Figure 8). We considered several hypotheses to explain its presence. First, we analyzed all of the solvents used in this work to determine whether it was a contaminant. GC-MS analyses indicated that it was not found in the solvents (data not shown). Second, we considered whether it could be a natural product; however, we could not find any published reports that support this idea. Lastly, we hypothesized that DMSO could be a rearrangement product that formed as an artifact during GC-MS analysis (113). Regardless, we hypothesized that the presence of DMSO increased the anti-biofilm activity of EEB by increasing its solubility; this idea was supported by laboratory

experiments (Figure 9A). DMSO was combined with HMP to assess if DMSO would result in enhanced anti-biofilm activity as occurred with EEB, but DMSO did not enhance HMP activity.

EEB and HMP treatments exhibited significant anti-biofilm activity but did not show antibacterial activity against log or stationary phase cells; these are hallmark characteristics of “anti-pathogenic” compounds (Figure 9 C-F). Anti-pathogenic therapeutics are compounds that target microbial pathogenicity while minimizing bactericidal activity (65). Anti-pathogenic and antivirulence compounds have gained attention in recent years because they apply less selective pressure against pathogens than bactericidal agents; these compounds may also delay the development of antibiotic resistance or restore sensitivity to antibiotic resistant, pathogenic strains (64, 65, 114). The anti-biofilm activity of HMP may derive from its structural similarity to autoinducer-2 (AI-2), a quorum sensing signaling molecule that affects diverse bacterial phenotypes including biofilm formation (115, 116). HMP may act as a competitive inhibitor of AI-2 signaling. Notably, HMP inhibited biofilm formation by *P. aeruginosa* PA01, although this activity was not observed with the crude gesho extracts (Figure 10). The mode of action behind the anti-biofilm activity of EEB is unknown, but its structural similarity to parabens suggest that it may influence biofilm formation through a common mechanism (117, 118).

There are several novel features in the presented work. This is the first research project to focus on gesho for its anti-biofilm activity. This work complements existing findings on the antibacterial (75, 85) and anti-parasitic (82, 83, 119) properties of gesho. Second, the extracts used in this research were unique compared to those used in prior works; they were prepared using ethanol rather than methanol in an effort to extract chemical compounds that were likely to be present in traditional brews and tinctures (76, 78). Third, this project tested both leaf and stem extracts independently for antibacterial and anti-biofilm activity, which proved informative as their

characteristics and efficacies were different. Overall, our findings support the traditional use of gesho for health benefits and also indicate that gesho-derived compounds could potentially have applications as therapeutics and as hygiene products.

2.4 Conclusion

Biofilm formation is a complex process, involving cells that switch from a planktonic to a sessile phenotype. The change to a surface-attached mode of growth may be induced by environmental cues or by signals produced by neighboring cells. Accordingly, there is a large network of genes involved with attachment. Moreover, the components involved in biofilm formation can vary greatly as microbes from diverse genera seek the benefits of a biofilm lifestyle. From a therapeutic perspective, this means that there are many targets by which to influence microbial biofilm formation.

Numerous anti-biofilm, anti-pathogenic and antivirulence compounds have been identified in plant essential oils (59, 61, 63, 120). Gesho, like other plant essential oils, is a mixture of diverse chemicals (74, 85). It is likely that some of the observed variation in gesho extract-induced activity is the result of different molecules contained therein affecting biofilm formation through diverse mechanisms. In this work we report two specific compounds with anti-biofilm activity derived from gesho. We predict that investigating additional constituents of gesho extracts will yield more novel molecules with anti-biofilm activity.

2.5 Figures and Tables

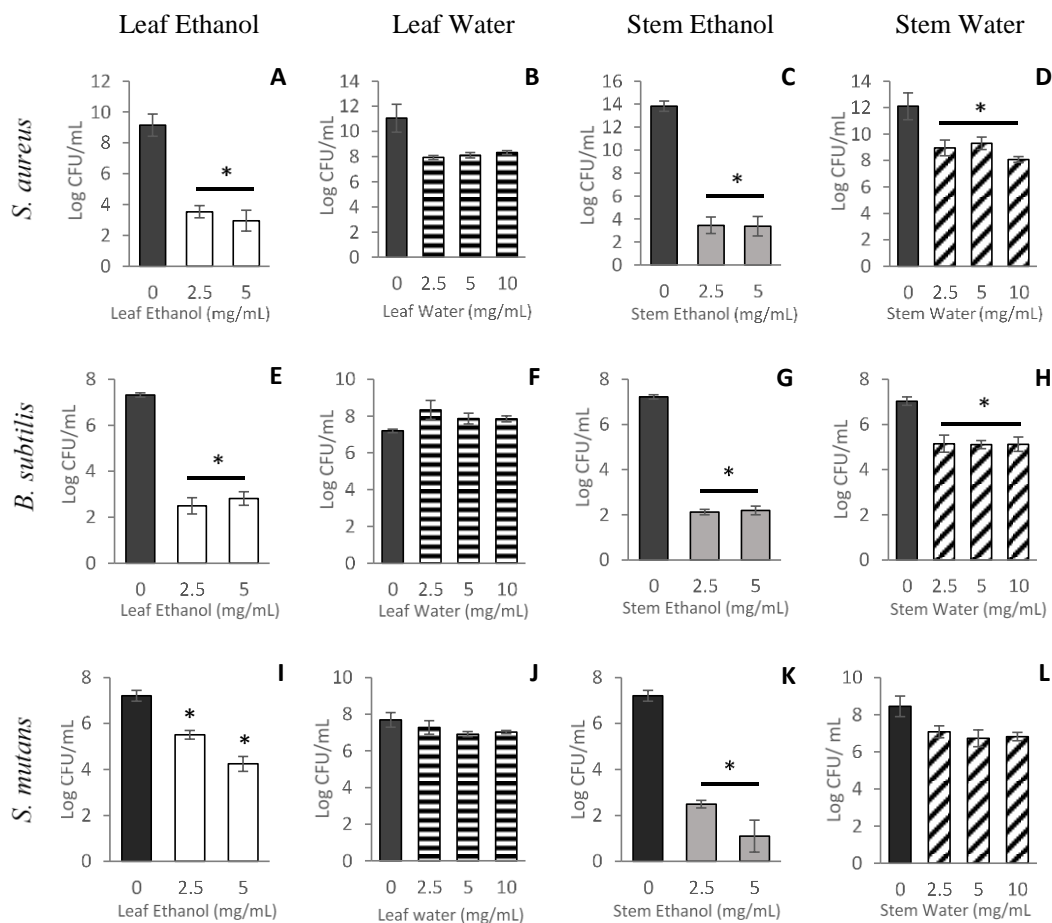


Figure 1. Bactericidal effect of aqueous and ethanol extracts on log-phase, planktonic cells. (A-D) Staphylococcus aureus (n = 3-6); (E-H) *Bacillus subtilis* (n = 3-4) and (I-L) *Streptococcus mutans* (n = 3-4). Bars indicate number of bacteria present in culture following exposure to extracts for 1 h. Legend: leaf ethanol (white bars), leaf aqueous (horizontal striped bars), stem ethanol (grey bars), stem aqueous (slanted striped bars). Error bars are standard error of the mean. ANOVA and Kruskal-Wallis tests were performed; (*) indicates a significant difference ($p < 0.05$) between treated samples and the untreated control.

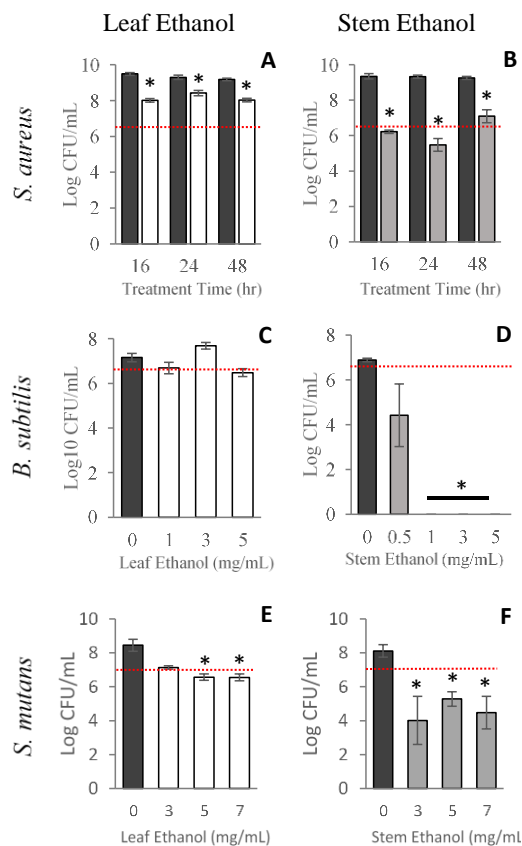


Figure 2. Effect of ethanol extracts on resting phase planktonic cell viability.

(A-B) *Staphylococcus aureus* (n = 3), (C-D) *Bacillus subtilis* (n = 3-4), (E-F) *Streptococcus mutans* (n = 3). Bars indicate the number of viable cells after growth for 24 h in the presence of stem (light grey bars) and leaf (white bars) ethanol extracts. Black bars represent untreated controls. The inoculum cell density is indicated by a red dotted line. Error bars are standard error of the mean. ANOVA, T-test, Kruskal-Wallis tests were performed; (*) indicates a significant difference ($p < 0.05$) between treated samples and the untreated control.

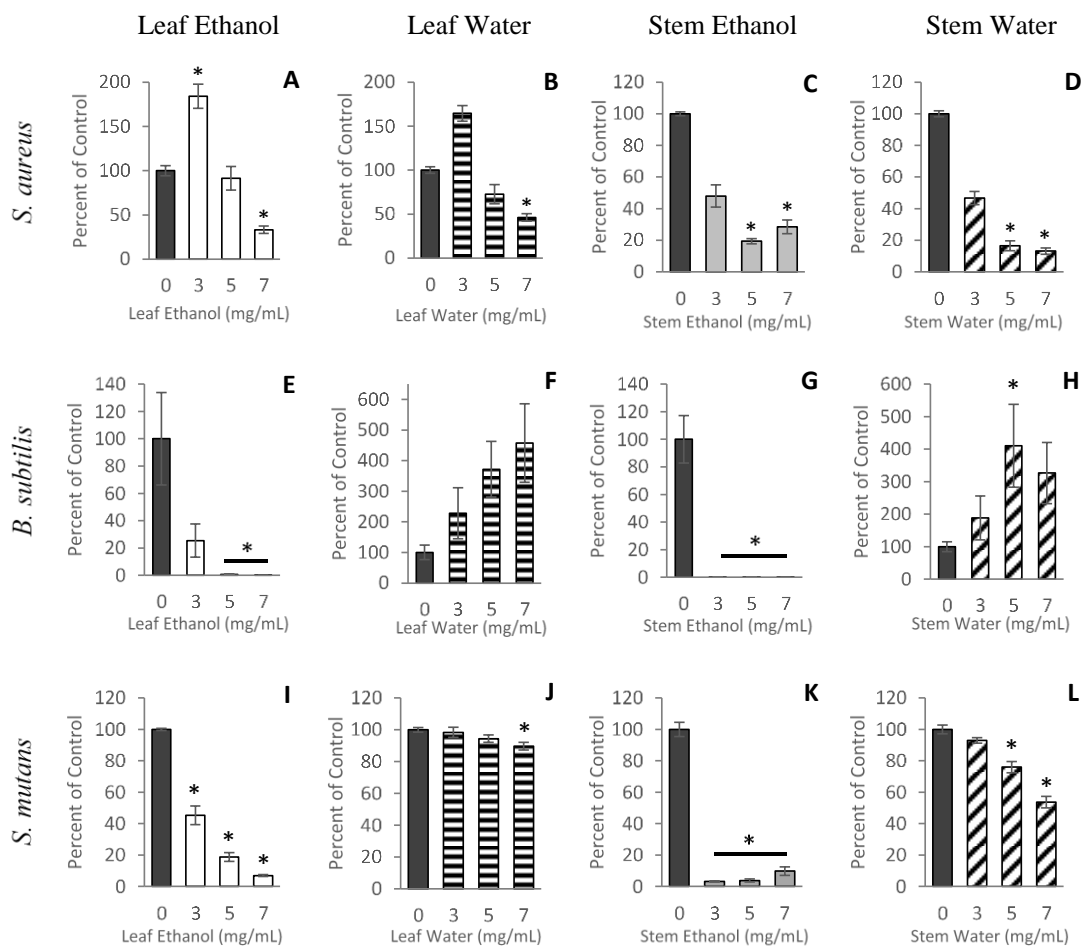


Figure 3. Effect of aqueous and ethanol extracts on Gram positive bacterial biofilm formation. (A-D) *Staphylococcus aureus* (n=10-15), (E-H) *Bacillus subtilis* (n=3-4) and (I-L) *Streptococcus mutans* (n=12). Bars indicate the extent of biofilm formation relative to untreated controls. Legend: leaf ethanol (white bars), leaf aqueous (horizontal striped bars), stem ethanol (grey bars), stem aqueous (slanted striped bars). Error bars are standard error of the mean. ANOVA and Kruskal-Wallis tests were performed; (*) indicates a significant difference ($p < 0.05$) between treated samples and the untreated control.

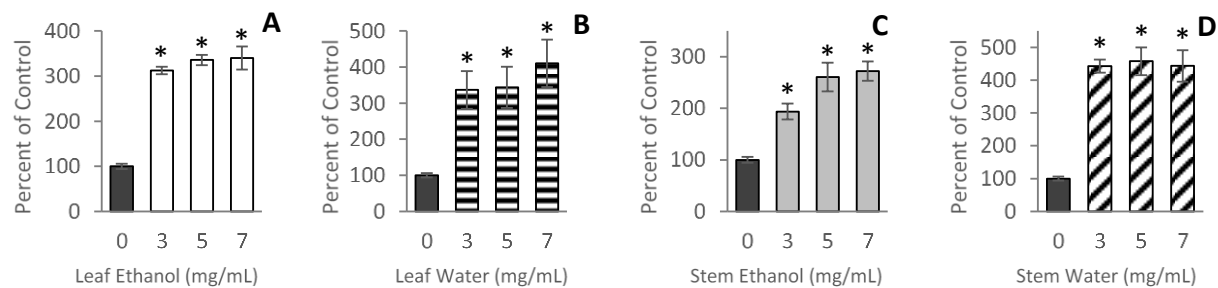


Figure 4. Effect of aqueous and ethanol extracts on Pseudomonas aeruginosa biofilm formation. Bars indicate the extent of biofilm formation relative to untreated controls. Legend: leaf ethanol (white bars), leaf aqueous (horizontal striped bars), stem ethanol (grey bars), stem aqueous (slanted striped bars). Error bars are standard error of the mean. Kruskal-Wallis tests were performed; (*) indicates a significant difference ($p < 0.05$) between treated samples and the untreated control.

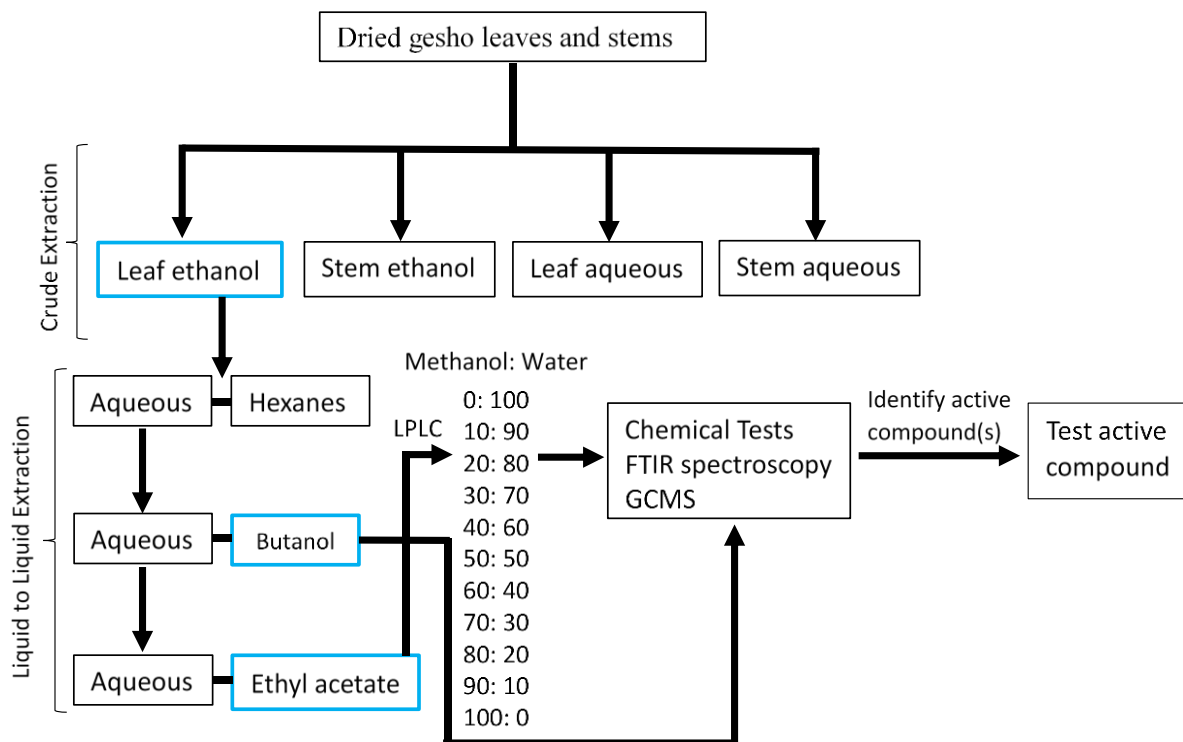


Figure 5. Diagram of gesho leaf ethanol extract fractionation and chemical analysis.

Crude ethanol extracts were prepared from ground leaves of *R. prinoides*, followed by liquid to liquid fractionation using hexanes, butanol and ethyl acetate. Butanol and ethyl acetate fractions were further separated via column chromatography and then analyzed via colorimetric chemical tests, Fourier transform infrared spectroscopy and gas chromatography-mass spectrometry (GCMS). Chemical compounds identified via GCMS were screened for antimicrobial and anti-biofilm activity.

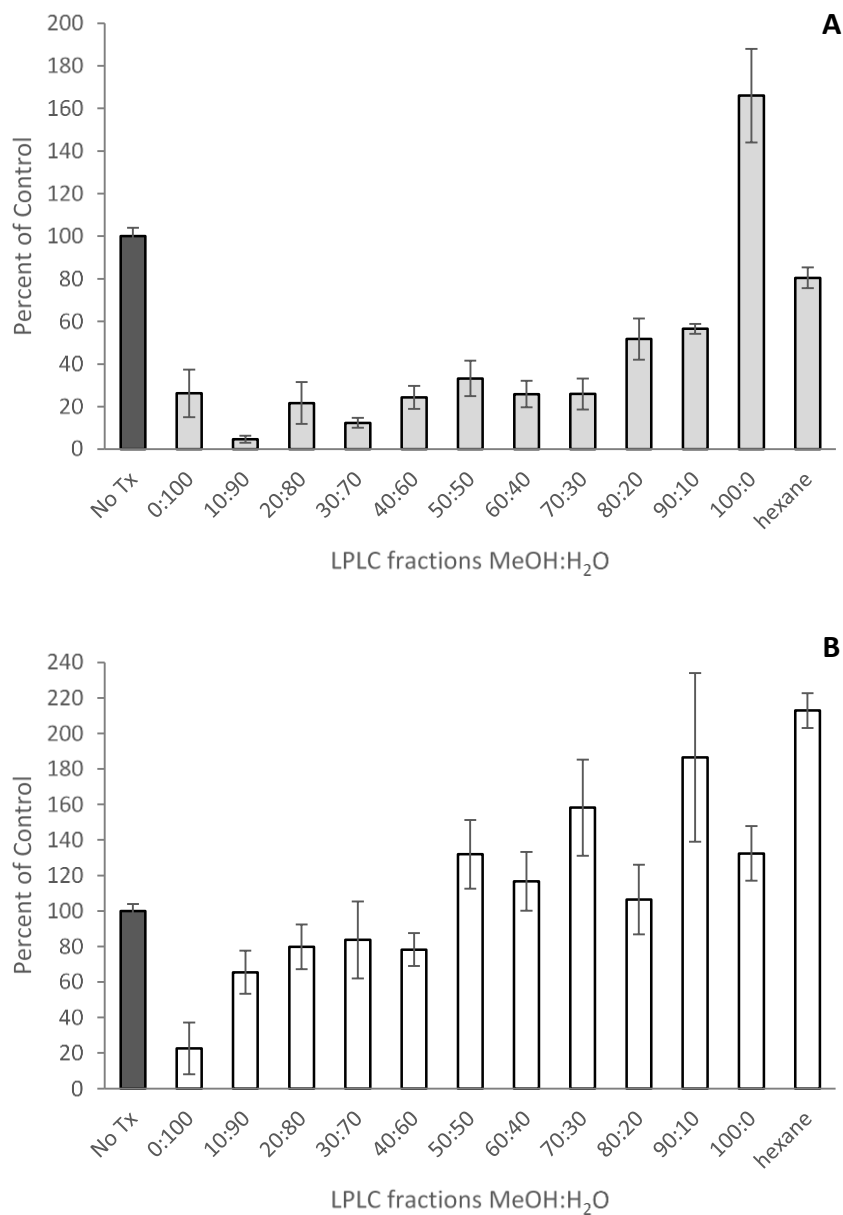


Figure 6. Effect of low pressure liquid chromatography fractions on Staphylococcus aureus biofilm formation.

Bars indicate the extent of biofilm formation relative to untreated controls. Legend: further separation of the butanol liquid to liquid fraction (grey bars), further separation of the ethyl acetate liquid to liquid fraction (white bars). Error bars are standard error of the mean.

Table 1. Chemical tests indicate the presence of alcohol- and phenol-containing compounds in the butanol and ethyl acetate fractions

Chemical Test	Functional group	Test result
Ninhydrin test	Amines	Negative
Folin-Ciocalteu test	Polyphenols	Positive
Beyer/ Potassium permanganate test	Alkenes and Alkynes	Negative
Jones Oxidation/ Sodium Chromate test	Alcohols	Positive
Carboxylic acid test	Carboxylic acid	Negative
Biuret test	Peptide bonds	Negative

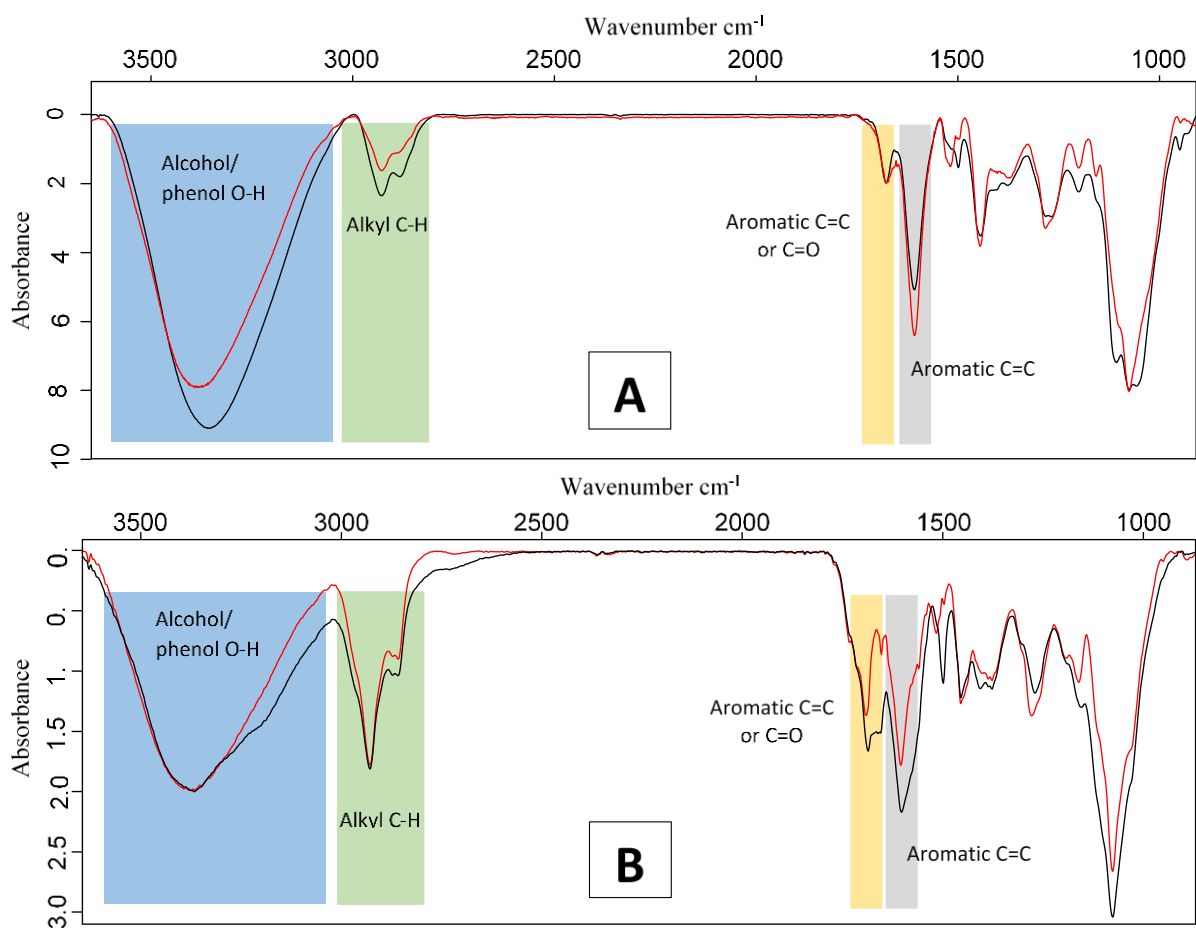


Figure 7. Fourier transform infrared spectroscopy of butanol and ethyl acetate fractions. Butanol (A) and ethyl acetate (B) fractions prepared on May 26th, 2017 (black) and June 15th, 2017 (red) exhibit strong hydroxyl (blue box), alkane (green box) and alkene (yellow and grey box) peaks. Peak strength was 4-5 times stronger in the butanol fraction chromatogram than the ethyl acetate.

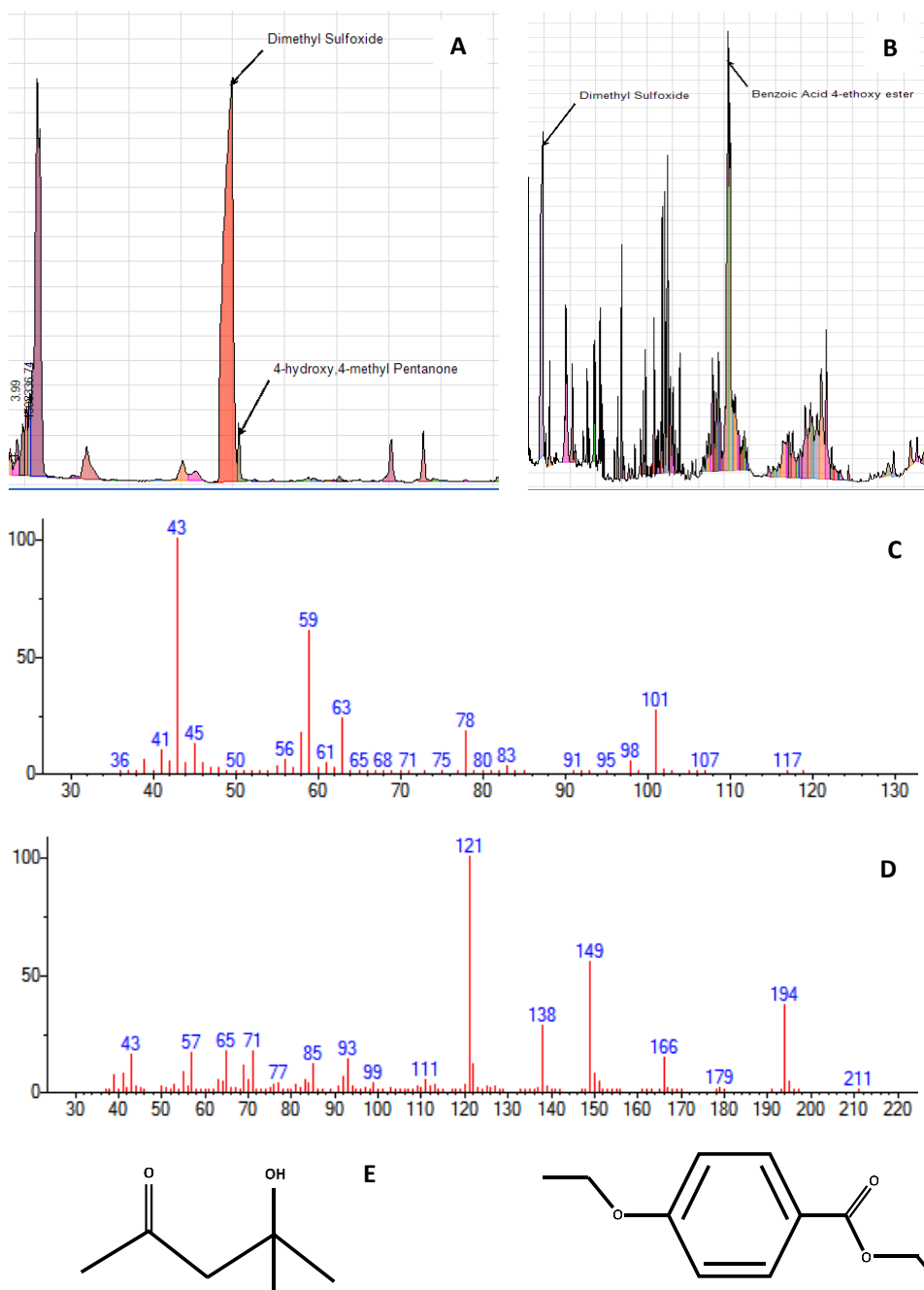


Figure 8. Gas chromatography mass spectrometry analysis of butanol fraction. 4-hydroxy-4-methyl-2-pentanone (A, C and E) and Ethyl 4-ethoxybenzoate (B, D and F) were identified as compounds present in the butanol fraction. There are numerous additional peaks in the mixture whose identities were not resolved or evaluated for antimicrobial or anti-biofilm activity.

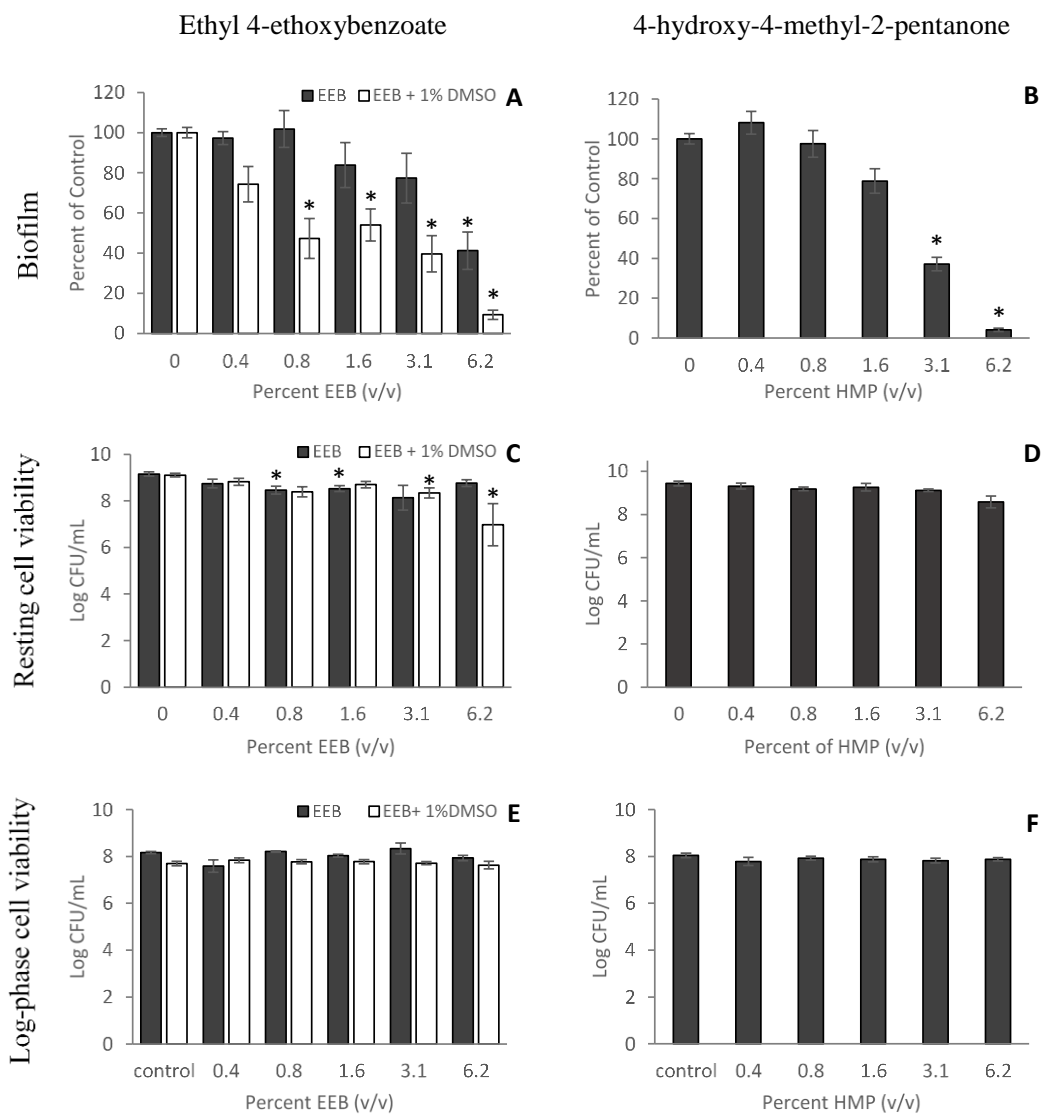


Figure 9. Effects of ethyl 4-ethoxybenzoate and 4-hydroxy-4-methyl-2-pentanone on S. aureus biofilm formation and planktonic cell viability.

Top row, effect on biofilm formation. Middle row, effect on resting phase cell viability. Bottom row, effect on log phase cell viability. Bars indicate the impact of treatments after 24 h. Legend: ethyl 4-ethoxybenzoate (EEB) and 4-hydroxy-4-methyl-2-pentanone (HMP) with (white bars) or without (black bars) the presence of 1% DMSO. Each assay was performed in triplicate and error bars represent the standard error of the mean. An ANOVA test was performed; (*) indicates a significant difference of ($p < 0.05$) between the treated samples and the untreated control.

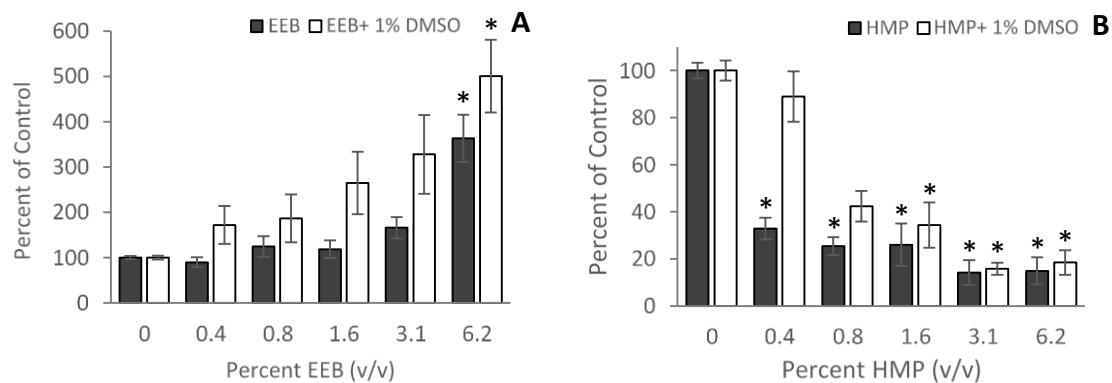


Figure 10. Effect of ethyl 4-ethoxybenzoate and 4-hydroxy-4-methyl-2-pentanone on Pseudomonas aeruginosa biofilm formation.

Bars indicate the extent of biofilm formation relative to untreated controls. Error bars are standard error of the mean. Kruskal-Wallis tests were performed; (*) indicates a significant difference ($p < 0.05$) between treated samples and the untreated control.

3 *RHAMNUS PRINOIDES* (GESHO) STEM EXTRACT PREVENTS CO-CULTURE BIOFILM FORMATION BY *STREPTOCOCCUS MUTANS* AND *CANDIDA* *ALBICANS*

3.1 Introduction

Streptococcus mutans and *Candida albicans* form robust polymicrobial biofilms in the oral cavity that are associated with a variety of oral and systemic illnesses. Growing alone, *Streptococcus mutans* biofilms are the major etiologic agent of dental caries and tooth decay while *Candida albicans* biofilms can result in oral candidiasis (121, 122). Working together, *S. mutans* and *C. albicans* dual-species biofilm infections are causal agents of disease including early-childhood caries, denture stomatitis, periodontitis, candidiasis, endocarditis and mucosal infections (123). In dual-species biofilms, these organisms exhibit a symbiotic relationship, exchanging metabolites and growth factors to ensure their mutual survival (124); this relationship can lead to enhanced biomass, exopolysaccharide production, resistance to stress and virulence gene expression (125, 126). This is a major concern as biofilms frequently have enhanced resistance to antibiotics, leading to treatment failure (7). In response, researchers have pursued plant extracts as a source of anti-biofilm therapeutics. There is a substantial body of research investigating the ability of plant extracts to control *S. mutans* (127, 128) or *C. albicans* (129, 130) mono-species biofilm; however, considerably fewer studies have focused on these organisms in polymicrobial biofilms (127). This is of concern because polymicrobial biofilms are generally more difficult to eradicate than their mono-species counterparts (131–133).

Rhamnus prinoides (gesho) has been used in traditional medicine throughout eastern Africa. The fruit, root, stems and leaves of gesho have been historically used to treat a variety of illnesses including ear, nose, and throat infections, tonsillitis, scabies, dandruff, rheumatism and pneumonia

(80, 81). Research on the antimicrobial activities of gesho have shown that extracts possess inhibitory activity against a variety of bacteria and plasmodia (75, 119), indicating that gesho is effective against both prokaryotes and eukaryotes. Based on this background information, we hypothesized that gesho extracts could be used to control *Streptococcus mutans* and *Candida albicans* biofilms, including co-culture biofilms.

3.2 Materials and Methods

3.2.1 Microbial strains and culture conditions

Streptococcus mutans and *Candida albicans* were used in this work. *Streptococcus mutans* was a gift from Margaret Gould-Bartlett, Georgia State University and *Candida albicans* was a gift from the laboratory of Sidney Crow, Georgia State University. The *S. mutans* 16S rDNA and 16S-23S intergenic spacer sequences were deposited to GenBank (NCBI, USA) under accession number MT318140; the GenBank accession number for the *C. albicans* 18S rDNA-ITS sequence was MT166273. The *S. mutans* and *C. albicans* strains used in this work shared over 99% similarity with the *S. mutans* T8 and *C. albicans* ATCC 18804 strains, respectively (Appendix A). *S. mutans* overnight cultures were grown in brain-heart infusion (BHI) (Becton, Dickinson, USA) broth while biofilms were formed in BHI broth supplemented with 0.5% sucrose. *C. albicans* overnight cultures were grown in yeast peptone dextrose (YPD) broth while biofilms were formed in 1X RPMI 1640 with L-glutamine (Corning, USA) supplemented with 165 mmol l⁻¹ morpholinepropanesulfonic acid (MOPS).

3.2.2 Genetic analysis

Genomic DNA was extracted from overnight cultures of *S. mutans* and *C. albicans* using a Quick DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA). Sequencing of the *S. mutans* 16S rDNA and 16S-23S intergenic spacer and the *C. albicans* 18S and ITS regions were conducted

using the primers listed in Appendix A. Polymerase chain reactions were conducted using the *Taq* DNA polymerase kit from New England Biolabs Inc. (USA). Thermal cycling conditions for *S. mutans* included: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 68° for 2 min; and a final extension at 72°C for 5 minutes. *C. albicans* thermocycler parameters included: initial denaturation at 95°C for 3 min; 30 cycles of 95°C for 30 sec, 55°C for 30 sec and 68° for 2 min 30 sec; and a final extension at 72°C for 5 minutes. Sanger sequencing was conducted at the Georgia State University core facility (Atlanta, GA USA) and Genewiz (South Plainfield, NJ USA). The resulting genetic sequences were assembled using DNA Baser Assembler v.5 (Heracle Biosoft, Arges, Romania). The contigs are found in GenBank under accession numbers MT318140 (*S. mutans*) and MT166273 (*C. albicans*).

3.2.3 Chemicals and reagents

The following reagents were purchased from Fisher Scientific (USA): crystal violet, potassium phosphate. Reagents purchased from Sigma Aldrich (USA) include: resazurin and sucrose. 95% ethanol was purchased from Decon Labs (USA). 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) was purchased from Invitrogen (USA) and RMP1 1640 with L-glutamine was purchased from Corning (USA). Direct Yellow 96 was purchased from AK Scientific.

3.2.4 Extract preparation

Rhamnus prinoides stems and leaves were purchased from a local Ethiopian market (Buford, Georgia, USA). The authenticity of the stems and leaves was verified by two local experts on Ethiopian foods (134). 24 g of ground leaves or fractured stems were added to 150 ml of sterile water or 95% ethanol in a 250 ml flask (95, 96). Flasks were shaken in the absence of light at 200 rpm at room temperature for four days. The extraction liquid was then collected and particulate

matter removed via centrifugation at 13,000 x g at 4 °C for 5 min; clarified supernatants were recovered for further processing. The extracts were air-dried under vacuum then collected in 50 ml of water and frozen prior to lyophilization. Extracts were stored at -80 °C. The percent yield (w/w) of the leaf and stem ethanol extracts were: 8 and 2%, respectively.

3.2.5 *Streptococcus mutans* biofilm formation assay

In vitro *Streptococcus mutans* biofilms were grown in filter sterilized BHI broth supplemented with 0.5% sucrose (BHI-sucrose). Biofilm formation was assessed using a polystyrene 96 well microtiter plate crystal violet assay (97, 98). Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 (~ 3.8 x10⁶ viable cells per milliliter of culture) in BHI-sucrose and combined with gesho extract to a final concentration of 7 mg ml⁻¹. Treatments were then serially diluted to final concentrations of 5, 3, 1, 0.5 and 0.25 mg ml⁻¹. No vehicles were added to solubilize the treatments. Negative controls consisted of diluted cells in BHI-sucrose without plant extract. 100 µl of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h (135). The next day, planktonic cells and spent media were removed from each well and the 96 well plates were washed 3 times with sterile water. Biofilms were then stained with 150 µl of 0.1% crystal violet for 15 minutes at 200 rpm. Excess dye was removed and the biofilms washed 3 times with sterile water. The plates were allowed to dry then the crystal violet was solubilized in 150 µl of 95% ethanol. Crystal violet absorbance measurements at 595 nm were taken using an MD SPECTRAMax plate reader (Molecular Devices Corporation, USA). This experiment was performed in triplicate and the sample sizes were between 12 and 48.

3.2.6 *Candida albicans* biofilm formation assay

Candida albicans biofilms were cultivated according to Pierce *et al.*, 2015 with modifications (136). Thawed *C. albicans* cultures were streaked on YPD agar and incubated overnight at 37 °C. The following day, 25 ml YPD broth was inoculated with a loopful of colonies and incubated at 30 °C for 14-16 h. Samples of overnight culture were stained with 0.1% v v⁻¹ methylene blue and cell densities were quantified using a hemocytometer. After quantification, *C. albicans* cells were centrifuged and washed twice with PBS and adjusted to a final concentration of 1x10⁷ cells in filter sterilized RMPI-MOPS. Diluted cultures were combined with gesho extract to a final concentration of 7 mg ml⁻¹. Treatments were then serially diluted to final concentrations of 5, 3, 1, 0.5 and 0.25 mg ml⁻¹. No vehicles were added to solubilize the treatments. Negative controls consisted of cells diluted in RMPI-MOPS without plant extract. 100 µl of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h. The next day, planktonic cells and spent media were removed from each well and the 96 well plates were washed 3 times with sterile water. Biofilms were then stained with 150 µl of 0.1% crystal violet for 15 min at 200 rpm (137, 138). Excess dye was removed, and the biofilms washed 3 times with sterile water. The plates were allowed to dry then the crystal violet was solubilized in 150 µl of 95% ethanol. Crystal violet absorbance measurements at 595 nm were taken using an MD SPECTRAmax plate reader (Molecular Devices Corporation, USA) (97). This experiment was performed in triplicate and the sample sizes ranged between 16 and 39.

3.2.7 *Streptococcus mutans* and *Candida albicans* dual-species biofilm formation assay

In vitro growth of polymicrobial biofilms was conducted according to de Oliveira *et al.*, 2017 with modifications (139). *S. mutans* and *C. albicans* inocula were prepared as stated above.

BHI-sucrose *S. mutans* and RPMI-MOPS *C. albicans* inocula were combined at equal volumes and mixed thoroughly. Co-cultures were combined with gesho extract to a final concentration of 3 mg ml⁻¹ and 100 µl of treated and untreated samples were added to each well of a microtiter plate. Plates were then incubated at 37 °C, 200 rpm for 24 h. No vehicles were added to solubilize the treatments. Negative controls consisted of diluted cells without plant extract. The following day, plates were washed, stained and measured as stated above. Experiments were performed in triplicate.

3.2.8 Planktonic growth assay

Streptococcus mutans and *Candida albicans* overnight cultures were prepared as stated above. Secondary overnight cultures of *Streptococcus mutans*, *Candida albicans* and combined *Streptococcus mutans* and *Candida albicans* were prepared in 20 ml of BHI, YPD and 1:1 BHI-YPD, respectively. Secondary overnight broth cultures were then diluted to an initial OD₆₀₀ of 0.01 (approximately 2.4 x10⁶ viable *S. mutans* cells and 1.5 x10⁴ viable *C. albicans* cells per milliliter of culture) in 15 ml of fresh 1:1 BHI-YPD broth. GSE was added to a final concentration of 3 mg ml⁻¹. Negative controls consisted of cells diluted in broth media without plant extract. Cultures were statically incubated at 37 °C for 9 h and samples were taken at every hour for agar plating. Time point samples were serially diluted (1:10) in PBS and radially plated on BHI agar plates. Agar plates were incubated overnight at 37 °C to allow for colony formation and plate counts were used to quantify cell density and growth. Co-culture *S. mutans* and *C. albicans* colonies were differentiated microscopically based on colony morphology. YPD and BHI were used for growth experiments to avoid bacterial flock formation which occurs when sucrose is present in the media.

3.2.9 *Streptococcus mutans* regrowth assay

Streptococcus mutans overnight cultures were prepared as stated above and secondary overnight cultures were prepared in 20 ml of BHI. Secondary overnight broth cultures were then diluted to an initial OD₆₀₀ of 0.01 (approximately 2.4×10^6 viable *S. mutans* cells per milliliter of culture) in 15 ml of fresh 1:1 BHI-YPD broth. GSE was added to the treatment flasks to a final concentration of 3 mg ml⁻¹. Negative controls consisted of cells diluted in broth media without plant extract. Cultures were statically incubated at 37 °C for 24 h. After 24 hours, 500 µl of each culture were added to a new flask of 15 ml BHI-YPB broth. Regrowth cultures were statically incubated at 37°C for 48 hours and samples were taken at t = 0, 8, 24 and 48 hours for agar plating. Time point samples were serially diluted (1:10) in PBS and radially plated on BHI agar plates. Agar plates were incubated overnight at 37 °C to allow for colony formation and plate counts were used to quantify cell density and growth.

3.2.10 *Biofilm killing assay*

Streptococcus mutans, *Candida albicans* and dual-species biofilms were formed as described above. Biofilms were allowed to form at 37 °C for 24 h. After incubation, spent media was removed and biofilms were washed twice with 150 µl of PBS. Gesho extracts were suspended in fresh, sterile media to a final concentration of 7 mg ml⁻¹. Treatments were then diluted to final concentrations of 5, 3, 1, 0.5, 0.25 and 0.12 mg ml⁻¹. No vehicles were added to solubilize the treatments. 100 µl of each treatment was added to each well of the microtiter plate and incubated at 37 °C for an additional 24 h. Negative controls consisted of 100 µl of fresh media without extract. After 24 h, planktonic cells and spent media were removed from each well and the biofilms were washed twice with 150 µl of PBS. 100 µl of resazurin (10 µg ml⁻¹) or XTT (0.5 mg ml⁻¹) in broth media were added to each biofilm. Plates were statically incubated at 37 °C for 1 h for *S.*

mutans or 3 h for *C. albicans* and co-cultures. Resazurin fluorescence intensity was measured at $\lambda_{\text{ex}}= 570 \text{ nm}$ $\lambda_{\text{em}}= 590 \text{ nm}$ using an Enspire fluorescence plate reader (PerkinElmer, USA) while XTT was measured at absorbance of 490 nm using a Victor plate reader (PerkinElmer, USA). Data were normalized to the untreated control. Experiments were performed in triplicate and the sample sizes ranged between 6-12 for treated samples and 14-24 for the untreated controls.

3.2.11 Streptococcus mutans polysaccharide production

Eight biofilms were formed for each treatment. *Streptococcus mutans* biofilms were formed as stated above. After 24 h of incubation, planktonic cells and spent media were removed from each well and the remaining biofilms were washed twice with 150 μl of PBS. 150 μl of 0.1% Direct Yellow 96 in PBS was then added to four biofilm wells and incubated in the dark, at room temperature for 30 min. Direct Yellow 96 binds polysaccharides such as beta glucans and was used for polysaccharide quantification. After incubation, unbound Direct Yellow 96 was aspirated and the biofilms were washed twice with 150 μl PBS. 100 μl of PBS were then added to each well and the fluorescence intensity was measured on a Victor plate reader at $\lambda_{\text{ex}}= 405 \text{ nm}$; $\lambda_{\text{em}}= 535 \text{ nm}$. Biofilm cells were scraped from the surface of the remaining four wells and collected into 400 μl of PBS. Biofilm cells were then serially diluted (1:10) in PBS and plated on BHI agar. Agar plates were incubated at 37 °C for 48 h and plate counts were used to measure cell density. Polysaccharide concentrations were calculated by adjusting the fluorescence intensity to the number of cells. Experiments were performed in triplicate.

3.2.12 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 software and VasserStats: website for statistical computation. Non-parametric (Kuskal-Wallis Test and Median Test) or parametric (ANOVA and t-tests) analyses were performed based on the characteristics of the data.

Comparisons were conducted between control (untreated) and gesho treated samples. Differences with a p-value < 0.05 were considered statistically significant.

3.3 Results and Discussion

3.3.1 *Gesho ethanol extracts inhibit Streptococcus mutans and Candida albicans mono-species biofilm formation*

Previously, our laboratory reported the inhibitory effects of gesho extracts on Gram positive mono-species biofilm formation (134). In that work, gesho ethanol extracts were found to significantly inhibit *Streptococcus mutans* biofilm formation at 3, 5 and 7 mg ml⁻¹ of treatment (134); however, the effects of extract treatment on polymicrobial biofilms or eukaryotic microorganisms were not assessed. The aim of this work was to expand on the anti-biofilm activities of gesho ethanol extracts and identify possible mechanisms of action. Gesho leaf and stem extracts significantly impacted *S. mutans* and *C. albicans* mono-species biofilm formation, resulting in up to 77 and 75 percent inhibition, respectively (Table 2). Treatment concentrations of 3, 5, and 7 mg ml⁻¹ of extract on *S. mutans* caused up to 97 percent inhibition and are reported in Campbell *et al.*, 2019. Both extracts were effective; however, the stem extract (GSE) exhibited overall greater percentages of inhibition when compared to the leaf extract (GLE) at the same treatment concentrations. The effects of treatment on each species were different; *S. mutans* treatments were concentration-dependent while *C. albicans* treatment efficacies leveled off at approximately 50 percent inhibition relative to the untreated control for all concentrations ≥ 0.5 mg ml⁻¹ GSE. The anti-biofilm activities of gesho were similar to those reported for other plant extracts (127, 129, 139, 140). Due to the superior inhibitory activity of GSE on mono-species biofilms, we evaluated the impact of GSE on *S. mutans* and *C. albicans* dual-species biofilm formation.

3.3.2 Gesho stem ethanol extract prevents Candida albicans and Streptococcus mutans dual-species biofilm formation

In co-culture, GSE not only impaired the ability of each species to form biofilms independently but also disrupted their synergistic relationship. *C. albicans* and *S. mutans* co-cultures were treated with 3 mg ml⁻¹ GSE due to its efficacy in treating *S. mutans* and *C. albicans* mono-species biofilms (Table 2). In the untreated controls, co-culture biofilm formation was enhanced more than 5-fold compared to mono-species biofilms, indicating a synergistic interaction (Figure 11). In contrast, GSE inhibited dual-species biofilm formation by 97 percent relative to the untreated control. These observations were similar to the inhibitory effects on *C. albicans* and *S. mutans* polymicrobial biofilms reported for *Rosmarinus officinalis* extract, *Camellia sinensis*-derived polyphenol 60, eugenol and thiazolidinedione-8 (139, 141).

3.3.3 Gesho stem ethanol extract inhibits Streptococcus mutans planktonic growth

We assessed whether GSE was inhibitory to planktonic growth and whether synergistic interactions were evident. In the absence of GSE, *S. mutans* and *C. albicans* in co-culture exhibited similar growth rates as when each species was grown alone, indicating that, in planktonic culture, no synergism occurred (Figure 12). This behavior is in contrast to what was observed in co-culture biofilms. 3 mg ml⁻¹ GSE did not impact the growth of *C. albicans* in mono- or co-culture. On the other hand, GSE prevented the growth of *S. mutans*, acting in a bacteriostatic manner (Figure 12). After 24 hours of exposure to 3 mg ml⁻¹ GSE, *S. mutans* was inoculated into fresh media with no GSE; there was an increase in cell density from 1.8x10³ to 2.7 x10⁸ CFU ml⁻¹ after 48 hours (Table 3).

3.3.4 *Gesho extracts impact the metabolic activity of biofilm dwelling cells*

Biofilm dwelling cells are frequently resistant to chemical treatments due to the presence of a dense extracellular matrix, metabolically attenuated cells and persister cells (7). GSE was effective in reducing the metabolic activity of *S. mutans* and *C. albicans* in established mono-species biofilms (Table 4). After 24 h of growth 3 mg ml⁻¹ GSE was added to wells containing growing biofilms; after an additional 24 h, the metabolic activity for *S. mutans* and *C. albicans* mono-species biofilms were 23 and 55 percent relative to untreated controls, respectively (Table 4). In contrast, adding 3 mg ml⁻¹ GSE to established *S. mutans*-*C. albicans* dual species biofilms increased metabolic activity more than 50 percent with respect to untreated controls. These data illustrate the strength of the synergism between the two species and demonstrate the challenge of treating polymicrobial biofilms.

3.3.5 *Gesho extracts decrease polysaccharide production by Streptococcus mutans biofilm cells*

Beta glucans are the major polysaccharide present in *S. mutans* biofilms and greatly impacts mono- and dual-species biofilm formation (142). Plant extracts and pure compounds have been found to inhibit *S. mutans* biofilm formation through preventing beta glucan production (128, 140); we hypothesized that gesho extracts could act in a similar manner. A fluorescent dye that binds to polysaccharides, Direct Yellow 96, was used to assess the effects of the stem ethanol extract on polysaccharide concentrations in the *S. mutans* biofilm matrix. A decrease in fluorescence indicated that extract treatments decreased polysaccharide production in a dose dependent manner with almost no glucans present at higher treatment concentrations (Figure 13). As beta glucans make up a significant proportion of biofilm matrix polysaccharides, these data indicated a potential mechanism by which GSE inhibited mono- and dual-species biofilm

formation. Beta glucans are synthesized by glucosyltransferase (Gtf) proteins through enzymatic conversion of sucrose (142). We hypothesize that compounds in the stem extract inhibit *S. mutans* Gtf glucan production, reducing *S. mutans* biofilm formation. It follows that the decrease in *S. mutans* and *C. albicans* dual species biofilm formation is also impacted by the reduction in glucans, as both organisms have been shown to use Gtf enzymes and glucans for attachment and biofilm enhancement (125, 126, 143). Overall, GSE promises to be a source of compounds that can inhibit *S. mutans-C. albicans* dual species biofilm formation.

Gesho is used in traditional medicine and our findings support its use as an antimicrobial and anti-biofilm agent. The data presented regarding GSE's efficacy against *S. mutans* and *C. albicans* biofilm formation and planktonic growth aligns with an earlier report regarding gesho's ability to control *S. aureus* and *B. subtilis* biofilms (134). Campbell *et al.*, 2019 identified two pure compounds from gesho leaf extract and together with this work, demonstrate that both the *R. prinoides* leaf and stem are sources of bioactive compounds useful for controlling microbial pathogens. The effect of GSE on *C. albicans* was notable in that it did not negatively impact growth but still inhibited biofilm formation. This combination of traits is indicative of an "anti-pathogenic" treatment. Anti-pathogenic therapeutics target microbial virulence but are non-biocidal; this reduces selective pressure resulting in antimicrobial resistance (65). To our knowledge, this work was the first to investigate the antimicrobial effects of *Rhamnus prinoides* extracts on *Candida albicans* or polymicrobial biofilms. To better understand the potential applications of gesho and gesho-derived compounds, further work into the chemical compositions and mechanisms should be conducted including investigations into Gtf binding and expression. Based on the data collected to date, gesho shows promise as a natural product that can be incorporated into oral hygiene products.

3.4 Figures and Tables

Table 2. Inhibition of biofilm formation by gesho ethanol extracts

Extract	Concentration (mg ml ⁻¹)	Percent inhibition ²	
		<i>S. mutans</i>	<i>C. albicans</i>
GLE ¹	0.0	0 ± 1	0 ± 3
	0.2	13 ± 1	52 ± 10*
	0.5	14 ± 2	47 ± 10*
	1.0	26 ± 7*	51 ± 10*
	3.0	Campbell <i>et al.</i> , 2019	59 ± 9*
	5.0	Campbell <i>et al.</i> , 2019	46 ± 14*
	7.0	Campbell <i>et al.</i> , 2019	51 ± 10*
GSE ¹	0.0	0 ± 3	0 ± 3
	0.2	33 ± 6	32 ± 9
	0.5	67 ± 5*	65 ± 3*
	1.0	77 ± 3*	73 ± 3*
	3.0	Campbell <i>et al.</i> , 2019	68 ± 6*
	5.0	Campbell <i>et al.</i> , 2019	75 ± 2*
	7.0	Campbell <i>et al.</i> , 2019	70 ± 2*

¹Leaf ethanol (GLE) and stem ethanol (GSE) treatments

²Percent inhibition are the mean of treated samples relative to the untreated control within the same species ± SEM

* indicates a significant difference (p< 0.05) between the treated sample and the untreated control

** Figure originated from the thesis of Raghda Ayman Ahmed Fathi, 2018, "*Rhamnus prinoides* (gesho) extract inhibits *Streptococcus mutans* and *Candida albicans* polymicrobial biofilm formation." Georgia State University. https://scholarworks.gsu.edu/biology_theses/82

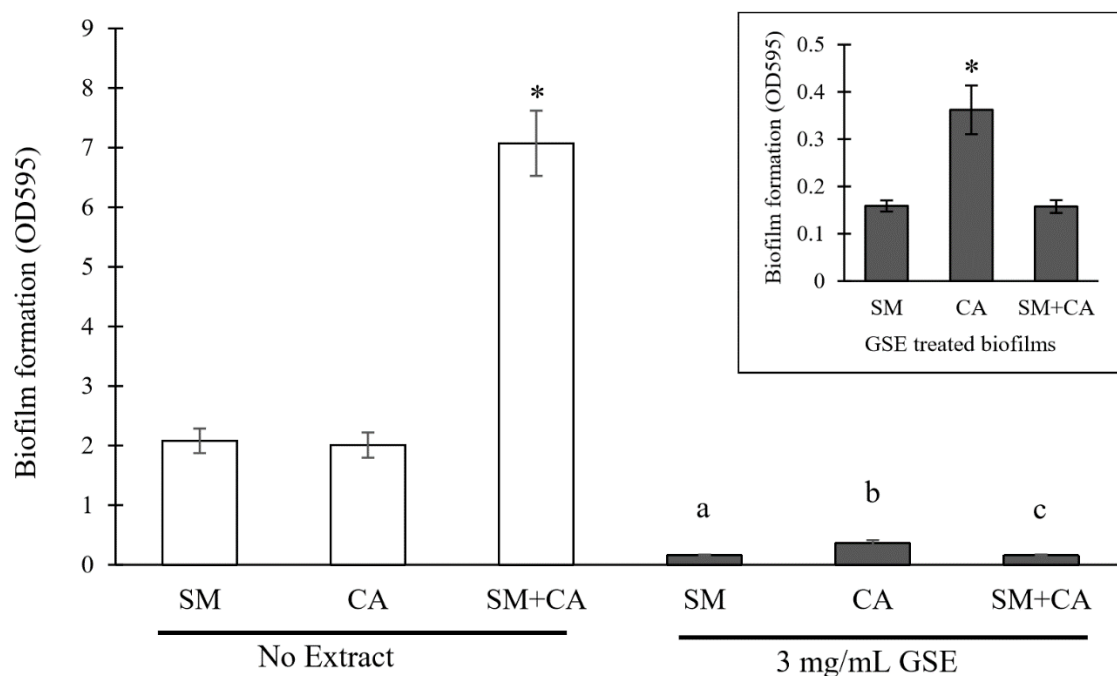


Figure 11. GSE inhibits Streptococcus mutans and Candida albicans dual species biofilm formation.

C. albicans (CA), *S. mutans* (SM) and dual species (SM+CA) biofilm formation were assessed after treatment with GSE (grey bars). Untreated biofilms acted as negative controls (white bars). Inset depicts biofilm formation of GSE treated samples only. All assays were performed in triplicate (n= 30). Error bars are standard error of the mean. Letters a, b and c indicate a significant difference ($p < 0.05$) between a treated sample and its corresponding untreated control. Asterisks indicate a significant difference among untreated or treated samples.

** Figure originated from the thesis of Raghda Ayman Ahmed Fathi, 2018, "*Rhamnus prinoides* (gesho) extract inhibits *Streptococcus mutans* and *Candida albicans* polymicrobial biofilm formation." Georgia State University. https://scholarworks.gsu.edu/biology_theses/82

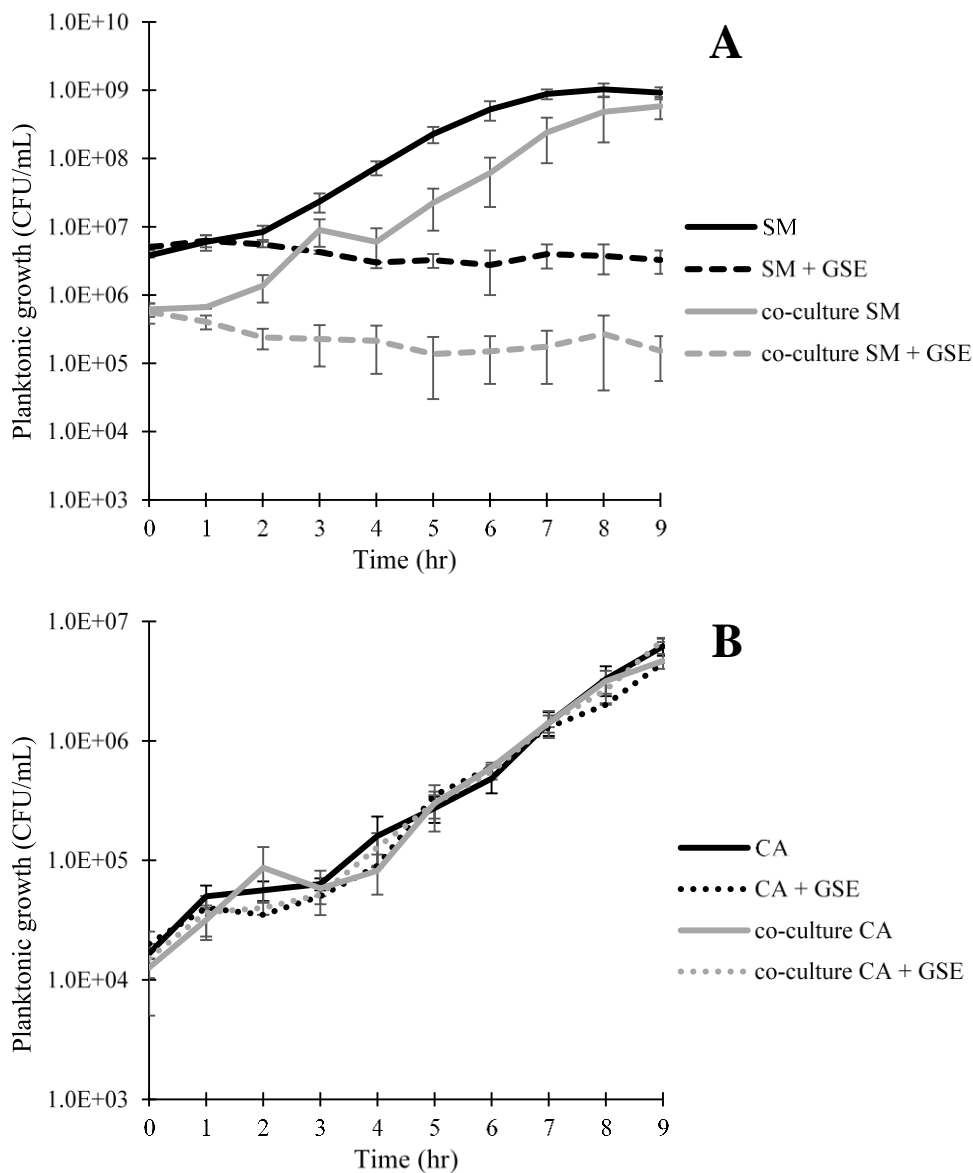


Figure 12. GSE arrests Streptococcus mutans growth.

The planktonic growth of *S. mutans* (SM) and *C. albicans* (CA) mono- and co-cultures were assessed in the absence or presence of 3 mg/mL GSE. Solid lines represent untreated cultures while treated cultures are represented by dashed lines.

Table 3. Streptococcus mutans regrowth after GSE removal

Time	Cell counts ($\times 10^4$)	
	Untreated control	3 mg ml ⁻¹ GSE
0	339 \pm 237	0.2 \pm 0.2
8	24523 \pm 16140	0.1 \pm 0.1
24	13250 \pm 8567	1668 \pm 1666
48	0 \pm 0	26500 \pm 3500

Table 4. Metabolic activity of biofilm dwelling cells after exposure to stem ethanol extracts

Concentration (mg/mL)	<i>Streptococcus mutans</i>	<i>Candida albicans</i>	<i>S. mutans and C. albicans</i>
0	100 ± 1.9	100 ± 0.9	100 ± 0.6
0.2	66 ± 6*	73 ± 3*	108 ± 4
0.5	51 ± 7*	67 ± 2*	121 ± 7*
1	37 ± 9*	60 ± 3*	131 ± 4*
3	23 ± 5*	56 ± 2*	145 ± 5*
5	31 ± 3*	53 ± 2*	152 ± 2*
7	23 ± 4*	54 ± 1*	164 ± 6*

Percent biofilm formation are the mean of extract treated samples relative to the untreated control for each species ± the standard error of the mean (SEM).

*indicate a significant difference ($p < 0.05$) between the treated samples

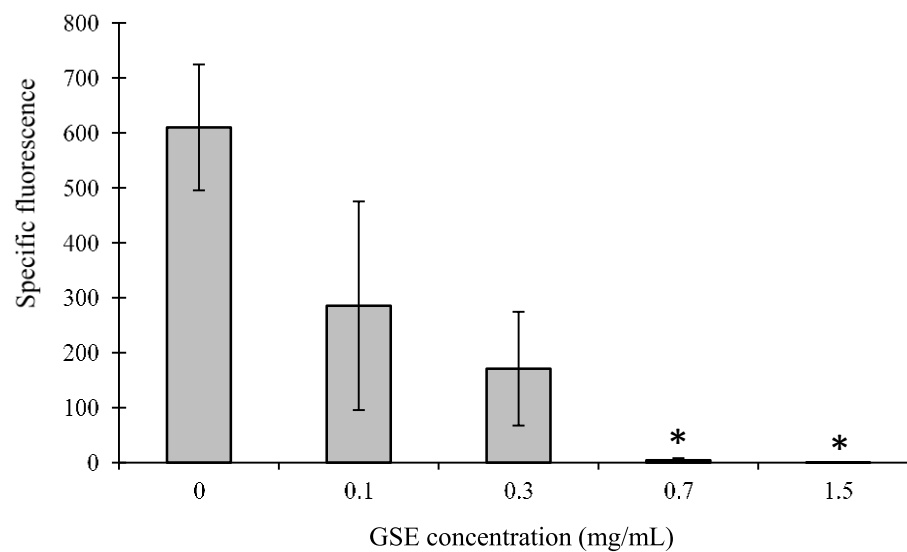


Figure 13. GSE decreases Streptococcus mutans glucan production.

Glucan concentration in *S. mutans* biofilms was assessed in the absence or presence of 3 mg/mL GSE. All assays were performed in triplicate. Error bars are standard error of the mean. Asterisk (*) indicates a significant difference ($p < 0.05$) between the untreated and treated samples.

4 4-ETHOXYBENZOIC ACID INHIBITS *STAPHYLOCOCCUS AUREUS* BIOFILM FORMATION AND POTENTIATES BIOFILM SENSITIVITY TO VANCOMYCIN

4.1 Introduction

Infections involving bacterial biofilms are notoriously difficult to treat due to their enhanced antibiotic resistance (7). Eradicating biofilms is further complicated by a global increase in antibiotic resistance among pathogens resulting from antibiotic misuse and overuse (144). A logical response to these interrelated problems is non-bactericidal therapeutic agents that prevent biofilm formation and also increase antibiotic susceptibility; such compounds would extend the longevity of currently used antibiotics and reduce selective pressure favoring resistance. These compounds are referred to as anti-pathogenic agents. An example of such a compound is hamamelitannin, which prevents *Staphylococcus aureus* biofilm formation and sensitizes it to vancomycin (71). Nonetheless, more anti-biofilm compounds with anti-pathogenic features are needed to diversify the existing arsenal (65).

In recent work, we identified two compounds from *Rhamnus prinoides*, an east African shrub used in traditional medicine, that prevented biofilm formation by Gram positive bacteria (134). Notably, ethoxy-4-hydroxybenzoic acid (EEB) reduced biofilm formation by *Staphylococcus aureus* by 60 percent with minimal inhibition of growth, a feature associated with anti-pathogenic compounds (134). Using EEB as a starting point, in this work, we screened the activity of compounds with related structures to identify more potent anti-biofilm compounds with anti-pathogenic characteristics. This work describes the effects of the compound 4-ethoxybenzoic acid (4EB) on *S. aureus* biofilm formation, antibiotic sensitivity and metabolite production.

4.2 Materials and Methods

4.2.1 Bacterial culture conditions and reagents

Staphylococcus aureus ATCC 6538 was grown in Luria-Bertani (LB) broth (Becton Dickinson and Company, USA). Compounds assessed in this study include: 2-hydroxybenzoic acid (salicylic acid), 3-hydroxybenzoic acid, 4-hydroxybenzoic acid (paraben), 4-hydroxybenzoic acid methyl ester (methyl paraben), 2,3-dihydroxybenzoic acid (pyrocatechuic acid), 2,5-dihydroxybenzoic acid (gentisic acid), 3,4-dihydroxybenzoic acid (protocatechuic acid), gallic acid, methyl gallate, ethyl gallate, propyl gallate, 4-ethoxybenzoic acid and ethyl 4-ethoxybenzoic acid. The following reagents were purchased from Fisher Scientific (USA): crystal violet, dimethyl sulfoxide, 4-ethoxybenzoic acid, ethyl gallate, gallic acid, hexadecane, 3-hydroxybenzoic acid, methyl gallate, potassium chloride, dibasic potassium phosphate, monobasic potassium phosphate and sodium chloride. Reagents purchased from Sigma, Aldrich or Sigma-Aldrich (USA) include: hexadecane, gentisic acid, p-hydroxybenzoic acid, methyl paraben, protocatechuic acid, pyrocatechuic acid, resazurin, salicylic acid, fluorescein 5(6)-isothiocyanate (FITC) and vancomycin. Bacto agar, yeast extract and tryptone were purchased from BD Biosciences (USA). Ethyl 4-ethoxybenzoic acid, 95% ethanol, propyl gallate, and rabbit's blood were purchased from Combi-Blocks (USA), Decon Labs (USA), Nutritional Biochemical Corporation (USA) and Hemostat Laboratories (USA). Florescent dyes SYBR Safe, propidium iodide, and Direct Yellow 96 were purchased from Invitrogen, Acros Organics and AK Scientific, respectively.

4.2.2 Biofilm formation assay

Biofilm formation was assessed using a polystyrene 96 well microtiter plate crystal violet assay (97). Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh LB broth and

combined with a target compound to obtain a final concentration of 7 mg/ml. Diluted cells ($OD_{600} = 0.01$) were then used to serially dilute the compound to final treatment concentrations of 5, 3, 1, 0.5 and 0.25 mg/ml. No vehicles were added to solubilize the treatments. Untreated cells served as negative controls. One hundred microliters of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h (98, 99). After 24 h, planktonic cells and spent growth media were removed from each well and the plate was washed 3 times in sterile water. Biofilms were then dried at room temperature or 37 °C and stained with 150 μ l of 0.1% crystal violet for 15 min at 200 rpm. Excess dye was removed via washing in sterile water and the plate was allowed to air dry. Crystal violet was solubilized in 150 μ l of 95% ethanol and absorbance measurements at 595 nm were taken using an MD SPECTRAmax plate reader (Molecular Devices Corporation, USA).

4.2.3 Resting cell viability assay

To quantify the viability of stationary phase planktonic cells, a growth agar-based assay was conducted. Spent media containing stationary phase planktonic cells were collected from microtiter plates after the conclusion of each biofilm formation assay. The collected cells were serially diluted (1:10) in PBS and dilutions were radially plated in 10 μ l volumes on agar growth medium. Agar plates were incubated at 37 °C overnight to allow for colony formation and plate counts were used to assess viability. Colony forming units per milliliter of culture (CFU/ml) was calculated by dividing the colony counts (C) by the volume added (V) multiplied by the dilution factor (D): $CFU/ml = C / (V \times D)$.

4.2.4 Vancomycin MIC assay

Overnight broth cultures were diluted to an initial OD_{600} of 0.01 in fresh LB broth and combined with vancomycin to a concentration of 1600 μ g/ml. Diluted cells ($OD_{600} = 0.01$) were

then used to serially dilute the vancomycin to final treatment concentrations of 800, 400, 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6 and 0.8 $\mu\text{g/ml}$. Untreated cells served as negative controls. One hundred microliters of treated and untreated samples were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h. Sample turbidities were then measured at an optical density 600 nm using an MD SPECTRAmax plate reader (Figure 16). The lowest concentration of vancomycin to inhibit planktonic growth (1.6 $\mu\text{g/ml}$) was used for subsequent growth curve assays (Figure 16).

4.2.5 *Staphylococcus aureus* growth curve

Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh LB broth and combined with 4-ethoxybenzoic acid (0.2 or 0.4 mg/ml), methyl gallate (0.2 or 0.4 mg/ml) or methyl paraben (0.8 mg/ml). Two milliliters of each treatment was added to 19 culture tubes and the samples were incubated for 24 h at 37 °C with shaking at 200 rpm. One culture tube from each sample was removed every hour and optical density measurements at 600 nm were taken. Time points were taken every hour for 9 hours. Final measurements were taken at 24 h the following day. Untreated samples and samples containing 1.6 $\mu\text{g/ml}$ vancomycin served as negative and positive controls, respectively. Specific growth rates were calculated based on measurements made during the first four hours.

4.2.6 *Resazurin* standard curve

Overnight broth cultures of *Staphylococcus aureus* were diluted to an OD₆₀₀ of 0.01 in fresh LB broth and incubated at 37°C, 200 rpm for 3 hr. After incubation, the optical density of the culture was measured and the culture was then diluted to an OD₆₀₀ of 1.0; this culture was then diluted to optical densities (600 nm) of 0.5, 0.25, 0.12, 0.06, 0.03, 0.01, 0.008, 0.004 and 0.002. Cellular respiration was measured by adding 50 μl of each dilution to a 96 well plate followed by

the addition of 50 μ l of 20 μ g/ml resazurin solution, prepared in LB broth. Resazurin is a blue, non-fluorescent oxidation-reduction indicator that is converted to the pink fluorescent intermediate resorufin upon reduction. The 96 well plate was statically incubated for 3 hr at 37 °C. During incubation, fluorescence intensity was measured at $\lambda_{\text{ex}}= 570$ nm $\lambda_{\text{em}}= 590$ nm every 6 minutes using an Enspire fluorescence plate reader (PerkinElmer, USA); these measurements were used to identify the time at which maximum fluorescence was achieved. Cell concentrations (CFU/ml) that correspond with resazurin samples were quantified using serial dilutions followed by plate counts. Samples were serially diluted (1:10) in PBS and dilutions were radially plated in 10 μ l volumes on growth medium. Agar plates were incubated at 37 °C for 24 hours to allow for colony formation.

4.2.7 Vancomycin MBC assay

Staphylococcus aureus biofilms were formed as described above. *Staphylococcus aureus* biofilms were grown in LB broth. Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh media and 100 μ l of culture was added to each well of a 96 well polystyrene microtiter plate; biofilms were allowed to form at 37 °C for 24 hrs. After incubation, spent media were removed and biofilms were washed twice with 150 μ l of PBS. Vancomycin was suspended in LB broth to a final concentration of 1600 μ g/ml. Treatments were then serially diluted to obtain concentrations of 800, 400, 200, 100, 50, 25, 12.5, 6.3, 3.1, 1.6 and 0.8 μ g/ml. Negative controls consisted of 100 μ l of fresh LB without vancomycin. One hundred microliters of each treatment were added to each microtiter plate biofilm and incubated at 37 °C for 24 h. After 24 h, planktonic cells and spent growth media were removed from each well and the biofilms were washed twice with 150 μ l of PBS. One hundred microliters of 10 μ g/ml resazurin in LB were added to each biofilm and the plate was statically incubated at 37 °C for 3 hrs.

During incubation, fluorescence intensity was measured at $\lambda_{\text{ex}}= 570 \text{ nm}$ $\lambda_{\text{em}}= 590 \text{ nm}$ every 6 minutes using an Enspire fluorescence plate reader (PerkinElmer, USA). Data were normalized to the emission reading at $t = 0$ and the rates of resazurin conversion to resorufin were calculated to identify the time at which maximum fluorescence was achieved. The number of viable biofilm cells were estimated using a resazurin standard curve that correlated bacterial cell density with the time required to reach maximum fluorescence intensity (Figure 18 and Table 8).

4.2.8 Staphylococcus aureus biofilm killing assay

Staphylococcus aureus biofilms were formed as described above. *Staphylococcus aureus* biofilms were grown in LB broth. Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh media and 100 μl of culture was added to each well of a 96 well polystyrene microtiter plate; biofilms were allowed to form at 37 °C for 24 hrs. After incubation, spent media were removed and biofilms were washed twice with 150 μl of PBS. Methyl gallate or 4-ethoxybenzoic acid were suspended in LB broth to a final concentration of 7 mg/ml. Treatments were then serially diluted to obtain concentrations of 3.5, 1.7, 0.8, 0.4, 0.2 and 0.1 mg/ml. Vancomycin treatments were diluted to a final concentration of 3.1 $\mu\text{g}/\text{ml}$. No vehicles were added to solubilize the treatments. Negative controls consisted of 100 μl of fresh LB without compound or antibiotic. One hundred microliters of each treatment were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h. After 24 h, planktonic cells and spent growth media were removed from each well and the biofilms were washed twice with 150 μl of PBS. One hundred microliters of 10 $\mu\text{g}/\text{ml}$ resazurin in LB was added to each biofilm and the plate was statically incubated at 37 °C for 3 hrs. During incubation, fluorescence intensity was measured at $\lambda_{\text{ex}}= 570 \text{ nm}$ $\lambda_{\text{em}}= 590 \text{ nm}$ every 6 minutes using an Enspire fluorescence plate reader (PerkinElmer, USA). Data were normalized to the emission reading at $t = 0$ and the rates of resazurin conversion to resorufin were

calculated to identify the time at which maximum fluorescence occurred. The number of viable biofilm cells were estimated by using a standard curve that correlated the rate of resazurin reduction (time to maximum fluorescence) with cell number per ml (Figure 18).

4.2.9 *Staphylococcus aureus* hemolysis assay

Hemolysis activity was assessed according to Lee, *et al.*, 2014 with a few modifications (61). Overnight *Staphylococcus aureus* broth cultures were diluted to an initial OD₆₀₀ of 0.01 in fresh LB containing 4-ethoxybenzoic acid at 0.2 or 0.4 mg/ml. Cultures were then incubated for 24 h at 37 °C with shaking at 200 rpm. One milliliter of each sample was transferred to 1.5 ml microfuge tubes and centrifuged at 10,000 rcf for 2 minutes. One hundred microliters of supernatant was added to 900 µl of 4% rabbit's blood (previously washed twice with 1X PBS via centrifugation at 800 rcf for 2 min). Hemolysis samples were incubated for 1 h at 37 °C, shaking at 200 rpm. PBS and 10% SDS treated samples served as negative and positive controls, respectively. Samples were then centrifuged at 2,000 x g for 5 min to pellet any intact blood cells. One hundred microliters of each supernatant were added to a 96 well microtiter plate and absorbance measurements at 450 nm were taken using an MD SPECTRAMax plate reader (Molecular Devices Corporation, USA).

4.2.10 *Hydrophobicity test (Microbial Adherence to Hydrocarbon Test)*

Hydrophobicity analysis was conducted according to Ciccio, *et al.*, 2015 with a few modifications (145). Three milliliters of *Staphylococcus aureus* cultures were grown in the presence of 4-ethoxybenzoic acid (0.2 or 0.4 mg/ml) for 24 h at 37 °C with shaking at 200 rpm. Cultures were diluted to an OD₆₀₀ of 0.6 and washed with 1 ml PBS. Five milliliters of the inoculum were produced, 1 ml was removed for plating on growth agar and 1 ml of hexadecane was applied to the air-liquid interface or the remaining 4 ml. Hexadecane samples were vortexed

for 1 min and the phases allowed to separate for 15 minutes at room temperature. One milliliter of the aqueous layer was removed and plated on growth agar. Agar plates were incubated at 37 °C overnight and plate counts were conducted the following day. Results were interpreted as the percent of cells present in the aqueous layer prior to (A_0) and after (A_1) the addition of hexadecane $[(A_0-A_1)/A_0]*100$.

4.2.11 Membrane integrity assay

Membrane integrity assays were conducted according to Brackman *et al*, 2016 with a few modifications (71). Overnight broth cultures of *S. aureus* were washed and diluted to an initial optical density (600 nm) of 1.0 in PBS. A 7 mg/ml stock solution of 4-ethoxybenzoic acid was prepared in diluted cells ($OD_{600} = 1.0$); the stock was then diluted to final treatment concentrations of 0.4 and 0.2 mg/ml in diluted cells ($OD_{600} = 1.0$). Two milliliters of each treatment were added to sterile polystyrene culture tubes and incubate at 37 °C, 200 rpm for 24 hrs. Untreated cells and heat-treated cells were used as the negative and positive control, respectively. Heat-treated cells were prepared by incubating 500 μ l of diluted cells ($OD_{600} = 1.0$) in a 98 °C water bath for 10 minutes. Fifty microliters of each sample was added to a 96 well plate followed by the addition of 50 μ l of 10 μ g/ml propidium iodide. 96 well plates were incubated in the dark for 15 minutes then read on an Enspire plate reader at $\lambda_{ex} = 535$ nm; $\lambda_{em} = 617$ nm.

4.2.12 Crystal violet standard curve

Staphylococcus aureus biofilms were formed as stated above. Overnight broth cultures were diluted to an initial OD_{600} of 0.01 and treated with 4EB at final concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.5 mg/ml. One hundred microliters of treated cells were added to each well of a microtiter plate and incubated at 37 °C, 200 rpm for 24 h. Five biofilms were tested for each treatment concentration. After 24 h of incubation, planktonic cells and spent media were removed

from each well and the remaining biofilms were washed twice with 150 μ l of PBS. One hundred microliters of PBS was added to two biofilm wells for each treatment and a sterile pipet tip was used to detach the biofilms from the wells. Detached cells were then serially diluted (1:10) in PBS and dilutions were radially plated in 10 μ l volumes on growth medium. Agar plates were incubated at 37 °C for 24 hours to allow for colony formation prior to conducting plate counts. Biofilms in the remaining 3 wells were stained with crystal violet as detailed above. A standard curve that correlated biofilm cell density (crystal violet absorbance) to *S. aureus* cell number (CFU/ml) was prepared and was subsequently used to normalize the measurements of proteins, polysaccharides and eDNA present in the biofilm EPS (Figure 20).

4.2.13 Staphylococcus aureus extracellular polysaccharide production

Staphylococcus aureus biofilms were formed as stated above. Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 and treated with 4EB at final concentrations of 0.2, 0.4 and 0.8 mg/ml. One hundred microliters of treated cells were added to each well of a microtiter plate and incubated at 37 °C for 24 h. Six biofilms were tested for each treatment concentration. After 24 h of incubation, planktonic cells and spent media were removed from each well and the remaining biofilms were washed twice with 150 μ l of PBS. One hundred-fifty microliters of 0.1% Direct Yellow 96, a fluorescent polysaccharide-binding dye, in PBS were then added to 3 biofilm wells and incubated in the dark, at room temperature for 30 min. After incubation, unbound Direct Yellow 96 was aspirated and the biofilms were washed twice with 150 μ l PBS. One hundred microliters of PBS was then added to each well and the fluorescence intensity was measured on a Victor V³ plate reader at λ_{ex} = 405 nm; λ_{em} = 535 nm (PerkinElmer, USA). Biofilms in the remaining 3 wells were stained with crystal violet as detailed above and the biofilm cell density was determined. For each sample, relative polysaccharide production was calculated by dividing the

measured fluorescence intensity by the corresponding number of biofilm cells. All experiments were performed in triplicate.

4.2.14 Staphylococcus aureus extracellular DNA production

SYBR Safe (Invitrogen, USA) is a fluorescent nucleic acid stain and was used to quantify biofilm extracellular DNA (eDNA). Twelve biofilms were formed for each treatment. *Staphylococcus aureus* biofilms were formed as described above with the addition of SYBR Safe to the growth medium (final concentration = 1X). After 24 h of incubation, planktonic cells and spent media were removed from each well and the remaining biofilms were washed twice with 150 μ l of PBS. One hundred-fifty microliters of PBS was then added to each well and the fluorescence intensity from the biofilm-associated cells was measured on a Victor plate reader at $\lambda_{\text{ex}} = 490$ nm; $\lambda_{\text{em}} = 535$ nm. Biofilms in the remaining 3 wells were stained with crystal violet as detailed above and biofilm cell density was calculated using a standard curve. For each sample, relative eDNA production was calculated by dividing the measured fluorescence intensity by the corresponding number of biofilm cells. Seven replicates of each condition were performed.

4.2.15 Staphylococcus aureus extracellular protein production

Protein quantification assays were conducted according to Stiefel *et al*, 2016 with a few modifications (146). *Staphylococcus aureus* biofilms were formed as described above. Nine replicate biofilms were formed for each treatment. Overnight broth cultures were diluted to an initial OD₆₀₀ of 0.01 and treated with 4EB at final concentrations of 0.2, 0.4 and 0.8 mg/ml. One hundred microliters of sample were added to wells of a microtiter plate and incubated at 37 °C for 24 h. After 24 h of incubation, planktonic cells and spent media were removed from each well and the remaining biofilms were washed twice with 150 μ l of PBS. One hundred-fifty microliters of 20 μ g/ml fluorescein 5(6)-isothiocyanate (FITC) in water was then added to 5 biofilm wells and

incubated in the dark, at room temperature for 30 min. FITC binds proteins and was used to quantify proteins in the biofilm matrix. After incubation, unbound FITC solution was aspirated and the biofilms were washed twice with 150 μ l of a 0.9 percent sodium chloride solution. One hundred microliters of water were then added to each well and the fluorescence intensity was measured on a Victor plate reader at $\lambda_{\text{ex}}=485$ nm; $\lambda_{\text{em}}=535$ nm. Biofilms in the remaining 4 wells were stained with crystal violet as detailed above and biofilm cell density was estimated using a standard curve. For each sample, relative protein production was calculated by dividing the measured fluorescence intensity by the corresponding number of biofilm cells. Four replicates of each condition were performed.

4.2.16 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 software. Non-parametric (Tukey's test and Independent samples median test) or parametric (t-test) analyses of variance were conducted based on the characteristics of the data. One-Way ANOVA and t-test analyses were conducted using VasserStats: Website for Statistical Computation at vasserstats.net. Comparisons were conducted between the compound-treated samples and the untreated control. Differences with a p-value < 0.05 were considered significant and are designated with an asterisk (*).

4.3 Results

4.3.1 4-ethoxybenzoic acid, methyl gallate and methyl paraben exhibit anti-pathogenic anti-biofilm activity

In previous work, ethyl 4-ethoxybenzoic acid (EEB) inhibited *Staphylococcus aureus* biofilm formation with minimal impact on planktonic growth (134). In an effort to identify compounds with greater anti-pathogenic anti-biofilm activity, structurally related compounds to

EEB were screened using two parameters (Figure 14). First, we measured the “BP50”, the concentration of compound required to inhibit biofilm formation by 50 percent relative to an untreated control biofilm. Second, we measured the “LC50”, the concentration of compound required to reduce the growth of planktonic cells by 50 percent relative to an untreated control sample. The two parameters were graphed on an x-y plot such that the anti-biofilm character (BP50) of each compound was presented on the x-axis and the planktonic growth toxicity (LC50) was presented on the y-axis (Figure 15). Examination of the graph indicated that the compounds clustered in three general categories: **low potency** ($BP50 \geq 7.5$ mM; $LC50 \geq 15$ mM), **biocidal** ($LC50 \leq 15$ mM) or **anti-pathogenic** ($BP50 \leq 7.5$; $LC50 \geq 15$) based on their BP50 and LC50 values (Figure 15A, Table 5-6). Molarity was used in this analysis to allow for direct comparisons of compound activity; BP50 and LC50 concentrations (molarity and w/v) for each compound are provided in Table 6.

3-hydroxybenzoic acid, paraben, pyrocatechuic acid, gentisic acid, protocatechuic acid, ethyl gallate and propyl gallate all possessed LC50 values less than 15 mM and thus were all categorized as biocidal regardless of their BP50 (Figure 15A). Low potency compounds included gallic acid, salicylic acid and EEB. BP50 and LC50 values for EEB were 104 mM and 818 mM respectively (note that EEB could not be seen on the graph with the other compounds). 4-ethoxybenzoic acid (4EB), methyl gallate and methyl paraben were categorized as anti-pathogenic (Figure 15A). The range of possible anti-pathogenic concentrations of 4EB and methyl gallate were between 0.1 and 0.8 mg/ml (0.5 mM and 5 mM); for methyl paraben, these concentrations were between 0.4 and 1.7 mg/ml (2 mM and 11 mM) (Figure 15B-D). Treatment concentrations that inhibited at least 40 percent biofilm formation without inhibiting growth were selected for subsequent investigations (Figure 15B-D).

4.3.2 4-ethoxybenzoic acid, methyl gallate and methyl paraben attenuate the growth of *Staphylococcus aureus*

The existence of concentrations of 4EB, methyl gallate and methyl paraben with no negative impact on planktonic growth after 24 hours of treatment (Figure 15B-D) led us to investigate the impacts of these compounds on the *S. aureus* log phase growth rate (Table 7 and Figure 17). *Staphylococcus aureus* cells were exposed to 0.2 and 0.4 mg/ml 4EB, 0.2 and 0.4 mg/ml methyl gallate or 0.8 mg/ml methyl paraben; these concentrations were found to exhibit anti-pathogenic inhibition of biofilm formation (Figure 15B-D). At the tested concentrations, growth was attenuated by no more than 50 percent relative to untreated cells (Table 7). Treatments with 0.2 mg/ml 4EB showed the least effect on growth, which was attenuated by 12 percent, followed by 0.8 mg/ml methyl paraben and 0.2 mg/ml methyl gallate, which decreased the growth rate by 25 and 30 percent, respectively. Higher concentrations of 4EB and methyl gallate enhanced the negative impacts on growth to approximately 46 percent attenuation for both compounds. Due to the high concentrations of methyl paraben required to observe an anti-pathogenic phenotype, further work was not continued with this compound.

4.3.3 4-ethoxybenzoic acid and methyl gallate enhance the anti-biofilm activity of vancomycin

Anti-pathogenic, anti-biofilm compounds like hamamelitannin have been found to potentiate the activity of vancomycin against biofilm-dwelling cells (71, 147). To determine whether 4EB or methyl gallate also had this activity, resazurin viability assays were used to quantify the number of live cells in an established *S. aureus* biofilm after 24 hours of treatment with 4EB or methyl gallate alone or in combination with 3.1 µg/ml vancomycin (Table 9). MBEC assays were conducted to identify the concentration of vancomycin required to significantly reduce

biofilm biomass without eradicating all biofilm cells (Table 8). Treatment with 3.1 $\mu\text{g/ml}$ vancomycin alone killed 85 percent of biofilm cells compared to untreated control biofilms. Treatments with only 4EB or methyl gallate resulted in no more than 16 percent reduction in viability compared to the untreated control. Combining 3.1 $\mu\text{g/ml}$ vancomycin with 4EB at concentrations ≥ 0.1 mg/ml reduced biofilm cell viability by an additional 44 to 85 percent compared to the vancomycin-only treated samples, indicating that 4EB potentiated vancomycin activity (Table 9). In contrast, methyl gallate in combination with vancomycin did not reduce biofilm cell viability compared to vancomycin alone. Given the lack of antibiotic potentiation, methyl gallate was excluded from further experiments.

4.3.4 4-ethoxybenzoic acid alters Staphylococcus aureus hydrophobicity and EPS production

The physiological impacts of 4EB on *S. aureus* were assessed by measuring its effects on membrane integrity, hydrophobicity, hemolysis activity and EPS production. Treatment with 0.4 mg/ml 4EB significantly decreased the percentage of hydrophobic planktonic *S. aureus* cells relative to the untreated and 0.2 mg/ml treated samples (Figure 19). The percent of hydrophobic cells decreased from 78 to 49 percent. In addition to the effect on hydrophobicity, 4EB treatments impacted the relative amount of extracellular polysaccharides and proteins in the biofilm matrix (Figure 21A-B). Treatments of 0.4 and 0.8 mg/ml 4EB significantly enhanced relative extracellular polysaccharide production up to 9-fold while the effect on extracellular protein production was more modest with a 1-fold increase. Relative extracellular polysaccharide production steadily increased as biofilm formation decreased. The impact of treatment on eDNA was the opposite; the amount of eDNA produced decreased as biofilm formation decreased (Figure 21C). No change in *S. aureus* membrane integrity or hemolysis activity were observed upon treatment (Figure 22-23).

4.4 Discussion

An increased prevalence of antibiotic resistance threatens to leave humanity with limited resources to combat microbial infections. Anti-pathogenic anti-biofilm compounds such as 4EB can potentially contribute to controlling this problem. To our knowledge, this work is the first to describe the anti-biofilm and antibiotic sensitization effects as well as anti-pathogenic character of 4EB. This information expands the functionality of 4EB, whose use has previously been associated with chemical synthesis or monooxygenase activation (148–150). We think that the BP50/LC50 method described herein to characterize the activity of 4EB is a novel and effective way to recognize anti-pathogenic anti-biofilm compounds. Additionally, this work establishes the previously unreported anti-biofilm activities of 3-hydroxybenzoic acid and gentisic acid and supports prior findings for protocatechuic acid, salicylic acid, and gallic acid (72, 151–157).

A structure-activity analysis of compounds related to EEB shed light on the contribution of specific molecular components to anti-biofilm activity. By comparing the BP50/LC50 profiles of 2-, 3- and 4-hydroxybenzoic acids, it was evident that a hydroxyl group in the ortho position significantly reduced both the anti-biofilm and antimicrobial activity. Alkylating the carboxyl group of 4-hydroxybenzoic acid to yield methyl paraben slightly reduced the antimicrobial effect but substantially increased the anti-biofilm activity. The antimicrobial activity of 4-hydroxybenzoic acid has been proposed to result from its effect on membrane stability and transport processes (72, 151, 158, 159). Although the specific reason remains to be determined, methylation enhanced anti-biofilm activity. This finding is significant because methyl paraben is widely used in food products and is the subject of controversy related to mammalian toxicity resulting from chronic exposure (158, 160).

A comparison of di- and trihydroxybenzoic acids indicated that the position of the hydroxyl groups impacted anti-biofilm activity. Notably, adding a second hydroxyl group at the 3- position to 4-hydroxybenzoic acid (yielding protocatechuic acid) significantly reduced the anti-biofilm activity. A third hydroxyl group at the 2- position (gallic acid) substantially reduced both the anti-microbial and anti-biofilm activity compared to protocatechuic acid. However, methylating the carboxyl group of gallic acid to form methyl gallate greatly increased the anti-biofilm activity; lengthening of the alkyl moiety (e.g. ethyl gallate and propyl gallate) increased biocidal activity. Several mechanisms of antimicrobial activity are known for the alkyl gallates including disruption of: membrane stability, polysaccharide production and oxidative phosphorylation (155, 161–163).

When elucidating the mechanism of activity of 4EB and structurally related compounds against *S. aureus* biofilm formation, two broad hypotheses must be considered. First, 4EB may inhibit biofilm formation through a physicochemical mechanism. In support of this hypothesis, 4EB reduced the hydrophobicity of *S. aureus*, which in turn could reduce adhesion and surface colonization to negatively charged surfaces such as polystyrene, which was used in the screening assay. Charge interactions are known to be a factor in early stage biofilm formation; disruption of these interactions negatively impacts biofilm initiation (1, 164). A common feature of the three compounds categorized as “anti-pathogenic” in this work (4EB, methyl gallate, methyl paraben) was the 4-hydroxybenzoic acid central structure and a single methyl or ethyl moiety on the molecule. We hypothesize that the methyl or ethyl group increases the association of the molecules with the hydrophobic *S. aureus* membrane while the negatively charged region of the molecules interferes with surface association.

Second, 4EB may reduce biofilm formation by influencing the transcription of requisite genes. Hamamelitannin (HAM), an anti-pathogenic compound with structural similarity to both

4EB and methyl gallate, has been reported to prevent *S. aureus* biofilm formation by altering the transcription of quorum sensing and stress response genes via the controversial TRAP transcriptional regulator (71, 165). Additionally, the structurally similar molecule gallic acid has been reported to act against *S. aureus* biofilm formation via the *ica* operon (155). These reports suggest that 4EB may also impact biofilm formation on a genetic level. In our work, 4EB was found to affect EPS production by *S. aureus*, increasing both extracellular polysaccharide and protein production; this could be the result of alterations in the expression of genes such as *ica*, *atl*, *bap* and *dltA* (151, 166). This physiological change appears to be contradictory to the overall anti-biofilm activity of 4EB. However, the increase in EPS production was only evident at high 4EB concentrations when the corresponding biofilm biomass was low, leading us to hypothesize that the increase was a response to stress (20, 167). In general, several plausible mechanisms exist by which 4EB can reduce *S. aureus* biofilm formation.

Another feature of 4EB is that it synergized the activity of vancomycin against *S. aureus* biofilm-dwelling cells. In contrast, methyl gallate did not affect vancomycin activity, indicating that the interaction between 4EB and vancomycin was not simply due to the presence of a secondary compound but rather a characteristic of 4EB. HAM was found to increase vancomycin sensitivity by thinning the peptidoglycan cell wall of *S. aureus* (71). The mechanism by which 4EB enhanced vancomycin sensitivity is presently unknown, however the effects of HAM on cell wall synthesis may act as a lead.

This work provides initial data on the anti-pathogenic and anti-biofilm activities of 4EB; however, additional effort is required to elucidate the full scope, applications and mechanisms of activity of this compound. The experiments reported here were conducted using a single, vancomycin-susceptible strain of *S. aureus*; to better understand the potential of 4EB as an anti-

biofilm agent, investigations using other *S. aureus* strains and microbial species are necessary, including antibiotic resistant strains. Experiments are also required to determine whether 4EB can sensitize pathogens to additional antibiotics beside vancomycin. Phenotypic analyses to elucidate the mechanism of 4EB activity revealed impacts on cell hydrophobicity and extracellular polysaccharide production. Molecular analyses, including transcriptomics and mutagenesis, will help identify and verify the various targets of 4EB. Overall, the anti-pathogenic and anti-biofilm activity of 4EB, along with its ability to potentiate vancomycin sensitivity, warrant its consideration as a therapeutic adjuvant to conventional *S. aureus* biofilm treatments.

4.5 Figures and Tables

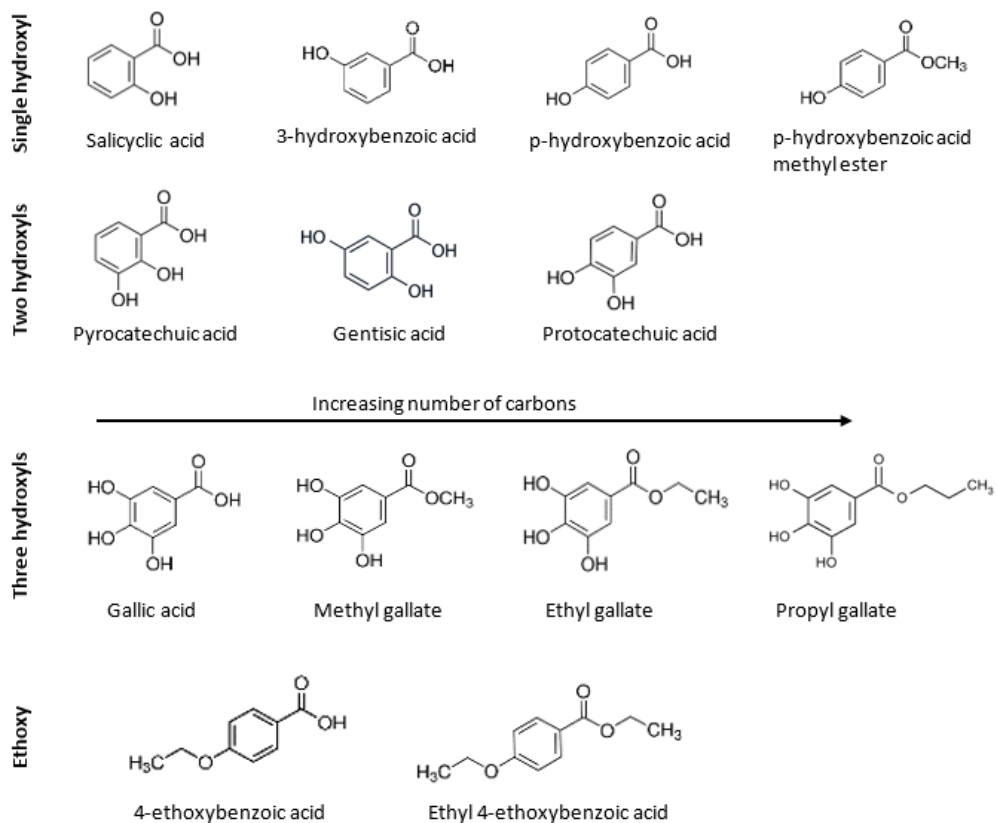


Figure 14. EEB-related compounds tested in this work

All compounds were structurally similar to the gesho-derived compound ethyl 4-ethoxybenzoic acid (EEB). Compounds were selected to investigate the effect of functional group location on anti-biofilm activity.

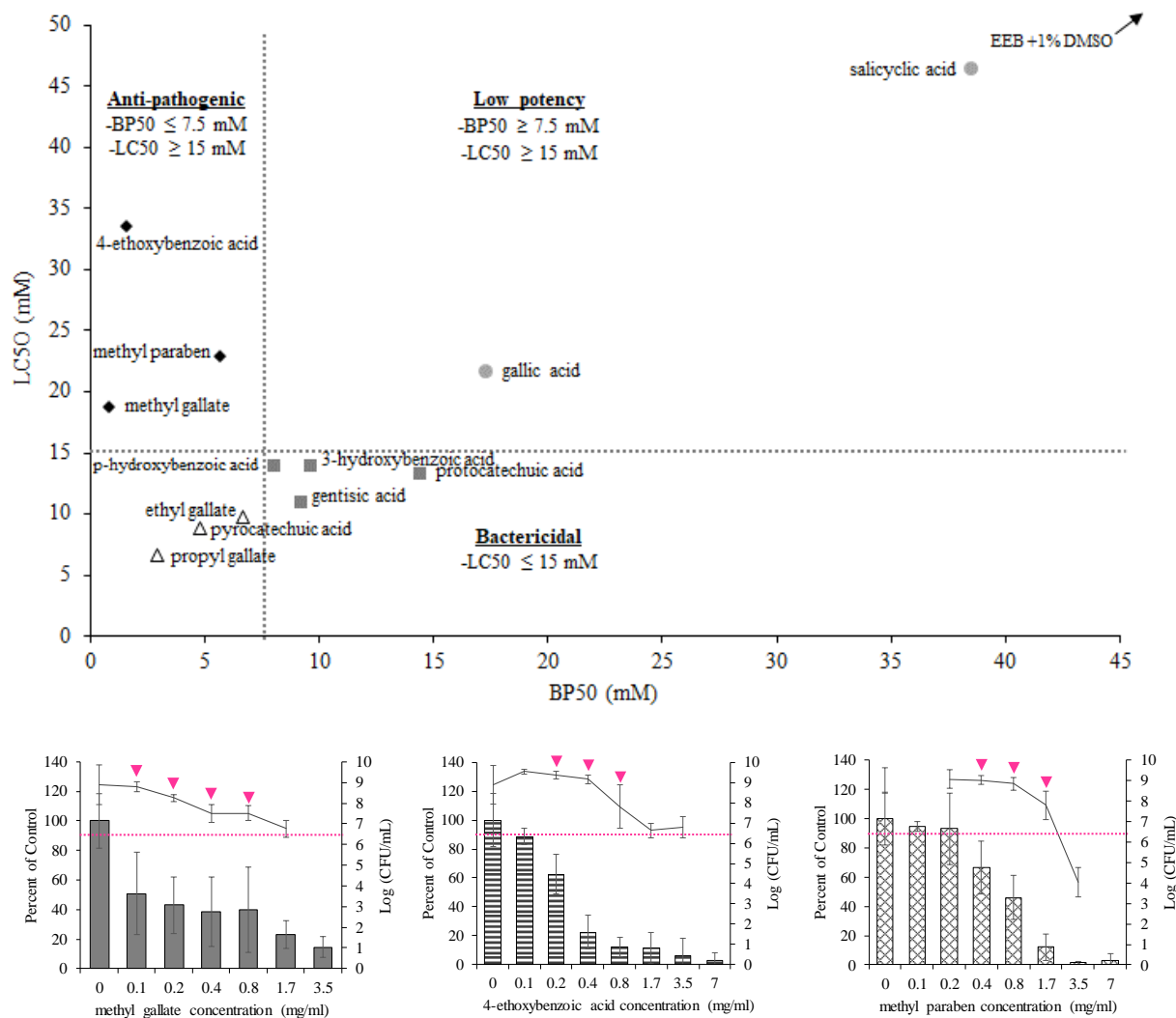


Figure 15. Effects of EEB-related compounds on *Staphylococcus aureus* biofilm formation and viability.

Data points represent the compound concentrations that resulted in half the maximum biofilm formation (BP50) and half the maximum planktonic growth (LC50). Compounds were characterized as anti-pathogenic, bactericidal or low potency (A). Anti-pathogenic concentrations of methyl gallate (B), 4-ethoxybenzoic acid (C) and methyl paraben (D) are indicated by arrow heads (B-D). The inoculum cell density is represented by the red dotted line. Error bars indicate standard deviation.

Table 5. Effect of phenolic acids on *Staphylococcus aureus* biofilm formation and stationary-phase cell viability

	Concentration		Biofilm		Stationary phase cells
	mg/mL	mM	% formation ¹	% inhibition ²	Log CFU/mL
Untreated control	0.0	0.0	100 ± 18.3 (68)	0	8.9 ± 1.0 (5)
Salicylic acid	7.0	50.7	20.7 ± 13.5 (17)	79	6.8 ± 0.2 (3)
	3.5	25.3 [#]	70.4 ± 28.2 (16)	30	8.2 ± 0.5 (3)
	1.7	12.3	137.4 ± 39.0 (16)	0	9.0 ± 0.3 (3)
	0.8	5.8	146.6 ± 59.0 (16)	0	9.5 ± 0.0 (1)
	0.4	2.9	132.2 ± 30.0 (17)	0	-
3-hydroxybenzoic acid	3.5	25.3	2.9 ± 3.1 (16)	97	0.0 ± 0.0 (2) [^]
	1.7	12.3	4.7 ± 7.2 (16)	95	5.3 ± 1.0 (2) [^]
	0.8	5.8	111.2 ± 42.3 (16)	0	8.4 ± 0.1 (2)
	0.4	2.9	173.1 ± 42.9 (16)	0	-
p-hydroxybenzoic acid	7.0	50.7	2.0 ± 0.2 (4)	98	-
	3.5	25.3	2.3 ± 0.9 (13)	98	1.9 ± 3.3 (3) [^]
	1.7	12.3	1.3 ± 0.9 (17)	99	2.8 ± 2.4 (3) [^]
	0.8	5.8 [#]	43.4 ± 34.0 (17)	57	7.4 ± 1.1 (3)
	0.4	2.9	145.1 ± 37.1 (17)	0	8.8 ± 0.2 (3)
	0.2	1.4	119.0 ± 19.6 (17)	0	-
	0.1	0.7	107.5 ± 25.4 (10)	0	-
p-hydroxybenzoic acid methyl ester	7.0	46.0	3.0 ± 4.8 (15)	97	-
	3.5	23	1.4 ± 1.2 (18)	99	4.0 ± 0.7 (2) [^]
	1.7	11.2 [#]	12.0 ± 8.9 (21)	88	7.8 ± 0.7 (5)
	0.8	5.3 [#]	46.1 ± 15.1 (21)	54	8.8 ± 0.3 (5)
	0.4	2.6 [#]	66.9 ± 18.1 (22)	33	9.0 ± 0.2 (4)
	0.2	1.3 [#]	93.3 ± 24.3 (22)	7	9.1 ± 0.4 (2)
	0.1	0.7	94.9 ± 3.3 (4)	5	-
Pyrocatechuic acid	3.5	22.7	4.2 ± 2.7 (12)	96	0.0 ± 0.0 (2) [^]
	1.7	11.0	3.9 ± 2.0 (12)	96	0.0 ± 0.0 (2) [^]
	0.8	5.2	30.5 ± 25.6 (12)	70	6.1 ± 0.7 (2) [^]
	0.4	2.6	160.2 ± 19.4 (12)	0	8.8 ± 0.0 (2)
Gentisic acid	3.5	22.7	6.8 ± 9.7 (12)	93	1.3 ± 1.8 (2) [^]
	1.7	11.0	4.2 ± 7.5 (15)	96	0.0 ± 0.0 (2) [^]
	0.8	5.2	151.9 ± 73.1 (15)	0	8.7 ± 0.2 (2)
	0.4	2.6	104.7 ± 27.9 (15)	0	9.3 ± 0.3 (2)
Protocatechuic acid	3.5	22.7	5.3 ± 10.7 (12)	95	0.0 ± 0.0 (2) [^]
	1.7	11.0	47.5 ± 32.8 (12)	53	6.0 ± 0.9 (2) [^]
	0.8	5.2	124.8 ± 49.4 (15)	0	8.9 ± 0.2 (2)
	0.4	2.6	97.1 ± 22.1 (15)	3	-
	0.2	1.3	112.7 ± 14.7 (9)	0	-

Table 5 continued.

	Concentration		Biofilm		Stationary phase cells
	mg/mL	mM	% formation ¹	% inhibition ²	Log CFU/mL
Untreated control	0.0	0.0	100 ± 18.3 (68)	0	8.9 ± 1.0 (5)
Gallic acid	3.5	20.6	25.5 ± 6.0 (16)	75	4.1 ± 0.2 (3) [^]
	1.7	10.0 [#]	102.3 ± 78.5 (16)	0	8.3 ± 0.7 (3)
	0.8	4.7	168.7 ± 31.4 (16)	0	8.8 ± 0.1 (3)
	0.4	2.3	173.3 ± 36.9 (16)	0	-
Methyl gallate	3.5	19.0	14.4 ± 7.0 (16)	86	-
	1.7	9.2 [#]	23.1 ± 9.6 (16)	77	6.8 ± 0.4 (2)
	0.8	4.3 [#]	39.9 ± 28.7 (16)	60	7.5 ± 0.3 (4)
	0.4	2.2 [#]	38.4 ± 23.7 (16)	62	7.5 ± 0.4 (4)
	0.2	1.1 [#]	42.9 ± 19.0 (12)	57	8.2 ± 0.2 (4)
	0.1	0.5 [#]	50.6 ± 27.8 (6)	49	8.8 ± 0.2 (4)
Ethyl gallate	3.5	17.7	15.4 ± 5.6 (16)	85	-
	1.7	8.6	24.9 ± 9.5 (16)	75	5.4 ± 0.0 (1) [^]
	0.8	4.0	100.7 ± 81.2 (16)	0	6.0 ± 0.5 (3) [^]
	0.4	2.0	75.5 ± 55.2 (16)	25	6.7 ± 0.3 (3)
	0.2	1.0	108.3 ± 86.1 (12)	0	7.6 ± 0.2 (3)
Propyl gallate	3.5	16.5	17.7 ± 6.9 (12)	82	-
	1.7	8.0	22.4 ± 24.7 (12)	78	5.6 ± 0.7 (2) [^]
	0.8	3.8	23.2 ± 12.0 (15)	77	6.2 ± 0.6 (3) [^]
	0.4	1.9	69.3 ± 58.7 (15)	31	6.9 ± 0.4 (3)
	0.2	0.9	146.4 ± 66.0 (12)	0	7.4 ± 0.1 (3)
4-ethoxybenzoic acid	7.0	37.3	2.5 ± 5.7 (3)	97	-
	3.5	21.1	6.2 ± 12.2 (16)	94	6.8 ± 0.5 (3)
	1.7	10.2	11.1 ± 10.6 (16)	89	6.6 ± 0.4 (3)
	0.8	4.8 [#]	12.1 ± 6.9 (16)	88	7.8 ± 1.1 (3)
	0.4	2.4 [#]	22.3 ± 11.7 (16)	78	9.2 ± 0.2 (3)
	0.2	1.2 [#]	62.6 ± 14.0 (12)	37	9.4 ± 0.2 (3)
	0.1	0.6 [#]	88.5 ± 5.7 (6)	11	9.5 ± 0.1 (3)

¹Percent biofilm formation are the mean relative to the untreated control ± the standard deviation (SD). Parenthetical values are the sample size (n).

²Percent biofilm inhibition is the difference between the untreated control and the treatment

[^] indicates plate counts below that of the inoculum 6.4 ± 0.1 (n=5).

[#] indicates treatment concentrations that inhibited biofilm formation without negatively impacting cell growth

Table 6. BP50 and LC50 of phenolic acid compounds on Staphylococcus aureus biofilm formation and viability

Compound	BP50		LC50	
	mM	mg/ml	mM	mg/ml
methyl gallate	0.82	0.2	18.8	3.5
ethyl gallate	6.7	1.3	9.8	1.9
propyl gallate	2.9	0.6	6.6	1.4
4-ethoxybenzoic acid	1.6	0.3	33.5	5.6
p-hydroxybenzoic acid	8.0	1.1	14.0	1.9
methyl paraben	5.7	0.9	22.9	3.5
3-hydroxybenzoic acid	9.6	1.3	14.0	1.9
gentisic acid	9.2	1.4	11.0	1.7
pyrocatechuic acid	4.8	0.7	8.9	1.4
protocatechuic acid	14.4	2.2	13.2	2.0
gallic acid	17.3	2.9	21.6	3.7
salicylic acid	38.5	5.3	46.4	6.4
EEB+ 1% DMSO	103.6	20.1	818.0	158.9

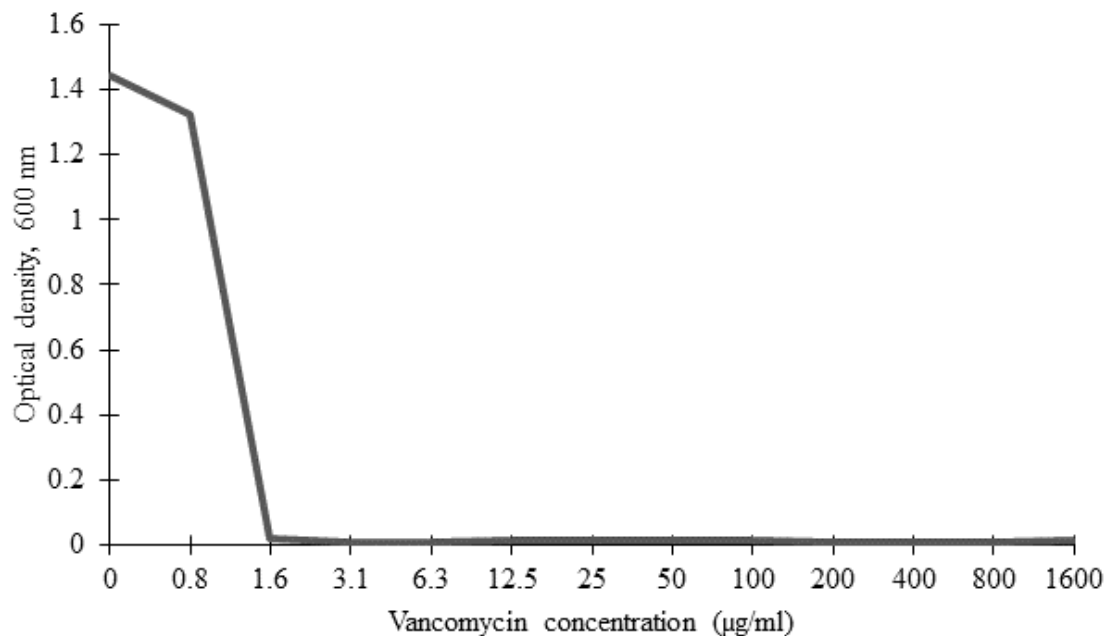


Figure 16. Effect of vancomycin on Staphylococcus aureus planktonic growth. Growth of *S. aureus* after 24 hours of treatment with vancomycin (0.8 µg/ml-1600 µg/ml) was assessed to identify the minimal inhibitory concentration (MIC). Details regarding this experiment can be found in the materials and method section 4.4 *Vancomycin MIC assay*. Untreated samples (0 µg/ml) served as a negative control.

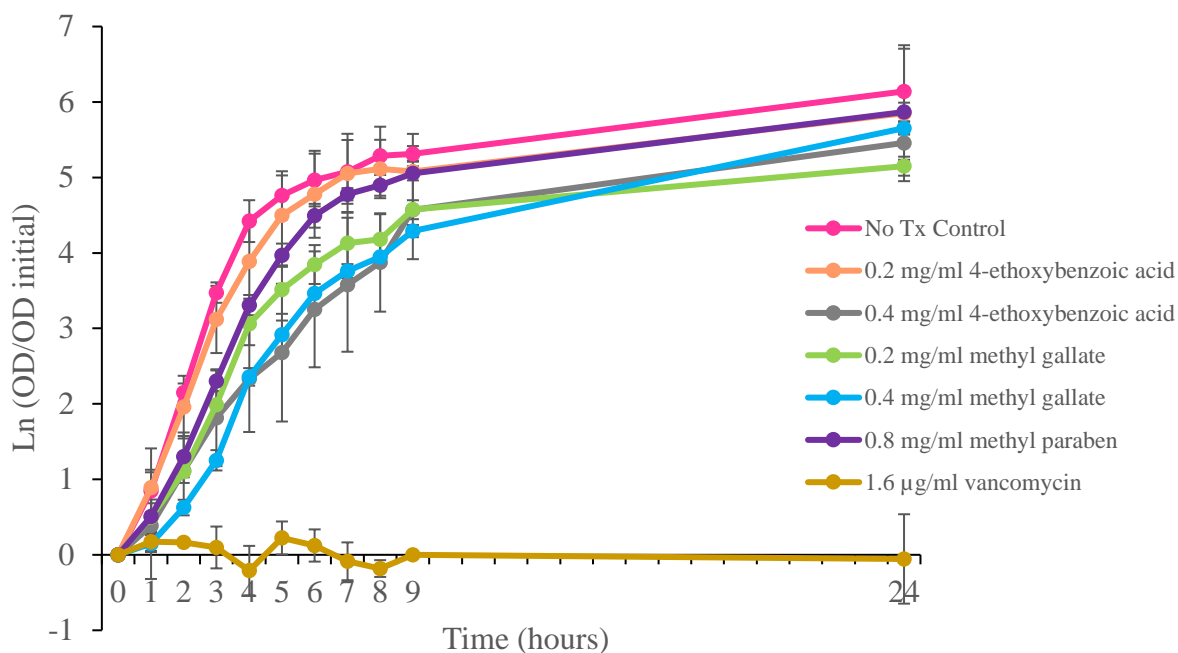


Figure 17. Effects of 4-ethoxybenzoic acid, methyl gallate and methyl paraben on *Staphylococcus aureus* growth.

Growth of *S. aureus* in the presence of 0.2 and 0.4 mg/ml 4-ethoxybenzoic acid, 0.2 and 0.4 mg/ml methyl gallate or 0.8 mg/ml methyl paraben was measured over 24 hours. Untreated (No Tx Control) and 1.6 $\mu\text{g}/\text{ml}$ vancomycin treated samples served as negative and positive controls, respectively. Growth rates were calculated from $t=0$ and $t=4$ and are presented in table 1 along with the percent attenuation. Error bars indicate standard deviation.

Table 7. Effects of 4-ethoxybenzoic acid, methyl gallate and methyl paraben on *Staphylococcus aureus* growth rate

Treatment	Growth rate (replications/ hr)	Percent attenuation
Untreated control	1.11	0
0.2 mg/ml 4-ethoxybenzoic acid	0.97	12
0.4 mg/ml 4-ethoxybenzoic acid	0.58	47
0.2 mg/ml methyl gallate	0.77	30
0.4 mg/ml methyl gallate	0.59	46
0.8 mg/ml methyl paraben	0.83	25
1.6 µg/ml vancomycin	0.0	100

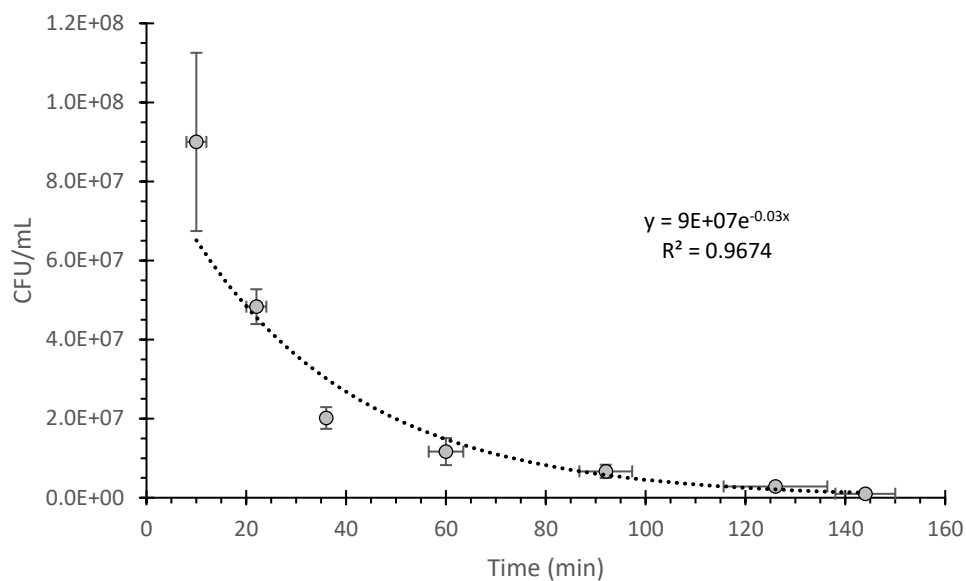


Figure 18. Resazurin standard curve.

The time required for various cell densities to reach maximum fluorescence was plotted and a line of best fit was generated. The equation of the curve was used to estimate the quantity of viable biofilm-dwelling cells after treatment with 4EB or methyl gallate alone or in combination with vancomycin.

Table 8. Biocidal effect of vancomycin on biofilm-dwelling cells

Vancomycin concentration ($\mu\text{g/ml}$)	Quantity of viable biofilm cells ($\times 10^4$) ¹
0	2581 \pm 463 (3)
0.8	2440 \pm 533 (3)
1.6	2015 \pm 203 (3)
3.1	773 \pm 447 (3)
6.2	3 \pm 3 (2)
12.5	2 \pm 1 (3)

¹Mean cell concentration \pm the standard deviation (SD). Parenthetical values are the sample size (n).

Table 9. Biofilm killing activity of compounds in combination with 2.1 mM vancomycin

Compound	Concentration (mg/ml)	Compound only ¹	Percent reduction compared to untreated control ²	Compound + 3.1 µg/ml vancomycin ¹	Percent reduction compared to untreated control ²
4EB	0	18.6 ± 2.8 (11)	-	2.7 ± 2.2 (11) ^b	-
	0.1	18.7 ± 1.8 (4)	0	3.3 ± 1.8 (12) ^b	0
	0.2	17.0 ± 5.3 (4)	9	1.5 ± 1.2 (12) ^b	44
	0.4	18.3 ± 5.0 (4)	2	0.4 ± 0.5 (9) ^{a,b}	85
	0.8	20.6 ± 3.7 (4)	0	1.0 ± 1.1 (12) ^{a,b}	63
Methyl gallate	0	18.6 ± 2.8 (11)	-	2.7 ± 2.2 (11) ^b	-
	0.1	15.7 ± 6.7 (4)	16	3.2 ± 1.9 (11) ^b	0
	0.2	15.8 ± 8.5 (4)	15	3.3 ± 2.2 (12) ^b	0
	0.4	16.2 ± 12.3 (4)	13	5.7 ± 4.2 (12) ^b	0
	0.8	28.1 ± 9.9 (4)	0	5.4 ± 7.6 (12) ^b	0

¹Mean colony forming units per milliliter ($\times 10^6$) \pm the standard deviation (SD). Parenthetical values are the sample size (n).

²Percent reduction are the mean of treated samples relative to the untreated or vancomycin only treated controls. Controls contain 0 mg/ml of compound.

(a) Indicated a significant difference ($p < 0.05$) between vancomycin and combination treated samples.

(b) Indicated a significant difference between the compound and combination treated samples.

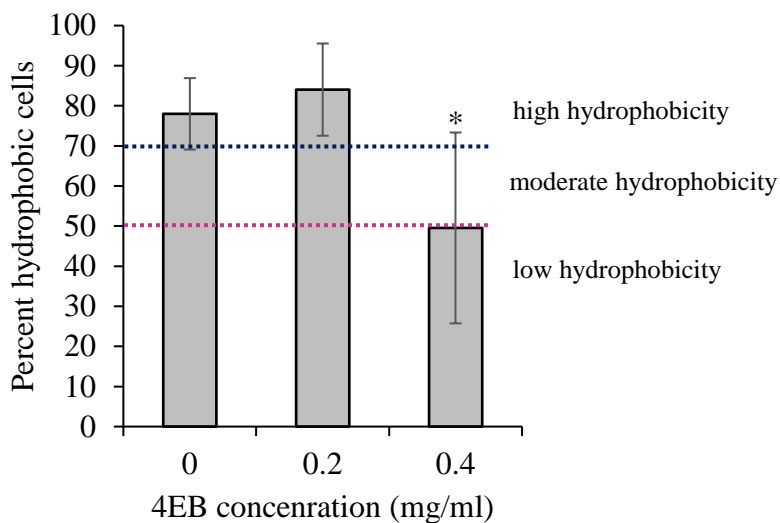


Figure 19. Effects of 4-ethoxybenzoic acid on Staphylococcus aureus hydrophobicity.

The hydrophobicity of cells treated with 4-ethoxybenzoic acid were categorized as low (≤ 50), moderate (50%-70%) or high ($\geq 70\%$). Untreated samples served as a negative control. Error bars indicate standard deviation. ANOVA Tukey's tests were performed; asterisks (*) indicated a significant difference ($p < 0.05$) between the treated samples and untreated control.

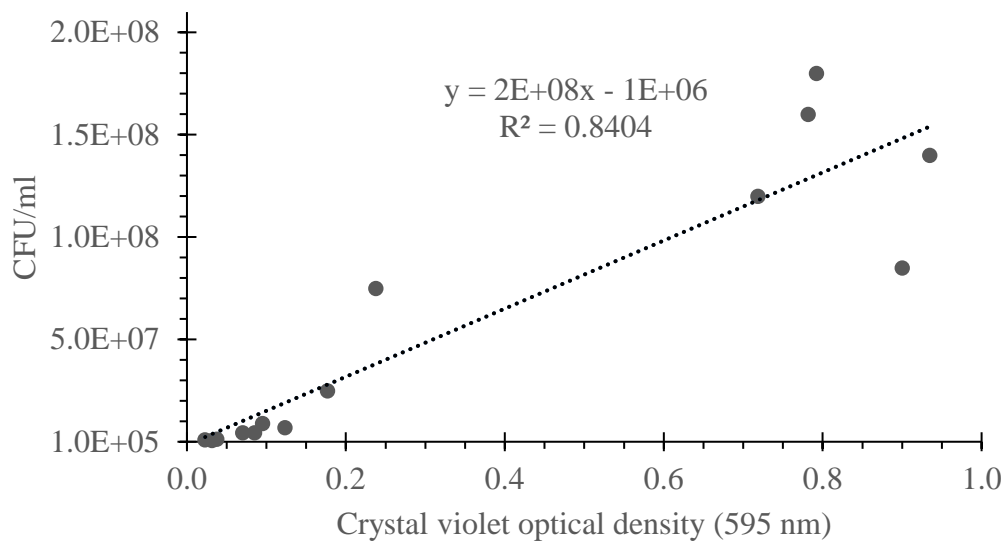


Figure 20. Crystal violet standard curve.

Colony counts that correlated to crystal violet measurements were plotted and a line of best fit was generated. The equation of the curve was used to estimate the quantity of biofilm-dwelling cells in EPS production assays.

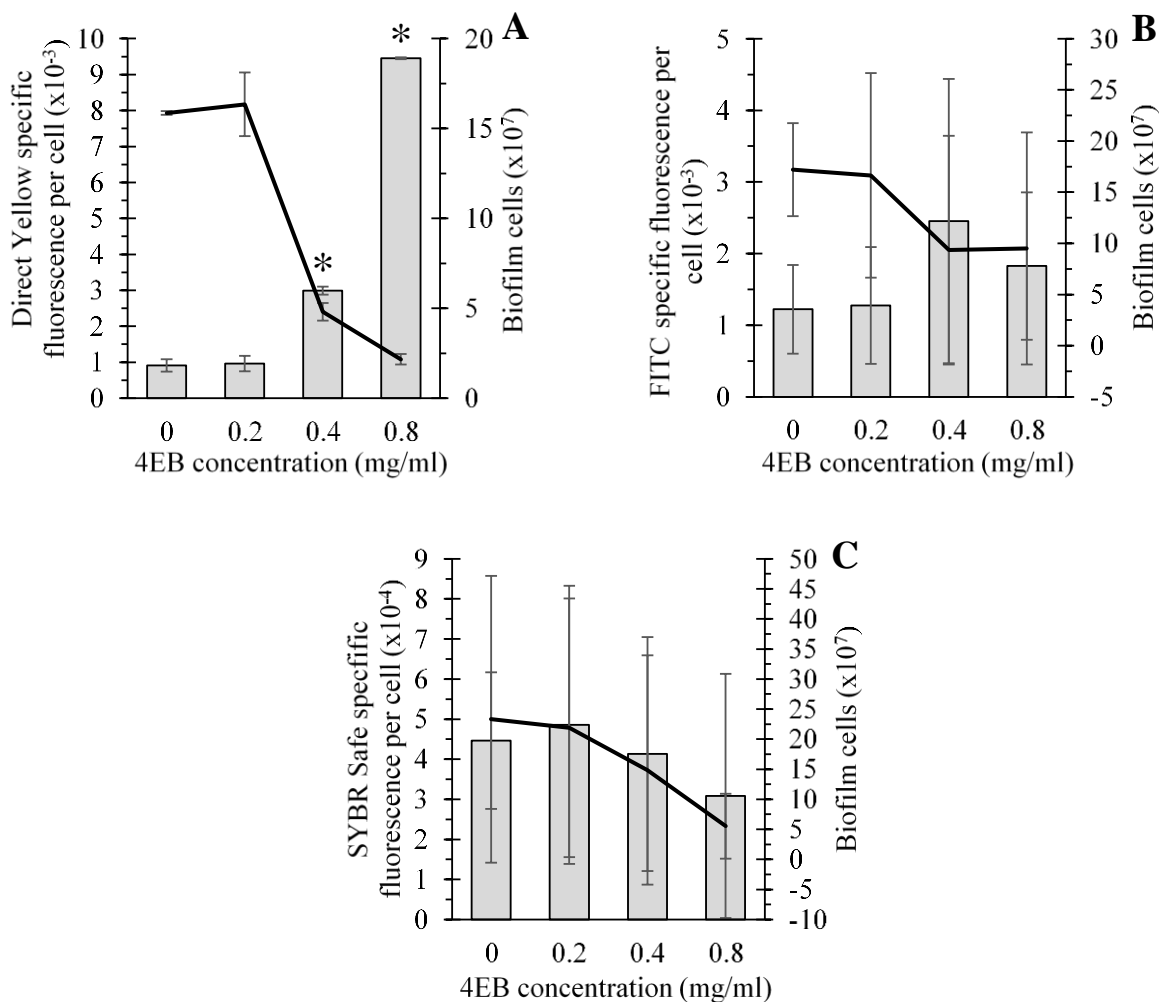


Figure 21. Effect of 4EB on *Staphylococcus aureus* extracellular polysaccharide, protein and DNA production.

Biofilm-associated polysaccharide (A), protein (B) and extracellular DNA (C) production was quantified using fluorescent dyes (grey bars). The corresponding biofilm cell counts are presented on the secondary y-axis (black line). Untreated samples served as negative controls. Error bars indicate standard deviation. ANOVA tests were performed; asterisks (*) indicated a significant difference ($p < 0.05$) in polysaccharide production between the treated samples and untreated control.

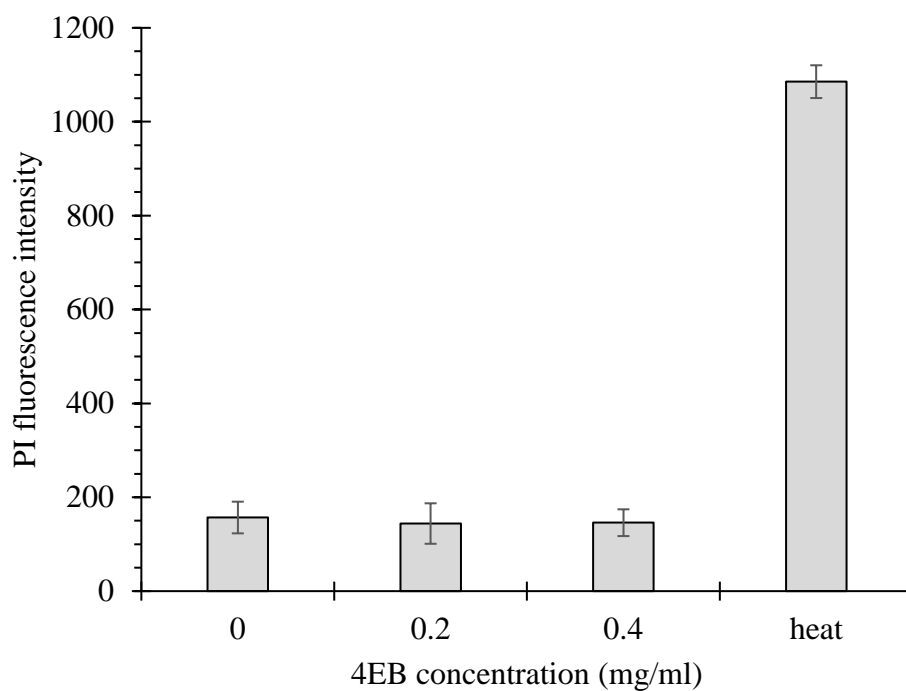


Figure 22. Effects of 4-ethoxybenzoic acid on Staphylococcus aureus membrane integrity. Bars represent propidium iodide fluorescence intensity which is an indicator of membrane permeability. The untreated and heat treated samples acted as negative and positive control, respectively. Error bars indicate standard deviation.

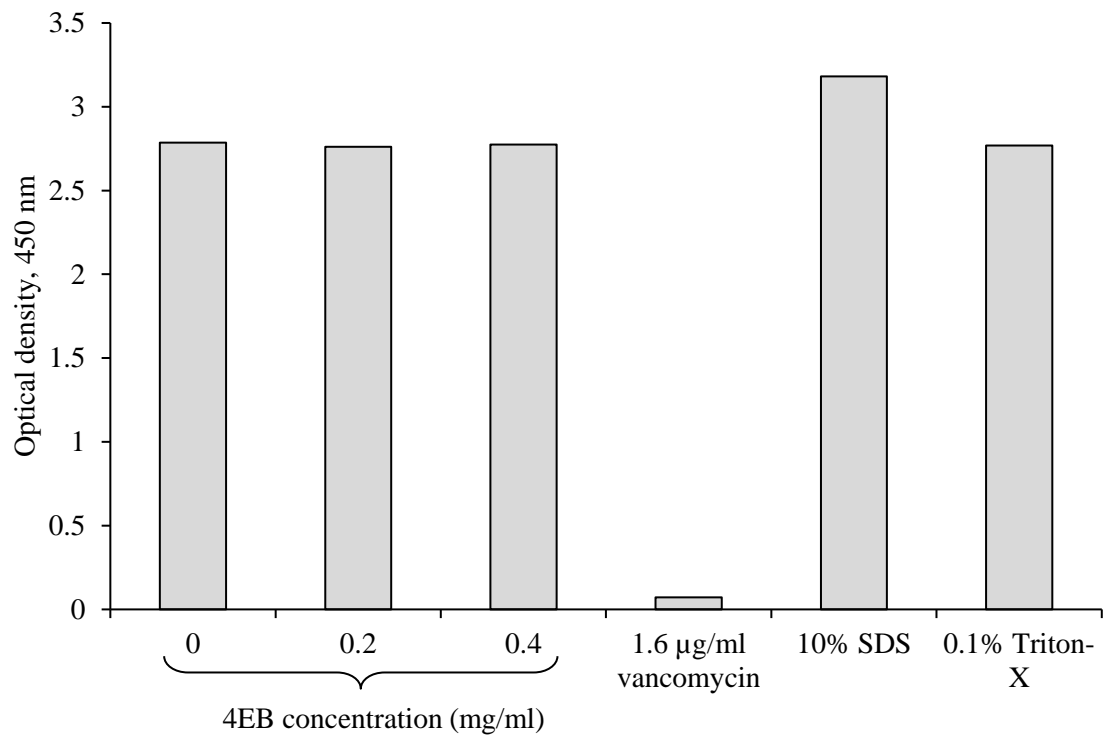


Figure 23. Effects of 4-ethoxybenzoic acid on Staphylococcus aureus hemolytic activity. Bars represent the presence of hemolytic activity on rabbit red blood cells. The untreated, 10% SDS treated and 0.1% TritonX-100 treated samples acted as negative controls and 1.6 µg/ml vancomycin was used as a the positive control.

5 CONCLUSIONS

Plants have been found to be a source of a variety of antimicrobial and anti-biofilm compounds (69, 70, 168, 169); *Rhamnus prinoides* is among these ethnopharmacologically relevant plant species. In this project, we identified and characterized the antimicrobial and anti-biofilm activities of gesho extracts and derived compounds on a variety of microorganisms including the opportunistic pathogens *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Candida albicans*. Gesho ethanol extracts were found to significantly inhibit mono-species biofilm formation via bactericidal, bacteriostatic and anti-pathogenic mechanisms in a species dependent manner. Additionally, GSE was found to inhibit *S. mutans* and *C. albicans* dual-species biofilm formation and to disrupt their synergistic relationship. Among these findings, the anti-pathogenic anti-biofilm activities of GLE on *S. aureus* and GSE on *C. albicans* were of great interest due to their possible applications as anti-pathogenic therapeutics. Further investigations into the chemical composition of GLE identified two compound of interest, ethyl 4-ethoxybenzoic acid (EEB) and 4-hydroxy-4-methyl pentanone (HMP); both compounds exhibited anti-pathogenic, anti-biofilm activity against *S. aureus* biofilms. Interestingly, HMP also significantly reduced biofilm formation of *P. aeruginosa*. Further investigations into this observation were not conducted, however the fact that HMP was able to inhibit biofilm formation of a resilient Gram negative bacterial species combined with the lack of antimicrobial studies on this compound warrants further investigations into its use as an anti-biofilm agent.

The anti-pathogenic, anti-biofilm activity of EEB on *S. aureus* led to a structure-activity analysis of structurally similar phenolic compounds. This analysis identified 4-ethoxybenzoic acid (4EB), methyl gallate and methyl paraben as three compounds that inhibited *S. aureus* biofilm formation in an anti-pathogenic manner; 4EB also exhibited synergism with vancomycin

significantly decreasing the number of biofilm-dwelling cells when compared to treatments with vancomycin alone. Investigations into the phenotypic effects of 4EB on *S. aureus* found that 4EB negatively impacted cell hydrophobicity and enhanced relative extracellular polysaccharide production. Taken together, this data suggested that 4EB may have future applications in topical antiseptic or surface disinfectant. Transcriptomic and mutagenesis experiments along with *in vitro* and *in vivo* toxicology studies will go a long way to achieving this goal. Additionally, the impacts of 4EB on *S. aureus* have led to current investigations into the effects of 4EB on biofilm formation by other bacterial and fungal species; preliminary data from these experiments have been quite promising and point to even more directions that this project can take.

The proliferation of antimicrobial resistance is a serious threat to human health and highlights the need for research on novel therapeutics such as anti-pathogenic compounds. Traditional medicine and phototherapeutics have been and continue to be a great source of antimicrobial agents as plants have evolved to produce a variety of compounds that prevent microbial colonization and discourage herbivory (170). Our work with *Rhamnus prinoides* extracts and derived compounds expands our repertoire of plant-based therapeutics and provides novel insights into applications of gesho. There are many directions that this project can be take going forward for both gesho extracts and gesho-derived compounds. Someday the fruits of the project may positively impact the lives of people around the world.

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APPENDICES

Appendix A

Table 10. PCR primers for *S. mutans* and *C. albicans* strain identification

Species	Primer name	Primer sequence	Source
<i>S. mutans</i>	8UA fwd	5'-AGAGTTTGATCCTGGCTCAG-3'	(Fujiwara <i>et al.</i> , 2001)
	1540R rvs	5'-AAGGAGGTGATCCAGCC-3'	(Fujiwara <i>et al.</i> , 2001)
	16S mid seq fwd	5'-AGATACCCTGGTAGTCCACG-3'	(Campbell <i>et al.</i> 2020)
	16S mid seq rvs	5'-CGTGGACTACCAGGGTATCT-3'	(Campbell <i>et al.</i> 2020)
	TF9911 fwd	5'-GAAGTCGTAACAAGGTAGCCGT-3'	(Fujiwara <i>et al.</i> , 2001)
	TF9912 rvs	5'-TGCCAAGGCATCCACC-3'	(Fujiwara <i>et al.</i> , 2001)
<i>C. albicans</i>	18S rRNA fwd	5'-TATCTGGTTGATCCTGCCAG-3'	(Campbell <i>et al.</i> 2020)
	18S rRNA rvs	5'-TCGATAGTCCCTCTAAGAAGTG-3'	(Teymuri <i>et al.</i> , 2015)
	ITS1 rvs	5'-CCGCAGGTTACCTACGGA-3'	(McCullough, Clemons & Stevens, 1999)
	ITS4 rvs	5'-TCCTCCGCTTATTGATATGC-3'	

>*S. mutans*_16S rRNA and 16S-23S intergenic spacer contig sequence (Genbank accession#: MT318140)

TTGCCAAGGGCACCACCGTGCGCCCTTATTAACCTTAACCTTATTTCTCGTTTCTCTGGCTTTTC
 AGCGTCTCGGTTTCTTTCTTGTTCCTATAGCTGCGCTATAGGGCTTTTACTAGCTATTCAAT
 TGCAATGAACCATCTCTAGGATACTTATCATATCCTAAGTGGATTTTATAGACTTCCTTGTC
 TTAAACAAGATATGAAGTTGAACTCCAGACTGACTTCTTAGAAAAATAGATCATCTTCTACA
 GAAGTATTCGCAAGCGAACCGTCTGTTAGTATCCTGTTTTAATGGAGCCTAGCGGGATCGAA
 CCGCTGACCTCCTGCGTGCAAAGCAGGCGCTCTCCAGCTGAGCTAAGGCCCCACTTACCCT
 CTCAAACCTAAACAAGAAGTTCCCCTAACGTGCTTTGCGTTTTTCCCTTAGAAAGGAGGTGAT
 CCAGCCGCACCTTCCGATACGGCTACCTTGTACGACTTCACCCCAATCATCCATCCCACCTT
 AGGCGGCTGGCCCCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTG
 ACGGGCGGTGTGTACAAGGCCCGGAACGTATTCACCGCGCGTGCTGATCCGCGATTACTA
 GCGATTCCGACTTCATGGAGGCGAGTTGCAGCCTCCAATCCGAAGTGAAGATCGGCTTTCAGA
 GATTAGCTTGCCGTCACCGGCTCGAACTCGTTGTACCGACCATTGTAGCACGTGTGTAGCC
 CAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCTCCGGTTTATTACCGGCA
 GTCTCGCTAGAGTGCCCAACTTAATGATGGCAACTAACAATAAGGGTTGCGCTCGTTGCGGG
 ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCTCCGATGTA
 CCGAAGTAACTTCCCTATCTCTAAGAATAGCATCGGGATGTCAAGACCTGGTAAGGTTCTTCG
 CGTTGCTTTCGAATTAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCCTTTGAGT
 TTCAACCTTGCGGTTCGTACTCCCCAGGCGGAGTGCTTATTGCGTTAGCTCCGGCACTAAGCC
 CCGGAAAGGGCCTAACACCTAGCACTCATCGTTTACGGCGTGACTACCAGGGTATCTAATC
 CTGTTTCGCTACCCACGCTTTCGAGCCTCAGCGTCAGTGACAGACCAGAGAGCCGCTTTCGCC
 ACTGGTGTTCCTCCATATATCTACGCATTTACCGCTACACATGGAATTCCACTCTCCCCTTC
 TGCCTCAAGTCAGACAGTTTCCAGAGCACACTATGGTTGAGCCATAGCCTTTTACTCCAGA
 CTTTCCTGACCGCCTGCGCTCCCTTTACGCCAATAAATCCGGACAACGCTCGGGACCTACG
 TATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGCTACCGTCACTGTGTG
 AACTTTCCACTCTCACACACGTTCTTGACTTACAACAGAGCTTTACGATCCGAAAACCTTCTT
 CACTCACGCGGCGTTGCTCGGTCAGACTTTCGTCCATTGCCGAAGATTCCCTACTGCTGCCTC
 CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCTCTCAGGTCGGCTAT
 GTATCGTCGCCTTGGTAAGCTCTTACCTTACCAACTAGCTAATAACAACGCAGGTCCATCTACT
 AGTGATGCGCTTGCATCTTTCAATCAATTATCATGCAATAATTAATATTATGCGGTATTAGCT
 ATCGTTTCCAATAGTTATCCCCCGCTAATAGGCAGGTTACCTACGCGTTACTCACCCGTTCCG
 GACTCAAGAAAACACGGTGTGCAAGCACAGTGTGTTCTCTTTCGCTCCCACTTGCATGTATTA
 GGCACGCCGCCAGCGTTCGTCCTGAGC

Figure 24. Streptococcus mutans 16S rRNA and 16S-23S intergenic spacer gene sequence.
 Segments of the *S. mutans* 16S rRNA and the 16S-23S intergenic region were amplified and sequenced using the 8UA fwd, MUT-R rvs, 16S mid seq fwd, 16S mid seq rvs, TF9911F and TF9912R primers. Sequences were then aligned using the analysis software DNA Base Assembler v5.15.0 to assemble a single contig.

>*C. albicans*_18S rRNA-ITS4 contig sequence (Genbank accession#: MT166273)

TTGAGGTCAAGTTTGAAGATATACGTGGTGGACGTTACCGCCGCAAGCAATGTTTTTGGTTA
 GACCTAAGCCATTGTCAAAGCGATCCCGCCTTACCACTACCGTCTTTCAAGCAAACCCAAGT
 CGTATTGCTCAACACCAAACCCAGCGGTTTGAGGGAGAAACGACGCTCAAACAGGCATGCC
 CTCCGGAATACCAGAGGGCGCAATGTGCGTTCAAAGATTTCGATGATTCACGAATATCTGCAA
 TTCATATTACGTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAACCAAGAGATCCGTTGT
 TGAAAGTTTTGACTATTAGTAATAATCTGGTGTGACAAGTTGATAAAAAATTGGTTGTAAGT
 TTAGACCTCTGGCGGCAGGCTGGGCCACCGCCAAAGCAAGTTTGTTCAAAAGAAAAACAC
 ATGTGGTGAATTAAGCAAATCAGTAATGATCCTTCCGCAGGTTACCTACGGAAACCTTGT
 TACGACTTTTACTTCTCTAAATGACCAAGTTTGACCAGCTTCTCGGTTCCAGAATGGAGTTG
 CCCCCTTTCCTAAACCAATCCGGAGGCCTCACTAAGCCATTCAATCGGTAGTAGCGACGGGC
 GGTGTGTACAAAGGGCAGGGACGTAATCAACGCAAGCTGATGACTTGCGCTTACTAGGAAT
 TCCTCGTTGAAGAGCAACAATTACAATGCTCTATCCCCAGCACGACGGAGTTTCACAAGATT
 TCCCAGACCTCTCGGCCAAGGCTTATACTCGCTGGCTCCGTCAGTGTAGCGCGCGTGC GGCC
 CAGAACGTCTAAGGGCATCACAGACCTGTTATTGCCTCAAACCTCCATCGACTTGAAATCGA
 TAGTCCCTCTAAGAAGTGACTATACCAGCAAATGCTAGCAGCACTATTTAGTAGGTTAAGGT
 CTCGTTTCGTTATCGCAATTAAGCAGACAAATCACTCCACCAACTAAGAACGGCCATGCACCA
 CCACCCACAAAATCAAGAAAGAGCTCTCAATCTGTCAATCCTTATTGTGTCTGGACCTGGTG
 AGTTTCCCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTGCCCTTCCGTCAA
 TTCCTTTAAGTTTCAGCCTTGGCACCATACTCCCCCAGAACCCAAAGACTTTGATTTCTCGT
 AAGGTGCCGATTGCGTCAATAAAAGAACAACAACCGATCCCTAGTCGGCATAGTTTATGGTT
 AAGACTACGACGGTATCTGATCATCTTCGATCCCCTAACTTTCGTTCTTGATTAATGAAAACG
 TCCTTGGTAAATGCTTTCGCAGTAGTTAGTCTTCAGTAAATCCAAGAATTTACCTCTGACAA
 CTGAATACTGATACCCCCGACCGTCCCTATTAATCATTACGATGGTCCTAGAAACCAACAAA
 ATAGAACCATAACGTCTTATTCTATTATCCATGCTAATATATTCGAGCAAAGGCCTGCTTTG
 AACACTCTAATTTTTTCAAAGTAAAAGTCCTGGTTTCGCCATAAATGGCTACCCAGAAGGAA
 AGGCTCGGCTGGGTCCAGTACGCATCAAAAAGATGGACCGGCCAGCCAAGCCCAAGGTTT
 AACTACGAGCTTTTTAACTGCAACAACCTTAAATATACGCTTTTGGAGCTGGAATTACCGCGG
 CTGCTGGCACCAGACTTGCCCTCCAATTGTTCTCTCGTTAAGGTATTTACATTGTACTCATTCC
 AATTACAAGACCCAAAAGGGCCCTGTATCGTTATTTATTGTCACTACCTCCCCGTGTCGGGA
 TTGGGTAAATTTGCGCGCCTGCTGCCTTCCCTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTC
 CGGAATCGAACCCTTATTCCCCGTTACCCGTTGAAACCATGGTAGGCCACTATCCTACCATC
 GAAAGTTGATAGGGCAGAAATTTGAATGAACCATCGCCAGCACAAAGGCCATGCGATTGCGAA
 AAGTTATTATGAATCATCAAAGAGCCCGAAGGCATTGATTTTTTATCTAATAAATACATCCC
 TTCCAAACAGTCGGGATTTTAAGCATGTATTAGCTCTAGAATTACCACGGTTATCCAAGTAG
 TAAGGTACTATCAAATAAACGATAACTGATTTAATGAGCCATTCGCAGTTTCACTGTATAAA
 TTGCTTATACTTAGACATGCATGG

Figure 25. *Candida albicans* 18S rRNA and ITS gene sequencing contig.

Segments of the *C. albicans* 18S rRNA and ITS1-4 genes were amplified and sequenced using the 18S fwd, 18S rvs, ITS1 rvs and ITS4 rvs primers. Sequences were then aligned using the analysis software DNA Base Assembler v5.15.0 to assemble a single contig.

Table 11. BLAST results for strain identification

	Description	Query Cover	E-value	Percent identity	Accession
<i>S. mutans</i>	<i>Streptococcus mutans</i> strain T8	99%	0.0	99.85%	CP044492.1
	<i>Streptococcus mutans</i> strain LAR01	99%	0.0	99.69%	CP023477.1
	<i>Streptococcus mutans</i> strain UA96	99%	0.0	99.69%	AF139600.1
	<i>Streptococcus mutans</i> strain UA140	99%	0.0	99.64%	CP044495.1
	<i>Streptococcus mutans</i> strain NN2025	99%	0.0	99.64%	AP010655.1
<i>C. albicans</i>	<i>Candida albicans</i> strain ATCC 18804	76%	0.0	99.82%	HQ876034.1
	<i>Candida albicans</i> strain SC5314-P0	100%	0.0	99.77%	CP025165.1
	<i>Candida albicans</i> strain SC5314-GTH12	100%	0.0	99.77%	CP025182.1
	<i>Candida albicans</i> strain SC5314	79%	0.0	99.75%	XR_002086442.1
	<i>Candida albicans</i> strain TIMM 1768	100%	0.0	99.77%	CP032012.1

Appendix B

Table 12. Metabolic activity of *Streptococcus mutans* and *Candida albicans* biofilm dwelling cells after exposure to ethanol and aqueous gesho extracts

Extract	Concentration (mg/mL)	<i>Streptococcus mutans</i>		<i>Candida albicans</i>		<i>S. mutans and C. albicans</i>	
		% metabolic activity ²	% biofilm inhibition	% metabolic activity ²	% biofilm inhibition	% metabolic activity ²	% biofilm inhibition
Control	0	100 ± 1.9	0	100 ± 0.9	0	100 ± 0.6	0
GLE ¹	0.2	87 ± 4	13	91 ± 2*	9	103 ± 3	0
	0.5	80 ± 6	20	88 ± 2*	12	102 ± 3	0
	1	76 ± 9	24	93 ± 1*	7	103 ± 4	0
	3	63 ± 11*	37	104 ± 1	0	107 ± 2	0
	5	59 ± 11*	41	102 ± 1	0	113 ± 2*	0
	7	55 ± 10*	45	103 ± 1	0	111 ± 5*	0
GLW ¹	0.2	94 ± 2	6	55 ± 3*	45	98 ± 2	2
	0.5	91 ± 2*	9	46 ± 3*	54	94 ± 3	6
	1	90 ± 3*	10	39 ± 2*	61	93 ± 3	7
	3	93 ± 2	7	23 ± 2*	67	87 ± 2*	13
	5	95 ± 2	5	30 ± 2*	70	84 ± 2*	16
	7	95 ± 3	5	29 ± 2*	71	85 ± 2*	15
GSE ¹	0.2	66 ± 6*	34	73 ± 3*	27	108 ± 4	0
	0.5	51 ± 7*	49	67 ± 2*	33	121 ± 7*	0
	1	37 ± 9*	63	60 ± 3*	40	131 ± 4*	0
	3	23 ± 5*	77	56 ± 2*	44	145 ± 5*	0
	5	31 ± 3*	69	53 ± 2*	47	152 ± 2*	0
	7	23 ± 4*	77	54 ± 1*	46	164 ± 6*	0
GSW ¹	0.2	95 ± 2	5	87 ± 2*	13	90 ± 3	10
	0.5	94 ± 2	6	78 ± 2*	22	99 ± 4	1
	1	96 ± 2	4	73 ± 2*	27	99 ± 3	1
	3	93 ± 2	7	62 ± 1*	38	104 ± 3	0
	5	93 ± 2	7	57 ± 1*	43	105 ± 2	0
	7	95 ± 3	5	49 ± 2*	51	109 ± 3	0

¹Leaf ethanol (GLE), leaf water (GLW), stem ethanol (GSE) and stem water (GSW) treatments

²Percent biofilm formation are the mean of extract treated samples relative to the untreated control for each species ± the standard error of the mean (SEM).

*indicate a significant difference (p < 0.05) between the treated samples