ANTI-INFECTIVE EFFECTS OF FRUIT PHYTOCHEMICAL EXTRACTS AGAINST *STREPTOCOCCUS PYOGENES*

by

Soheila Abachi

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
December 2015

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Group-A-streptococci (GAS) is the common cause of acute bacterial pharyngitis in children and adults. Streptococcal pharyngitis is initiated by successful colonization followed by establishment of biofilm. The aim of this research was to investigate the anti-infective, anti-biofilm, and anti-adhesion properties of aqueous and ethanolic extracts of ten selected fruits against *Streptococcus pyogenes* and its virulence factors. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) for the six effective fruit extracts ranged from 0.25 to 4 mg/mL and 4 to 16 mg/mL, respectively. Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradicating Concentration (MBEC) of cranberry and sumac berry extracts ranged from 1 to 2 mg/mL and 2 to 8 mg/mL, respectively. Minimum concentrations against the adhesion of GAS to uncoated and fibronectin coated substratum ranged from 0.25 to 16 mg/mL. Cranberry and sumac extracts possess notable inhibitory effects against growth, adhesion, biofilm formation and biofilm eradication of GAS.

LIST OF ABBREVIATIONS AND SYMBOLS USED

List of abbreviations and symbols used

19615	*S. pyogenes* ATCC 19615
49399	*S. pyogenes* ATCC 49399
μL	Micro liter
A	Absorbance
Ae. hydrophila	*Aeromonas hydrophila*
A. viscosus	*Actinomyces viscosus*
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
B. cereus	*Bacillus cereus*
BHI	Brain heart infusion
BSA	Bovine serum albumin
C carbohydrate	Cell wall polysaccharide of streptococci
C. albicans	*Candida albicans*
Ci. freundii	*Citrobacter freundii*
C4BP	Complement component 4b-binding protein
CD44	Cluster of differentiation 44
CD46	Cluster of differentiation 46
CDC	Center for disease control and prevention
CFU	Colony forming unit
Clinical	*S. pyogenes* clinical strain
CLSI	Clinical laboratory standards institute
CO_2	Carbon dioxide
Conc.	Concentration
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSM	Deutsche sammlung von mikroorganismen
En. aerogenes	*Entrobacter aerogenes*
E. coli	*Escherichia coli*
EPS	Exopolysaccharide
F protein	Fibronectin-binding protein
F1/Sfb1	Streptococcal fibronectin binding protein 1
Fba	Fructose-bisphosphatealdolase
FBP54	Fibronectin-binding protein 54
FDA	Food and Drug Administration

List of abbreviations and symbols used

Fn	Fibronectin
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GABHS	Group A β-hemolytic *Streptococcus*
GAS	Group A streptococci
GCBHS	Group C β-hemolytic streptococci
GMSM-K	Immortalized human oral epithelial
GPS	Global positioning system
H. alvei	*Hafnia alvei*
HaCaT	Transformed aneuploid immortal keratinocyte cell line
has	Hyaluronan synthase
HEp-2	Human epithelial type 2 cell line
HIV	Human immunodeficiency virus
HTEpiC	Human normal tonsil tissue cell line
HVR	Hypervariable region
Kl. lactis	*Kluyveromyces lactis*
Kl. marxianus	*Kluyveromyces marxianus*
K. pneumoniae	*Klebsiell pneumoniae*
KB	KERATIN-forming tumor cell line hela
kDa	Kilodalton
L. monocytogenes	*Listeria monocytogenes*
Lac. rhamnosus	*Lactobacillus rhamnosus*
Lbp	Laminin-binding protein
LTA	Lipoteichoic acid
M protein	emm protein
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
mg	MilliGram
Mga	Multigene activator
mL	Milliliter
MRSA	Clinical methicillin-resistant *St. aureus* (MRSA)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng	Nano Gram
nm	Nano meter
NoV P	Norovirus particles
OD	Optical density
P	Probability or *P-value*

List of abbreviations and symbols used

P. aeruginosa	*Pseudomonas aeruginosa*
Pr. mirabilis	*Proteus mirabilis*
Pi. pastoris	*Pichia pastoris*
Pr. vulgaris	*Proteus vulgaris*
PAC	Proanthocyanidin
PBP	Penicillin binding proteins
PBS	Phosphate-buffered saline
Pep M5	Peptic fragment of the group A streptococcal serotype 5
PFBP	*S. pyogenes* fibronectin-binding protein
PG	Peptidoglycan
qPCR	Quantitative real-time polymerase chain reaction
r	Pearson's correlation coefficient
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sa. enteritidis	*Salmonella enteritidis*
Sa. agona	*Salmonella agona*
St. aureus	*Staphylococcus aureus*
Sac. cerevisiae	*Saccharomyces cerevisiae*
S. criceti	*Streptococcus criceti*
St. epidermidis	*Staphylococcus epidermidis*
Sh. flexneri	*Shigella flexneri*
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*
S. sanguinis	*Streptococcus sanguinis*
St. saprophyticus	*Staphylococcus saprophyticus*
S. sobrinus	*Streptococcus sobrinus*
S. typhimurium	*Salmonella typhimurium*
Scl	Streptococcal collagen-like protein
SEDS	Shape elongation division and sporulation proteins
Sfbx	*Streptococcus pyogenes* fibronectin-binding protein
SOF	Serum opacity factor
SpeB	Streptococcal pyrogenic exotoxin B
T. cutaneu	*Trichosporon cutaneu*
TA	Teichoic acid
TCA	Tricarboxylic acid
USA	United States of America
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
Y. enterocolitica	*Yersinia enterocolitica*

List of abbreviations and symbols used

μg	MicroGram
μM	Micro molar

ACKNOWLEDGEMENT(s)

Here, I would like to thank Dr. Vasantha Rupasinghe, my supervisor, for his guidance, and patience throughout my graduate studies. I also would like to express my sincere thanks to the committee members, Dr. B. Rathgeber and Dr. S. Lee for their continuous support. Individuals who have assisted me in collecting the plant material for my study include Mr. Jeffrey Morton, Mr. Kelly Cheverie, Dark Tickle Company, Prince Edward Island Preserve Company, Forbes Wild Foods, Village of Bible Hill and others. Thank you all for lending a hand.

I am also grateful for the support and companionship I have received from my friends, Indu Parmar, Qingya Zou, Mahboobeh Zamani Babgohari, Sabrina Macé, Satvir Sekhon-Loodu, Jennifer Spence, and other group members.I am grateful to Dr. Hossein Elahi Ghomshei for his most invaluable spiritual teachings and conveying Shams Tabrizi's messages making me a stronger person. Foremost, I extend my sincere appreciation to most important people in my life, my father, my mother, Soheil and Farshad for the indefinite love they have spread over my years.

A big appreciation goes to government of Nova Scotia for awarding me the Nova Scotia Graduate Entrance Scholarship and Graduate Research Training Initiative Scholarship. My gratitude also goes to Robert P. Longley's family for granting me the Graduate Scholarship of year 2014 and to University of Ottawa for the 2015 NHPRS of Canada Travel award. This project was made possible by the funds received from NSERC CRD and Island Abbey Foods.

CHAPTER 1 INTRODUCTION

The human body carries a diverse population of microbes (excluding viruses and parasites) as normal microbiota soon after birth until death (*1*). In healthy individuals, normal microbiota on human skin, respiratory and gastrointestinal tract, nails, eyes, and genitalia are harmless, however when environmental elements permit they often cause infection in compromised hosts (*1*). Streptococci species form a significant portion of the normal bacterial flora of humans and animals and are found in the oral cavity and nasopharynx (*2*).

Opportunistic and pathogenic group A streptococci (GAS) cause infections that range from none life-threatening conditions such as mild skin infection or pharyngitis to life-threatening and severe conditions such as necrotizing fasciitis, rheumatic fever and highly lethal Streptococcal toxic shock syndrome (*3*). Streptococci have very specific virulence factors, enabling them to cause such diverse infections (*4-6*).Pharyngitis is the most common form of GAS infections.

Many microbes, bacteria or virus, induce pharyngitis. Briefly, rhinovirus accounts for approximately 20% of the viral cases followed by, coronavirus 5%, adenovirus 5%, herpes simplex virus 4%, parainfluenza virus 2%, and influenza virus 2% (*7*). Among bacterial causes, group A *Streptococcus* is responsible for 15-30%, and group C β-hemolytic streptococci for about 5% of the cases (*8*).Accurate identification of the acute pharyngitis causative bacteria is especially important when it comes to its antimicrobial therapy.

Streptococcus pyogenes(group A β-hemolytic *Streptococcus* (GABHS)) is the common cause of acute bacterial pharyngitis also known as strep throat or sore throat.

Recorded cases of GAS bacterial pharyngitis are 15-36% in children and 5-15% in adults (*9, 10*). School aged children in seasons of spring and winter harbor the bacterium up to 20% whereas this rate is much lower in adults (*11*). On a global scale, over 616 million new cases of GAS pharyngitis occur every year (*12*). Not only is this bacterium responsible for hundred millions of pharyngitis cases but also approximately 10,000 to 15,000 cases of invasive GAS cases are reported annually in the United States, accounting for 10% to 13% mortality rate (http://www.cdc.gov/ncidod/dbmd/abcs/forinvasiveandnoninvasivediseaseincidence) (*13*). Acute infections can lead to rheumatic fever and post-streptococcal glomerulonephritis (kidney inflammation), which distress children worldwide with disability and death, if antibiotic treatment fails or if the disease is left unattended (*14, 15*). Rheumatic fever and rheumatic heart disease are known to be the leading causes of cardiovascular death during the first five decades of life in underdeveloped countries mainly concerning children (*16*). Tissues contributing to the GAS nosocomial infections are upper respiratory tract, skin, vaginal and anal area, although latter cases are rare (*17*).

GAS has several surface proteins and produces numerous extracellular products that facilitate permeation and successive evasion of the host's immune system (*16*). Streptococcal pharyngitis results from the proliferation of GAS in the pharynx (*16*). Virulence associated factors enable *S. pyogenes* to attach to host tissues, elude the immune response, and spread by penetrating the host tissue layers followed by colonization (*4-6, 18, 19*).

There are several important steps for initiation of GAS infectious diseases. Bacteria's capacity to adhere to host tissues and then competing with the normal

microbial flora of nasopharynx plays an important role. After successful attachment, bacteria establish interaction with salivary glycoproteins, extracellular matrix, serum components, host cells and other microbes and then assemble in cell aggregates. Bacteria then begin to multiplying and forming microcolonies. Lastly, microcolonies differentiate into exopolysaccharide (EPS)-encased communities which are called mature biofilm (*20, 21*). Biofilms' role in GAS pathogenesis has recently been proposed and experimentally supported in a number of recent publications (*8, 21, 22*).In addition to biofilm, M protein is also responsible for GAS virulence and complications (*16, 23*). Bacterial activities within biofilms are regulated by the occurrence of quorum sensing, and according to this communication bacteria release chemical signals leading to the expression of virulence genes. Proliferation of GAS in the pharynx leads to the invasion of the host tissues. It has been reported that streptococci adhere in two steps: first weak reversible adhesion probably mediated by hydrophobic interactions using lipoteichoic acid (LTA), second firm irreversible adhesion step mediated by composite multivalent interactions (*24-29*).

It has been suggested that EPS, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community (*30*).CDC report says 65% of human bacterial infections involve biofilms and treatment of these biofilm-associated nosocomial infections costs more than $1 billion annually (*4, 30*).

Humans as the main reservoir of GAS has certain defense mechanism to prevent the colonization and adhesion of the bacteria including but not limited to (i); secretion of fatty acids by skin, (ii) entrapment of bacteria by muco-ciliary blanket of the upper respiratory tract, and (iii) induction of aggregation and reducing adhesion by salivary mucins (*17*). However treatments are required to prevent the infection. Penicillin,

amoxicillin, first-generation cephalosporins and erythromycin are the recommended antibiotic treatment of streptococcal sore throat however, erythromycin resistant strains have been repeatedly reported (*31-33*).To prevent exposing the patient to inappropriate therapy and to avoid formation of antimicrobial resistant strains health experts are responsible for the true identification of the strain. According to the researchers of internal medicine at the University of Missouri, "current GAS antibiotic treatments interfere with critical pathogenic biological processes to kill or stop its growth leading to endurance of stronger strains of harmful bacteria and prosper of resistant bacteria" (*34*).

Generally, use and misuse of antibiotics not only put the subject at risk but also influence the entire population. Antimicrobial resistance is compromising the treatment of invasive severe infections including severe streptococcal infections (*4*). Bacteria resistant to multiple types/classes of antibiotics are of most serious debilitating health threats. This threat becomes significant in vulnerable patients (e.g., individuals undergoing chemotherapy, dialysis and organ transplants) due to infection-related complications. This puts healthcare providers in the position to use antibiotics that may be more toxic to the patient, and frequently more expensive, leading to an increased risk of long-term disability with lower survival rates (*4*).

Bacteria employ some basic mechanisms to resist an antimicrobial agent. Resistant bacterium (i); alters the drug receptor by making the target insensitive to inhibitor (antibiotic), (ii); decreases the physiologic importance of target molecule to the bacteria's pathogenicity, (iii); produces new enzyme molecule that could replace the inhibited target, (iv);decreases the amount of drug that reaches the receptor by altering

drug's entry or increasing its removal by efflux pumps, and (v);destroys or inactivates antibiotic drug so it loses its ability to bind to targets (*23*).

Some penicillin-resistant streptococci have developed resistance by altering the penicillin-binding proteins. In Gram-positive species, β-lactamases enzymes (penicillinase) that are primarily exoenzymes and are excreted into the milieu around the bacteria, mediate this mechanism. GAS is not yet resistant to penicillin but some Gram-positive bacteria have grown resistant to penicillin and ampicillin through β-lactam hydrolysis (*2*). Beta-lactamases similarly attack other β-lactam compounds such as cephalosporins (*35*).

Streptococci species have become resistant to macrolides such as clindamycin and erythromycin through altering 23S RNA and as emphasized earlier, U.S. center for disease control and prevention (CDC) has prioritized erythromycin-resistant GAS as concerning threat in 2013 report (*4*). Macrolide resistance among GABHS clinical isolates in the United States is on rise, possibly because of azithromycin overuse (*36*). This climb in certain areas of the United States and Canada reaches 8-9 percent (*37*). Most guidelines recommend reserving erythromycin for patients who are allergic to penicillin (*38*).

According to Dr. Frieden, director CDC, antimicrobial resistance is a serious health threat in the 21st century. Infections caused by resistant bacteria are now on the rise and their resistance to in-use antibiotics is worrisome (*4*). The decrease in the rate of pathogen's susceptibility to available antibiotics has made it much more difficult to combat the infectious diseases.

All these antibacterial mechanisms exert strong selective pressure that favors emergence of antibiotic-resistant strains. An alternative strategy is to suppress the pathogen's virulence without inhibiting its growth, thus downgrading the strong selection for resistance followed by practice of traditional bacteriostatic or bactericidal plant based/derived antimicrobials (*34*).

Plants produce diverse secondary metabolites, most of which are phenols or their oxygen-substituted derivatives such as tannins that could be the raw materials for future drugs. These agents appear to have structures and modes of action that are distinct from antibiotics, thereby suggesting that cross-resistance with agents already in use may be minimal (*39*).

In general, fruits and especially berries are a good source of phytochemicals, particularly anthocyanins, proanthocyanidins, and terpenoids. These phytochemicals are considered anti-inflammatory, anti-carcinogenic, and antioxidant agents as well as antimicrobials (*40, 41*).Various medicinal plants have been tested for their antimicrobial activity and all have proven that phytochemicals exhibit significant antibacterial activity against *Streptococcus* species particularly polyphenols. Natural antimicrobials like their synthetic counterparts (antibiotics) target different molecules and processes to inhibit the colonization, and viability of the bacteria. Phytochemicals inactivate bacterial toxins, modulate the molecules and processes pre-requisite for bacteria's metabolic pathways, and or reduce the rate of protein synthesis (*41*).It is worth noting that natural antimicrobial products do not need to be bactericidal to be able to suppress such processes and activities. It is plausible that a compound is likely to be an effective bacterial growth inhibitor if it can deteriorate the cytoplasmic pH, increase the

permeability of plasma membrane, prevent extracellular and intracellular microbial enzyme production, interrupt bacterial metabolic pathways, or disrupt adherence and biofilm formation (*41*). As observed, there is a fair amount of scientific evidence that phytochemicals exert significant anti-streptococcal effects and apart from their bactericidal effects, their main bacteriostatic strategy is their anti-adhesiveness attribute (*42-46*). More research is required to elucidate the antibacterial activities of berries against GAS.

Thesis objectives

In this study, it was hypothesized that phytochemicals-rich extracts of selected wild and cultivated berries/fruits of Atlantic Canada will suppress or inhibit the growth of *S. pyogenes* in a specific approach of adherence and biofilm formation inhibition or eradication under experimental conditions. All the selected berries had been studied elsewhere recently for their antimicrobial effects against various Gram-positive, Gram-negative, and fungi except *S. pyogenes* leading us to the hypothesis that phytochemicals-rich extracts of Atlantic Canada berries possess inhibitory effects against the growth of *S. pyogenes*. The wild berries under study grow abundantly in the region and they all have the potential to be cultivated and produced commercially.

Fruits of Crowberry *(Empetrum nigrum),*Blackcurrant*(Ribes nigrum),*Sumac berry*(Rhus typhina),*Squash berry *(Viburnum edule),*Cranberry *(Vaccinium macrocarpon),*Blueberry *(Vaccinium corymbosum),* Haskap*(Lonicera caerulea),* Partridgeberry*(Vaccinium vitis-idaea),* Buckthorn *(Rhamnus cathartica),*and Mountain ash*(Sorbus decora)* contain substantial amount of secondary metabolites, therefore suggesting their anti-infective properties against GAS. Different strains of GAS with

relatively dissimilar virulence scale were included in the assays to make a conclusive statement about the efficacy of berries.

CHAPTER 2 LITERATURE REVIEW

2.1. Streptococcus pyogenes

Group A *Streptococcus* (GAS) or *Streptococcus pyogenes* is of the kingdom of bacteria belonging to the Firmicutes phylum (*23*). GAS is classified as a Bacilli falling under the order of Lactobacillales (*23*). This bacterium is one of the species of genus *Streptococcus* within the family of *Streptococcaceae* (*23*). GAS displays group A antigen in the cell wall, is non-motile and non-sporulating (*47*). GAS is a Gram-positive bacterium, 0.6-1.0 micrometer in diameter, spherical to ovoid in shape, nutritionally fastidious, with fermentative metabolism (*48*).

GAS ferment sugars, mainly glucose as carbon source, to lactic acid for growth and energy production but could also grow abundantly in a medium with no protein nor peptide (*49*). The bacterium is a facultative anaerobe therefore, it can be cultured and grown at 37°C in either ambient air or in 5–10% CO_2 (*50*).GAS lacks the necessary respiratory chains for a functional tricarboxylic acid (TCA) cycle and oxidative-cytochromes for electron transport(*50*). To make up for this deficiency GAS expresses a variety of primary transport systems, (i); synthesizing a cytochrome-like respiratory chain, or(ii); formation of ATP from ADP and inorganic phosphate by coupled reaction of NADH oxidation and phosphorylation reaction (*51-53*).

GAS divides in one parallel plane (*54*). Enzymes that are involved in the synthesis of peptidoglycan and the peptide bridge cross linkages between them are called penicillin binding proteins (PBP)and accordingly this would determine the susceptibility of the bacterium to penicillin (*55*). Unlike rod shaped bacteria like *Escherichia coli* that carry 12 PBPs, cocci have about 4-7 of them *(55).*The shape, elongation, division and

sporulation (SEDS) proteins as described by Zupan et al. are as necessary as PBPs for the cell wall synthesis of a cocci (*55*).The cell wall of streptococci is among the most studied bacterial cell walls (*55*). Streptococci growth starts at the old pole where new pole is synthesized (*55*). Doubling time of GAS is 36 minutes (*23*). Peptidoglycan synthesis usually starts from middle of the cell and new pole is made before the previous pole's cell wall is completed resulting in long chains of bacteria that had grown out of a single unit (*55*).

GAS produces extracellular proteins that have been shown to give rise to the remarkable virulence of the organism, triggering a nonspecific host immunological response (*56*). Significant genetic diversity among different strains allow the bacterium to cause variety of diseases at several tissue sites (*57*). Specific virulence factors assist *S. pyogenes* to attach to the host tissue, escape phagocytosis, and spread by infiltrating the host epithelial layers followed by colonizing (*4-6, 19, 58*). Complete genome of *S. pyogenes* ATCC 19615, M1 strain, and partial genomes of M3, M5, M28, M44, M49, and M83 are available (*59-62*). M1 serotype is the most widespread serotype found in severe invasive infections (*56*). M1 serotype is mainly involved in post-streptococcal sequelae namely rheumatic heart disease as well as glomerulonephritis (*15*). According to another study more than 70% of GAS-caused severe invasive infection relate to serotypes M1 and M3(*63*).

The genome study of GAS showed that 83% of its open reading frames could have identifiable homologue from other bacterial species(*56*). The majority of protein similarity in the currently available genome databases was specifically related to *B. subtilis, Lactococcus lactis*, and various streptococci (*56*).According to protein–coding

sequences, the metabolic pathways identified in the genome were (i); glycolytic pathway, (ii);fatty acid synthesis, (iii);nucleotide synthesis and transport, and (iv); carbohydrate transport and metabolism. Tricarboxylic acid cycle pathway and its complementary electron transport was not present however as said earlier, bacteria makes up for this deficiency (*56*).

2.1.1. Pharyngitis, causes, and pathophysiology of streptococcal sore throat

Any painful condition in the oropharynx is called sore throat (*11*). Over the years, this term has become associated with pharyngitis or tonsillitis. Secondary to infection with hemolytic streptococci, tonsillitis could occur in about half of the subjects (*11*). Sore throat as one of most common illnesses worldwide can be caused by viral or bacterial infections (*7*). Taken all together upper respiratory tract infections account for 200 visit to physician per 1000 population in USA yearly (*7*). Day-care attending children are at higher risk and they may have 8-9 episodes of respiratory tract infections per year of which half are associated with pharyngitis (*11*).Even though GAS is the common cause of bacterial pharyngitis, but other microorganisms namely *Actinomyces* spp , *Arcanobacterium haemolyticum*, *Bacteroides* spp., *Borrelia* spp., *Bordetella pertussis*, *Chlamydophila pneumoniae*, *Chlamydia trachomatis*, *Corynebacterium diphtheria*, *Corynebacterium pyogenes* could cause acute pharyngitis (*7*).

Signs of GAS pharyngitis overlap the symptoms of non-streptococcal pharyngitis meaning that proper diagnosis of the disease on the basis of clinical presentation is often impossible (*9*).Clinical presentations suggestive of GAS pharyngitis in children aged 5–15 years are: sudden onset of sore throat, tonsillopharyngeal inflammation, fever, vomiting, headache, nausea, abdominal pain, patchy tonsillopharyngeal exudates, anterior

cervical adenitis (tender nodes), palatal petechiae, winter and early spring presentation, and scarlatiniform rash, however, for true identification of the condition microbiological tested should be performed (*64, 65*).

2.1.2. Surface characteristics of GAS

A general rule is that the surface of the bacteria is an important factor in host–pathogen interactions. Many virulence associated genes identified in the genome have been linked to cell surface as well as to the extracellular proteins (*56*). Among all distributed virulence genes (including mitogenic exotoxin Z (a novel acidic superantigenic toxin), mitogenic factor (heat-stable nuclease), Mga regulon (transcriptional activator) and etcetera)the *emm* region may be called the pathogenicity island as described by Suvorov et al. although this has not been experimentally examined (*63*).

In addition to the capsule, several factors embedded in the cell wall facilitate attachment of the bacteria to various host cells, including M protein, lipoteichoic acid, and fibronectin-binding protein(s) (*66*). M protein not only helps bacteria to attach to the host tissue but also inhibits opsonization by an alternative complement pathway, binding to host complement regulators and to fibrinogen (*15*). Pilli of *S. pyogenes* (fuzzy appearance outside the cells) promotes pharyngeal cell adhesion, aggregation to human cells and biofilm formation (*47, 67, 68*). The virulence factors ruled by transcriptional regulators are controlled in a way to respond to intracellular, extracellular and growth related signals (*63*).

2.1.3. Adhesion

Colonization will be initiated after successful entry and adhesion of the pathogen into the host and its mucosal surfaces of the pharynx cavity, if the physiological conditions allow, consequently producing more than one class/type of adhesins by the surviving streptococci (*69*). Moreover, successful adhesion depends on hydrophobic interactions (*70*). Most bacterial pathogens, including streptococci, have long filamentous structures known as pilli or fimbriae, involved in the initial adhesion of bacteria to host tissues, and in bacteria–bacteria interactions facilitating DNA interchange between organisms, and in formation of biofilm (*67*). Certain proteins such as extra cytoplasmic proteins, including surface proteins, have to be synthesized prior to the formation of a biofilm (*71*). Many clinical isolates of *S. pyogenes* have been reported as hydrophobic while their avirulent counterpart strains lack that feature (*72, 73*). According to Hasty et al., there are multiple mechanisms of bacterial adhesion that is largely dependent on the type of target substratum and the strain (*69*). The two-step model proposed back in 1992 by Hasty at al. consist of primary weak reversible adherence that could be inhibited by multiple washings and next is the firm irreversible attachment that could resist washes (*69*). Latter is mediated by receptor-specific adhesins which differs among niches in the upper respiratory tract (*69*).In Gram-positive bacteria, hydrophobic components can be found (i); covalently bound to cell wall, such as streptococcal M and F proteins, and or (ii); in the cytoplasmic membrane (e.g., LTA of GAS). The bacterial cells in the biofilms are adhered to one another by an extracellular matrix made of DNA, proteins and polysaccharides (*74*).

Adhesion of *S. pyogenes* to various host cells is facilitated by capsule and several other elements of cell wall including: M protein, lipoteichoic acid and F protein (*66*). A

recent study has demonstrated that *S. pyogenes* pilus promotes pharyngeal cell adhesion and biofilm formation (*67*). Altering surface hydrophobicity by sub-minimum inhibitory concentration of penicillin and rifampin reduces the adhesion of *S. pyogenes* to epithelial cells suggesting that surface-associated LTA will determine the surface hydrophobicity content of GAS, which consequently affects the bacterium's interaction with mammalian host cells (*75*-*77*).

2.1.3.1. Lipoteichoic acid

In addition to M protein, some believe that LTA assists in adhesion of the organism to the fibronectin of host epithelial cells. Wicken and Knox introduced LTA as a new class of bacterial antigen back in 1975 (*78*). Specific to Gram-positive bacteria, LTA is simply a teichoic acid attached to a lipid (a polymer phosphodiester-linked glycerol phosphate covalently bound to glycerophosphoryldiglucosyldiglyceride) (*79*). LTA's lipid moiety embeds in membrane while its charged backbone, glycerol phosphate, is in cell wall (*78*). Negatively charged backbone of glycerol phosphate and the hydrophobic lipid give this molecule its amphipathic characteristic (*79*). Previous studies have tested the purified M protein, peptidoglycan, LTA and C carbohydrate for their ability to inhibit the adhesion of GAS to epithelial cells and results suggest LTA significantly possess the inhibitory activity (*80*).

2.1.3.2. M-protein

To establish a relationship between different strains of GAS and the infection outbreaks worldwide, M serotyping is invaluable (*13*). M protein is one of the most well studied virulence factors of the bacterium. To date, 124 serotypes have been proposed since Dr. Rebecca Lancefield introduced the method to classify GAS strains based on a

variable heat stable surface protein, called M protein (*13*). It is worthy of note that new methods of classification are based on (i) analyses of 5' *emm* gene sequences, (ii) expression of serum opacity factor, and (iii) T and R proteins (*17*).The M proteins share identity to some extent (*81*).Complete and partial sequence studies of M5, M6 and M24 have discovered dissimilarity among serotypes however homology also was evident (*81*). Slight deviation among the same serotype, namely M5 isolated from different strains have been observed (*81*). The difference and the similarity is caused by the residue N-terminal hypervariable region (*82*). Usually M protein has about 50-residue N-terminal hypervariable region (HVRs) varying extensively in sequence among the different serotypes (e.g. M4-N and M22-N) (*82*). M protein may adhere by N-terminal hypervariable or the C-terminus conserved domains to the host cells (*82*). The adhesion by variable domain will depend upon expression of different receptors by the host however, adhesion by conserved domain will be independent of the M type (*5*).

While different in sequence, M4-N and M22-N (C4BP binding domains) employ similar folds (*82*). HVRs of M4 are folded as coiled coils and folded nucleus of the M4 HVR has a length of about 27 residues (*82*).Although M proteins variable in HVR sequences, they mostly bind to human C4BP (complement component 4B-binding protein) (*82*). This bound-form helps bacteria resist phagocytosis (*82*). Peptic fragment of the group A streptococcal serotype 5(Pep M5), M5 protein, have been sequenced (*81*). Pep M5 protein consist of some matching repeating sequences: four 7-residue segments and two of 10-residue segments (*83*). The anti phagocytic activity of the M protein may relate to the amino acid sequences of the fragments (*15*). Attempts of the bacteria to survive in the host's environment from the immunological pressure have given rise to the

variation of the amino acids of different serotypes of M protein, according to Dr. Lancefield (*15*).Aside from sequence variation, M proteins also vary in size, from 41 to 80 kDa (*56, 83*). This variation is strain dependent. This size divergence is evident within the serotype 6 (M6),isolated from different strains that may be explained by the extensive repeats at DNA level (*84*). Long reiteration of DNA sequences lead to deletions and duplications within the M-protein gene and consequently M proteins of varying sizes are formed (*83*).

2.1.3.3. Other adhesins

Since the suggestion of lipoteichoic acid as the foremost adhesin of the bacterium, back in 1975, at least 17 more adhesins and their relative receptors have been acknowledged (*5*). The adhesins are; M protein, Protein F1/Sfb1 (streptococcal fibronectin binding protein 1), Protein F2, PFBP (*S. pyogenes* fibronectin-binding protein), FBP54 (fibronectin-binding protein 54), 28 kDa protein, G3PDH (glyceraldehyde-3-phosphate dehydrogenase),vitronectin-binding protein, galactose-binding protein, hyaluronic acid, C-carbohydrate, collagen-binding protein, R28 ,Scl1 (streptococcal collagen-like protein 1), Scl2 (SclA, SclB),SpeB (streptococcal pyrogenic exotoxin B),Sfbx (*Streptococcus pyogenes* fibronectin-binding protein), Lbp (laminin-binding protein),and Fba (Fructose-bisphosphatealdolase, a novel fibronectin-binding protein). Receptors proposed for the previously mentioned adhesins are; fibronectin, integrins, fibrinogen, galactose, laminin, collagen, cytokeratin, macrophage scavenger receptor, CD46 (cluster of differentiation 46, a complement regulatory protein), fucose/fucosylated glycoprotein, sialic acid containing receptors, CD44, SOF (Serum opacity factor),and Heparin sulfate (*5*).

2.1.3.4. Capsule

Another major virulence factor proposed for GAS according to studies is the hyaluronic acid capsule composed of repeating subunits of β1,4-linked disaccharides of glucuronic acid β1,3-linked to N-acetylglucosamine identical to the one found in the connective tissues of human (*85*). Production of capsule is a general attribute among all GAS strains although the production rate may differ (*85*). Capsule will elude the immune system to help bacteria resist phagocytosis and scientific reports indicate only highly encapsulated strains are associated with acute rheumatic fever and severe invasive infections (*86*). The *has* gene cluster (*has*A, *has*B, *has*C), a locus vastly conserved among GAS strains encodes the enzymes essential for hyaluronic acid synthesis (*86*). Experimental approaches report the production of hyaluronic acid by GAS only at the exponential phase (*85, 86*). This production is stopped at stationary phase with loss of synthase activity of the membrane (*85, 86*). Highly encapsulated strains have mucoid appearance when cultured onblood agar plates(*48*).In heavily encapsulated GAS strains capsule is responsible for resistance to phagocytosis not M protein (*87*). Acute pharyngitis is developed typically by non-mucoid GAS strains and only 3% have been morphologically mucoid (*88*). In the same study, conversely strains that had mucoid appearance accounted for 21% of serious streptococcal infections and 42% of rheumatic fever cases (*88*).

2.1.4. Biofilm

Studies show biofilms are primary component of ancient prokaryotic life cycle and can be found abundantly on the face of earth as single or multispecies form (*89*). To form biofilm, planktonic bacteria attach to either inert or coated surface that could be

mediated by electrostatic contacts or bacterial surface adhesins (*89*). Attachment is followed by proliferation of the primary colonizers and their coaggregation with their own kind or strange planktonic bacteria, production of EPS which stabilizes the architecture, leading to the maturation of the biofilm (*89*). Sessile bacteria then could detach and form biofilm at different site. Intraspecies and interspecies communication systems are responsible for regulation of developmental, metabolic, genetic, and physical properties of a biofilm (*89*).

Biofilm is defined as a thin film-like structure composed of a layer of bacterial mass that adheres to surfaces by the bacterial glycocalyx and then starts colonizing (*22*). Biofilm formation is not an attribute only specific to few species but a general ability of all microorganisms. Biofilm building pathways are species specific, diverse, and dependent on environmental conditions. Although diverse, there are common features among all biofilms; (i); cells in the biofilm are glued together by an extracellular matrix made of exopolysaccharides (EPS), proteins, and occasionally nucleic acids, (ii); biofilm formation is initiated by environmental and bacterial signals, and (iii); biofilms offer bacteria protection from antibiotics and environmental stresses including immunological responses of host (*90*). Bacteria increase the expression of their outer cell surface adhesins when environmental conditions allow promoting cell-cell and cell-surface interaction (*91*). Bacterial biofilms can build up on abiotic (plastic, glass, metal, etc.) or biotic (plants, animals, and humans) surfaces (*92*). In microbial biofilms, bacterial cells start to aggregate on the surface forming micro-colonies and these embed in an extracellular matrix shaping the biofilms (*71, 93*). Clinically, biofilms are important because they reduce susceptibility of the bacteria to antimicrobials, and increase invasion

ability of the pathogen (*94*). For the prevention of biofilm, quorum sensing and adhesion shall be inhibited.

2.1.4.1. GAS biofilm

Quorum-sensing systems are utilized by streptococci to regulate several physiological properties, including the ability to tolerate acids, incorporate foreign DNA, form biofilm, and become virulent. Aside from adherence, biofilms are of significant importance as approximately 65% of human bacterial infections involve biofilms also pertinent to streptococci species (e.g. *S. pyogenes)* (*74, 95, 96*).

A general model for biofilm formation of non-motile species such as *S. pyogenes* has been proposed (*90*). Chronic and recurrent streptococcal infections are linked to biofilm formation. Biofilm like structures called micro colonies have been reported for GAS *in vivo* and *in vitro* studies (*97*). Existence of microcolonies have been documented for GAS isolates from patients with impetigo and atopic dermatitis, sore throat, acute glomerulonephritis, post-streptococcal glomerulonephritis, and etc. (*97*). It has been suggested that the formation of biofilm in soft tissues of host (throat, skin, heart valves, and kidney) play a significant role in GAS pathogenesis (*15*). A feature that has been observed in all GAS infections is the growth of bacteria in close association and interaction with the affected soft tissue and formation of rough surface aggregates (*97*).These glycocalyx-encased communities are referred as biofilm in GAS (*22*). The affinities of GAS serotypes to different adherence substrates differ from one another and this applies even to isolates of the same serotype (*97*). For cell-surface interaction of GAS presence of M protein is vital and the hyaluronic capsule becomes important for subsequent maturation of the biofilm (*98*). Inclusively appropriate regulation of both is

critical for the biofilm developmental process (*98*). DNA microarray analysis shows significant differences in expression between biofilm and planktonic lifestyles of *S. pyogenes* HSC5 (*emm* type 14) at logarithmic and early stationary phase growth (*98*). A through comparison between the expression of logarithmic phase growth and biofilm revealed that of the total 1517 genes in the genome 227 genes (15%), 225 genes (15%) were upregulated and downregulated respectively (*98*). The top percentage change in up-and-downregulated combined versus total number of genes in logarithmic phase growth and biofilm comparison are energy production and conversion (56%), carbohydrate transport and metabolism (46%), secondary metabolites biosynthesis, transport and catabolism (45%), lipid transport and metabolism (44%) and nucleotide transport and metabolism (36%) (*98*).

Almost the same numbers were recorded in comparison of biofilm and stationary phase culture, 212 genes (14%) upregulated, and 203 genes (13%) downregulated(*98*).The main changes for the latter comparison were carbohydrate transport and metabolism (35%), intracellular trafficking and secretion (36%), replication, recombination and repair (34%), post-translational modification, protein turnover, chaperones (34%), and amino acid transport and metabolism (32%)(*98*). Conclusion can be made that metabolism and virulence factor expression in mature GAS biofilm is noticeably different from that of exponential and stationary phases of planktonic growth.

2.2. Antibiotics

Throughout history, infectious diseases have claimed more lives than any other diseases. Authors of "antibiotic discovery and development" have called antibiotics,

wonder drugs that could cure the deadliest of all (*35*).Discovery of antibiotics started with the contaminating mold growing on one of Fleming's experimental discarded petri dishes that unpredictably had inhibited the growth of staphylococci (*35*). The mold was of *Penicillium* genus and so this magical antibiotic back in 1929 was named penicillin. Though magical, they also could lose their effect. Bacteria could resist the antibiotic through mutations or genetic exchange mechanisms(*30*). Resistant bacteria, especially significant for healthcare-associated infections, are on rise but antibiotic discovery efforts have slowed down.

Antimicrobials' mechanism of action can briefly be described based on disruption of several activities and processes including cell wall synthesis, plasma membrane integrity, nucleic acid synthesis, ribosomal function, and folate synthesis (*23*). The mechanisms of action of these antibiotics differ from one another. Macrolides are capable of inhibiting a number of protein synthesis stages (*35*). Macrolides mostly disrupt one of the events occurring on the ribosome however they have no inhibitory effect on amino acid activation or attachment to a particular tRNA (*35*). Most of the macrolides have an affinity or specificity for 70S ribosomes not 80S, resulting in macrolides selective effectiveness (*35*). Erythromycin and clindamycin all interfere with ribosome function (*16, 23, 99*).

2.2.1. Streptococcal pharyngitis antibiotic treatment

Penicillin or one of its derivatives (e.g., amoxicillin and ampicillin) are the recommended first-line treatment (penicillin V at 50 mg/kg of body weight/day for 10 days) for non-allergic patients diagnosed with Group A *Streptococcus* infections (*4, 100*). For allergic individuals, azithromycin and clarithromycin are recommended and in fact,

azithromycin is prescribed more commonly than penicillin in USA, which has consequently, according to some studies, caused GAS resistance to azithromycin (*36*). For severe GAS infections like toxic shock syndrome and necrotizing fasciitis, a combination of penicillin and clindamycin are prescribed. Minimum bactericidal concentrations of penicillin against GAS have been reported to be 0.003-0.12 μg/mL with no resistance documented so far (*3, 101, 102*). The effectiveness of penicillin depends on the prolonged treatment regimen rather than high dose therapy (*4, 100, 103*). The therapy's efficacy is maximal when started within 9 days of onset of symptoms of GAS pharyngitis (*103*). Azithromycin and clarithromycin have been reported for relatively higher efficacy compare to penicillin in the treatment of GAS pharyngitis (*103*). Table 1 summarizes the *in vitro* susceptibilities of GAS to common antibiotics. Patient compliance treated with macrolides, due to their easier dosing schedule (3 - 5 days regimen) is significantly improved (*38, 100*). Adequate penicillin therapy compliance is defined as consumption of 80% of the recommended dosage within 10-day regimen (*100*). Amoxicillin, a derivative of penicillin, has higher efficacy in eradicating GAS than penicillin itself. Studies have reported oral cephalosporin resistant to β-lactamase producing organisms in the pharynx resulting in high efficacy than penicillin (*104*). For treatment of streptococcal pharyngitis several factors are taken into consideration when prescribing an antibiotic: effectiveness, spectrum of activity, safety, dosing schedule and cost (*38*). It is worthy of mention that according to studies, M serotypes play no to little difference in the penicillin treatment outcomes (*105*).

For patients with penicillin allergy, U.S. treatment guidelines recommend erythromycin. In instances where gastrointestinal side effects of erythromycin is

observed, physicians prescribe the FDA-approved second-generation macrolides azithromycin and clarithromycin (*38*).Penicillin derivatives (ampicillin or amoxicillin), clindamycin, and cephalosporins and macrolides are all effective against GABHS (Table 1). According to a survey, from 1997 to 2003 U.S. physicians prescribed antibiotics to 53% of children with sore throat. Amoxicillin was mostly prescribed (26% of visits), followed by penicillin (7%), first-generation cephalosporins (3%), and erythromycin (2%). The most commonly prescribed non-recommended antibiotics were other cephalosporins (6% of visits), extended-spectrum macrolides (5%), and amoxicillin/clavulanate (3%).Beta-lactam and macrolide class of antibiotics are recommended and prescribed for GAS pharyngitis.

Table 1: Summary of Minimum Inhibitory Concentration (MIC) of common antibiotics for *S. pyogenes*

	Concentration (µg/mL)				
Antibiotic	**MIC50**	**MIC90**	**MIC range**	**Mechanism of action**	
Erythromycin	0.016	0.031	0.0078-8.0	Bind to the 50S ribosomal subunit	Protein synthesis inhibitor
Azithromycin	0.016	0.031	0.0078-4.0		
Clarithromycin	0.0078	0.016	0.0039-4.0		
Chloramphenicol	4	4	2-8		
Clindamycin	0.125	0.125	0.06-0.125		
Tetracycline	0.25	2	0.0039-8.0	Bind to the 30S ribosomal subunit	
Oxacillin	0.06	0.06	≤0.03-0.25	Inhibit PBP cross-links	Cell envelope
Cephalothin	0.1	0.1	0.0125-0.2		
Cefoxitin	1	1	1-4		
Cefixime	0.25	0.5	0.078-0.5		
Cefuroxime	≤0.03	≤0.03	≤0.03		
Cefotaxime	≤0.03	≤0.03	≤0.03		
Ceftriaxone	≤0.03	≤0.03	≤0.03-0.125		
Penicillin	0.006	0.012	0.003-0.024		
Vancomycin	0.25	0.5	0.25-0.5	Inhibit PG chain elongation	
Rifampin	0.5	0.5	≤0.03-0.5	Act upon DNA-dependent RNA polymerase	RNA inhibitor
Ciprofloxacin	0.256	0.5	0.016-2.0	Inhibit DNA gyrase	DNA gyrase inhibitor
Cotrimoxazole	≥64	≥64	≥64	Block folate metabolism	Folic acid synthesis inhibitor

Modified table from Stevens et al. (*3*).
Definitions for MIC50 and MIC90 are minimum inhibitory concentrations at which 50% and 90% of the isolates are inhibited, respectively.

2.2.1.1. Streptococcal pharyngitis antibiotic treatment failure

Antibiotic treatment failure is the reason for multiple episodes of pharyngitis in up to 30% of affected individuals (*97*). Up to one-third of the patients treated for streptococcal pharyngitis fail to respond to antibiotic therapy (*100*).

Multiple episodes of GAS pharyngitis can be eradicated by a 10-day regimen of amoxicillin, clindamycin, or oral cephalosporin (*3*). GAS is not resistant to penicillin, but over time they have become resistant to clindamycin, tetracycline, vancomycin and macrolides (e.g., erythromycin, azithromycin and clarithromycin) (*4, 106*)however the most immediate concern of health care system for Group A *Streptococcus* (GAS) infections are clarithromycin and clindamycin resistant strains (*4*).

Erythromycin resistant GAS was first reported back in 1950s in England. High incidence (>80%) of resistance to erythromycin was recorded back in 1970s in Japan due to distribution of newly evolved serotypes (*106*). Although resistance to erythromycin is relatively low but increased rates have been observed in countries like Finland, Taiwan, and Italy (*106*). To address the issue of erythromycin resistant bacteria and to correlate the *in-vitro* data with the clinical outcome, an international surveillance collaborative study of antibiotic resistance took place in Italy with 3,227 children enrolled in the research. Antibiotics were prescribed to all patients with positive streptococcal rapid test (1,048 of the 3,227(32.5%)): macrolides; 46.3%, penicillins; 34.3%, and cephalosporins; 19.4%. The breakdownof each class of the antibiotics were as follows; azithromycin and clarithromycin accounted for >90% of macrolides, amoxicillin/clavulanate and amoxicillin accounted for >90% of penicillins, and cefaclor accounted for>60% of cephalosporins (*106*).

Throat swabs of 93.1% (934 isolates) were cultured in laboratory. All isolates were susceptible to penicillin and 42.8% (400 isolates) were erythromycin resistant (*106*).Of the 668 follow-up patients (time between the first and the follow-up visit was approximately 16.9 days)77.8% (520 of 668) had eradication of the bacterium and the remaining were non-eradicated (accounting for 22.2% (148 of 668)) (*106*).

Penicillins are incapable of penetrating the cell membrane thus failing to eradicate the internalized (into respiratory epithelial cells) GAS (*107*). Fibronectin-binding proteins F1 and F2 not only mediate adherence but also facilitate entry into epithelial cells. F1 protein, M6 protein and the N-terminal A and B repeats regions of M1 have been suggested to activate the invasion of eukaryotic cells by GAS (*108*). Internalized GAS is protected from antibiotics and the immunological responses of the host leading to persistence of the bacterium (*107*). Fibronectin attached to protein F1 turns into a bridging molecule towards host cell integrins initiating invasion (*107*). The bridge to alpha5beta1 integrins mediates the cytoskeletal rearrangement and the ingestion of GAS by eukaryotic cells (*109*).

2.3. Phytochemicals as antimicrobials

There are approximately 250,000 to 500,000 species of plants on earth of which either humans or other animal species use only up to 10% as food (*41*). Humans have always benefited from the therapeutic power of medicinal plants, dating back to prehistoric era (*41*). Since the discovery of antibiotics of bacterial or fungal origin, the folklore antimicrobial plants have often been disregarded (*41*). Approximately 25-50% of the marketed pharmaceuticals in USA have higher plant origins but few are intended to

fight the infectious diseases (*41*). According to Moerman's estimation, native American groups have used 1,625 species of plants as food and 2,564 species as drugs (*110*).

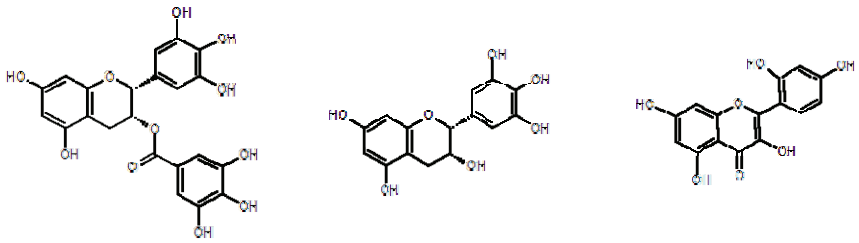
Plants produce secondary metabolites with defensive roles that are usually aromatic like phenols or their oxygen-substituted derivatives (*41*). To date studies and reports have documented the discovery of nearly 10% of the secondary metabolites, accounting for 12,000 compounds (*41*). In his review, Cowan has divided antimicrobial phytochemicals into several categories; (i) terpenoids, essential oils, (ii) alkaloids, (iii) lectins and polypeptides (iv) polyacetylenes, and (v) phenolics with subclasses of (a) simple phenols, (b) phenolic acids, (c) quinones, (d) flavonoids, (e) flavones, (f) flavonols, (g) tannins and (h) coumarins. Their major antimicrobial mechanism of action could be summarized as (i) substrate deprivation, (ii) membrane disruption, (iii) binding to adhesins and proteins, (iv) formation of complex with cell wall, (v) inactivation and inhibition of enzymes, (vi) inhibition of HIV reverse transcriptase, (vii) metal ion complexation, (viii) interaction with eukaryotic DNA (antiviral activity), (ix) intercalation into cell wall and/or DNA, (x) blocking viral fusion or adsorption, and (xi) formation of disulfide bridges (*41*).

Phytochemicals have shown antimicrobial effects against Gram-positives, Gram-negatives, fungi, and viruses (Table 2).Natural products extracted from mainly medicinal plants as well as fruits of wild type, are the subject of many studies as alternative substitutes to currently used antibiotics for the treatment of infectious diseases and antibiotic-resistant human pathogens. With more in depth studies of the potent components of these plant extracts and their mode of action, new drugs and antibiotics can be developed for the soon to be post-antibiotic era.

2.3.1. Phytochemicals effective against GAS infections

Folklore medicinal plants such as cashew plant, stickwort, mountain daisy, bayberry and others have been used for centuries to treat streptococcal infections and respiratory tract infections including pharyngitis in many forms such tea, gargle, drop, and infusion (*111*). The anti-adhesive properties of root extract of *Pelargonium sidoides* have been studied against GAS attachment to human epithelial type 2 (HEp-2) cells (*45*). Results have shown that after pre-treatment of GAS with 30 μg/mL methanol insoluble and methanol soluble fractions, adhesion of the pathogen to HEp-2 cells was inhibited up to 30 to 35% (*45*). To characterize the anti-adhesive proportion of these fractions comparative chemical studies were performed (*45*). The study revealed that the proanthocyanidins content of the fraction was of prodelphinidin nature, and the inhibition of adhesion was specific rather than non-specific (*45*). Successful inhibition of adhesion and hydrophobic interactions could reduce and or prevent sore throat caused by *S. pyogenes* (*45*). Crude extract of *Eleutherine americana* at 250 mg/mL partially inhibited the quorum-sensing of a clinical isolate of *S. pyogenes*, while at the same concentration *Rhodomyrtus tomentosa* had stronger inhibition activity (*112*).Various medicinal plants have recently been tested for antimicrobial activity and all have proven that phytochemicals particularly polyphenols exhibit significant antibacterial activity against different strains of GAS such as HITM 100, ATCC 19615 and clinical isolates. Few examples of these plants are wild maracuja (*Passiflora foetida*), white weed (*Ageratum conyzoides*), calabash tree (*Crescentia cujete*), bush-banana (*Uvaria chamae*), ginger (*Zingiber officinale*), bitter kola (*Garcinia kola*), little gourd (*Coccinia grandis*), and others (*42-44, 113-122*). Adhesion reduction of *S. pyogenes* DSM2071to HEp-2 cells

have been tested with (-)-epigallocatechin(Figure 1:(2))and (-)-epigallocatechin-3-*O*-gallate (Figure 1:(1))at concentration of 30 µg/mL and the reported results are 15% and 40% respectively (*45*). Also Morin(Figure 1:(3)), a flavonol, at concentration of 225µM reduced the biofilm biomass of *S. pyogenes* MGAS 6180 by 60% (*46*).

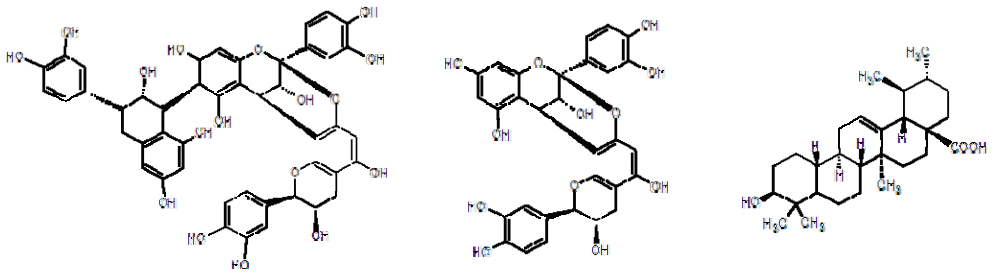


(1) **(2)** **(3)**

Figure 1: Chemical structure of phytochemicals with **adhesion inhibition** and **biofilm biomass reduction** activity against **GAS** **(1)**: (-)-Epigallocatechin-3-*O*-gallate,**(2)**: (-)-Epigallocatechin,**(3)**: Morin

2.3.1.1. Phytochemicals of berries with antimicrobial activity

Berries are good source of micronutrients, fiber and polyphenols, especially anthocyanins. Cranberry, an evergreen shrub native to North America, is one of the most well studied fruits for its biological activities. Among all its biological activities, antimicrobial activity has also been documented. The exerted beneficial activities may relate to their phytochemical composition (e.g. phenolic acids, flavanols, anthocyanins, proanthocyanins, catechins, and triterpenoids). Cranberry consists of phenolic acids and these compounds repeatedly have been studied for their antimicrobial activities.



(1) (2) (3)

Figure 2: Chemical structure of selected phytochemicals of berries
(1): Epicatechin-(4β→8, 2β→O→7)-epicatechin-(4β→8)-epicatehin (A-type proanthocyanidins) cranberry,(2): Procyanidin A2 of cranberry,(3): Ursolic acid of bearberry

Phytochemical profiling of *Scrophularia frutescens* and *Scrophularia sambucifolia* also known as figworts commonly used in folk medicine for treatment of inflammation, pain and most notably tuberculosis indicates presence of ferulic, isovanillic, p-hydroxycinnamic, p-hydroxybenzoic, syringic, caffeic, gentisic, protocatechuic, p-coumaric, and vanillic acids in these species (*123*). Study has shown that these species possess antibacterial activity particularly against Gram-positive bacteria (*123*). In addition tarragon and thyme, reported for their antimicrobial activity, consist of phenolic acids mainly caffeic acid (*41*). *Rhus aromatic*(fragrant sumac)extract with antiviral activity against herpes simplex virus, consists primarily of gallic and betulinic acid (phenolic acids), quercetin (a flavonoid), quercitrin (a glycoside formed from the flavonoid quercetin and the deoxy sugar rhamnose), and beta-sitosterol (a phytosterol) (*124*).

Table 2: summary of antimicrobial activity of selected berries/ or their family members

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
	Vaccinium macrocarpon		
P. aeruginosa	A549 lung epithelial	anti-adhesion and	(*125*)

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
	cell infection model	anti-invasion	
St. aureus	Static microplate biofilm model, microbroth dilution	anti-biofilm activity	(*126*)
E. coli, *Pr. mirabilis*	HT1376 cell line model, CDM-modified silicone	Adhesion, motility and urease activity reduction, downregulating the expression of flagellin gene	(*127, 128*)
C. albicans	Artificial urine, static microplate biofilm, and silicone disc biofilm models	Adherence and biofilm inhibition, iron chelation	(*129*)
Pr. mirabilis	Urease reporter and swimming, swarming motility assays, comparative qPCR	Interruption of motility and expression of important virulence factors	(*130*)
E. coli, *L. monocytogenes*, *Lac. rhamnosus*	LIVE/DEAD viability assay, micro broth dilution method	Antibacterial, Hyperpolarization and depolarization (membrane permeability) effect on the membrane by different fractions	(*131*)
S. mutans	Saliva-coated hydroxyapatite biofilm model, transcriptome response analysis	Impairment of biofilm formation, downregulating the expression of genes involved in adhesion, acid stress tolerance, and glycolysis	(*132*)
Norovirus	Saliva-binding enzyme-linked immunosorbent assay	Specific binding ability of human NoV P particles reduced	(*133*)
P. aeruginosa	Petri dish swarming assay	Inhibition of swarming motility	(*134*)
Porphyromonas	Fluorescein isothiocyanate-labelled	Inhibition of growth, adhesion and biofilm	(*135*)

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
gingivalis	*P. gingivalis* and human oral epithelial cell line GMSM-K	formation	
St. epidermidis, *St. aureus*, MRSA, *St. saprophyticus*	Microtitre plate static biofilm model, micro broth dilution method	Inhibition of growth and biofilm formation	(*136, 137*)
	Viburnum opulus		
Ae. hydrophila, *B. cereus*, *En. aerogenes*, *E. coli*, *K. pneumoniae*, *Pr. vulgaris*, *P. aeruginosa*, *S. typhimurium*, *St. aureus*, *Y. enterocolitica*	Agar diffusion method	Antibacterial	(*138*)
P. aeruginosa, *E. coli*, *S. typhimurium*, *St. aureus, B. subtilis*, *L. monocytogenes*, *E. faecalis*, *Micrococcus luteus, St. epidermidis, Trichosporon cutaneum*, *Kl. marxianus*, *Sac. cerevisiae*	Agar well diffusion method	Antibacterial, antifungal	(*139*)
St. aureus, MRSA, *B. subtilis*, *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *C. albicans*	Disc diffusion, and tube dilution technique	Antibacterial	(*140*)
St. aureus, St. epidermidis, S. pyogenes	Disc diffusion assay	Antibacterial	(*141*)
S. typhimurium, *Sa. agona*, *St. aureus*, *L. monocytogenes*, *E. faecalis*	Agar plate count	Antibacterial	(*142*)
E. coli, *S. typhimurium*, *E. faecalis*, *L. monocytogenes*, *St.*	Agar well diffusion method	Antibacterial	(*143*)

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
aureus, *B. subtilis*			
	Rhus coriaria*, *R. typhina		
B. cereus, *B. megaterium*, *B. subtilis*, *B. thuringiensis*, *L. monocytogenes*, *St. aureus*, *Ci. freundii*, *H. alvei*, *E. coli*, *Pr. vulgaris*, *Sa. enteritidis*	Gradient plate method	Antibacterial	(*144*)
B. cereus, *B. subtilis*, *St.aureus*, *L.monocytogenes*, *E. coli*, *S.typhimurium*, *H. pylori, Sac.cerevisiae*, *Pi. pastoris*, *Kl. lactis*	Agar dilution and disc diffusion	Antifungal, antibacterial	(*145*)
B. cereus, *St. aureus*, *E. coli*, *Pr. vulgaris*, *S. typhi*, *Sh. flexneri*	Disc and well diffusion	Antibacterial	(*146*)
B. cereus, *B. megaterium*, *B. subtilis*, *B. thuringiensis*, *L. monocytogenes*, *St. aureus*, *Ci. freundii*, *E. coli*, *H. alvei*, *Pr. vulgaris*, *Sa. enteritidis*	Cup Method	Antibacterial	(*147*)
	Sorbus aucuparia		
B. cereus, *St. aureus*, *P. aeruginosa*, *C. jeujuni*	Agar well diffusion, growth curve in liquid cultures	Antibacterial	(*148, 149*)
B. subtilis, *B. cereus*, *E. coli*, *S. marcescens*	Micro dilutionplate	Antibacterial	(*150*)
	Empetrum nigrum		
St. aureus, *C. albicans*, *E. coli*, *B. subtilis*	Disk diffusion method	Antibacterial, antifungal	(*151, 152*)
	Ribes nigrum		
Respiratory Syncytial Virus (RSV), *influenza virus A and B*,	Virus adsorption on the cell surface and plaque reduction assay, agar	Anti-viral, antibacterial	(*153*)

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
adenovirus (AdV), *herpes simplex virus type*, *Haemophilus influenzae type B*, *S. pneumoniae*	plate count method		
Candida species	Broth dilution	Anti-candidal	(*154*)
B. cereus, *L. monocytogenes*, *St. aureus*, *E. coli*, *P. aeruginosa*, *Sa. enteritidis*, *C. albicans*, *A. niger*	Microwell dilution	Antibacterial, antifungal	(*155*)
	Lonicera caerulea*, *L. lanceolata		
C. parapsilosis, *St. epidermidis*, *E. coli*, *E. faecalis*, *S. mutans*	Micro dilution, CFU/disk count, crystal violet staining	Antibacterial, adherence and biofilm formation inhibition	(*156*)
B. subtilis, *K. rhizophila*, *L. monocytogenes*	Agar well-diffusion	Antibacterial	(*157*)
K. pneumonia, *E. coli*, *P. aeruginosa*, *E. cloacae*, *St. aureus*, *M. luteus*, *A. niger*	Agar dilution, viable cell count	Antibacterial, antifungal	(*158, 159*)
	Vaccinium vitis-idaea		
P. gingivalis, *P. intermedia*	Agar dilution	Antibacterial	(*160*)
Herpes simplex virus	XTT assay, African green monkey kidney cells (Vero) (ATCC CCR-81)	Antiviral, attachment inhibition	(*161*)
	Rhamnus catharticus*, *R. orbiculatus		
M. tuberculosis	Tube dilution method	Anti-tuberculosis	(*162*)
St. aureus, *P. aeruginosa*, *E. coli*, *C. albicans*, *A. niger*, *M. gypseum*	Micro broth dilution	Antibacterial, antifungal	(*163*)

Tested microorganism	Assay / model / cell line used	Conclusion	Ref.
	Vaccinium corymbosum*, *V. angustifolium		
S. Typhimurium, *C. jejuni*, *L. monocytogenes*, *E. coli 157:H7*	Micro broth dilution	Antibacterial	(*164*)
Sa. enteritidis, *L. monocytogenes*, *S. Typhimurium*	Agar dilution and diffusion, agar plate count	Antibacterial	(*165-167*)
E. coli	Agglutination suppression of human red blood cells (HRBC)	Anti-adhesion	(*168, 169*)
V. parahaemolyticus, *St. epidermidis*	Crystal violet staining, plate count, agar dilution	Biofilm formation reduction	(*170, 171*)

There is a fair amount of scientific evidence that phytochemicals for example A-type proanthocyanidins of cranberry exert significant anti-streptococcal effects at as low concentration as 500 μg/mL in a specific approach of ATPase activity inhibition (85%) (*25*). Apart from phytochemicals' bactericidal effects, their main bacteriostatic strategy is their anti-adhesiveness attribute such as ursolic acid (Figure 2:(3)) that completely inhibit the adherence of *S. mutans* at 1024 μg/mL and this penta-cyclic triterpene acid is found in bearberry (*172, 173*). Table 2 summarizes selected studies that suggest berries and their extracts/constituents could potentially be considered antimicrobials.

CHAPTER 3 MATERIALS AND METHODS

3.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO), penicillin G sodium salt, sodium chloride, and Triton X-100 from were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Life Technologies (Burlington, ON, Canada). Bovine serum albumin, ethanol, glycerol, human fibronectin, and phosphate-buffered saline (PBS: 1X without calcium and magnesium) were purchased from Fisher Scientific (Ottawa, ON, Canada). Bacteriological agar and Brain Heart Infusion (BHI) were purchased from Oxoid Ltd. (Nepean, ON, Canada).

3.2. Plant material

The source of wild berries are given below (if the berries were collected locally the GPS location is noted). *Empetrum nigrum* (Crowberry) and *Viburnum edule* (Squash berry) were provided by Dark tickle company (Saint Lunaire-Griquet, Newfoundland). *Rhamnus cathartica* (common Buckthorn) was harvested the 1st week of September, 2014 at GPS location of 40 Cox Rd, Truro, NS B2N 2R8, 45°22'25.3"N 63°15'49.7"W.*Sorbus decora* (Mountain ash or Rowanberry) was harvested the 3rd week of September 2014 at GPS location of 69 Pictou Rd, Truro, NS B2N 2R9, 45°22'32.2"N 63°15'51.9"W. *Rhus typhina* (Sumac or Staghorn) was harvested the 4th week of September, 2014 at GPS location of 221 Farnham Rd, Truro, NS B2N 2X6, 45°23'00.4"N 63°15'52.1"W.

Cultivated berries were collected in the following order with their location (if applicable); *Ribes nigrum L. cv. Titania* (Blackcurrant), provided by a PEI commercial grower, *Vaccinium corymbosum* (Blueberry, wild type), university experimental plot

(Debert, Nova Scotia), *Vaccinium macrocarpon* (Cranberry), commercial farm of Cranberry Acres (Berwick, Nova Scotia), *Lonicera caerulea* (Honeysuckle, variety of Borealis) (Nova Scotia), and *Vaccinium vitis-idaea* (Partridgeberry, wild type) (southern Labrador, Newfoundland and Labrador). All were stored at -20°C until further use.

3.3. Bacterial strains and growth conditions

S. pyogenes namely ATCC 19615, ATCC 49399 and clinical strain were included in the study. *S. pyogenes* ATCC 19615 and ATCC 49399, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), were isolated from pharynx of child following episode of sore throat and human throat, correspondingly. Clinical strain was kindly provided by Dr. R. J. Davidson's Bacteriology laboratory, Division of Microbiology, Pathology & Laboratory Medicine, Nova Scotia Health Authority, Halifax, NS (isolated from a positive strep. throat patient). Bacteria were stored in BHI broth containing 20% glycerol at -50°C for further use. Bacteria were routinely grown in BHI broth and were incubated overnight statically at 37°C.

3.4. Preparation of bacterial cultures

Before each experiment, bacterial cultures were prepared fresh. Bacteria stored in glycerol were minimally thawed and ~10 µL was transferred using an inoculation loop to a 15 mL tube containing fresh BHI broth of about 10-12 mL. The tube was incubated overnight at 37°C. Thoroughly vortexed overnight culture was diluted 2 times and its absorbance was read at 600 nm by microplate reader (medium was subtracted).

Simultaneously 1.5 mL of the overnight culture was centrifuged (13,000 rpm, 10 minutes) and the supernatant was aspired and discarded carefully. The volume of BHI broth required to resuspend the pellet was calculated by the following formula;

Volume (mL) = (Absorbance 600_{nm}× 2 × 1.5mL) / 1.0

Theoretically, the stock bacterial culture's density equals 10^9 CFU/mL. Dilutions (10-fold) specific for each assay was made from the stock. Exact density of the bacterial inoculum for each assay was routinely confirmed by plating the dilutions of the bacterial culture onto BHI agar plates.

3.5. Preparation of phytochemicals-rich extracts

The extracts were prepared according to the method of Gunathilake et al. (*174*) with modifications. Two types of extracts were prepared, aqueous extracts and ethanolic extracts. One hundred Grams of each of the wild/cultivated berries were weighed in triplicates and homogenized with 100 mL of solvent (95% ethanol or deionized water) in the ratio of 1:1 for few minutes using ethanol resistant commercial blenders. The homogenized berries were filtered through Whattman No.5 filter paper under vacuum. The residue consecutively was re-extracted with another 100 mL of solvent (deionized water and ethanol (1:1 ratio)) and filtered under vacuum. Filtrates were combined and stored at 4 °C.

The ethanolic extracts were rotary evaporated (Heidolph Rota Chill LM6 120 Volt, Illinois, USA) under reduced pressure to obtain the crude extract. The crude extracts were transferred to plastic cups and frozen at -20°C, overnight. The frozen crude extracts were lyophilized in a freeze drier (Dura-Stop MP by FTS systems, Philadelphia, USA) under 3600 mT vacuum, first 36 hours at -20°C and then for 14 hours at 20°C.

The freeze dried samples were scraped out from the plastic cups and stored in pre-weighed plastic centrifuge tubes at -80 °C until further analysis. Aqueous extracts were not rotary evaporated however they were lyophilized similar to the ethanolic extracts.

Exception was made for the extraction of sumac. Berry: solvent ratio was significantly different (1:4) from other berries because of extra dry nature of sumac.

After dissolution of the extracts with the appropriate solvent, filter sterilization method was used to remove any contaminating microbes from the extracts. To confirm that the extracts are contamination free, 100 µL of the extract was plated on BHI agar plate by spread plate method and incubated for several days at 37°C. Clear plates are an indication of contamination free preparations.

3.6. Determination of minimum inhibitory and bactericidal concentrations of extracts against *S. pyogenes*

Minimum inhibitory and bactericidal concentrations were determined using micro-broth dilution method as described by the Clinical Laboratory Standards Institute (CLSI). Briefly, overnight grown bacterial cultures of *S. pyogenes* ATCC 19615, ATCC 49399, and clinical strain were standardized using the method described previously (see section 3.4.) yielding about 10^8 CFU/mL, and diluted (10-fold dilution) further up to approximately 5×10^4–5×10^5 CFU/mL using saline water and BHI broth.

Stock solutions of the extracts / compounds were prepared using sterile distilled water. Aqueous extract, ethanolic extract and penicillin G were first dissolved in sterile distilled water, filter sterilized and then diluted to various concentrations. Working solutions were prepared using BHI media. Working solutions of the extracts / compounds were further diluted (2-fold serial dilution) in the well yielding different concentrations, generally ranging 0.06–128 mg/mL.

To the 100 µL of the diluted extract, bacteria were added to the final volume of 200 µL in each well (5×10^4–5×10^5 CFU/mL). The 96-well plate was then incubated at

37°C for 24 hours. Sterile distilled water was used as solvent control. Wells without extract were considered as growth control. Background color was subtracted from the readings (wells with extracts and without bacterial inoculum). Both aqueous and ethanolic extracts of *Rhamnus cathartica* were viscous and therefore concentration of >16 mg/mL could not be tested. Readings were taken at 600 nm with micro-plate reader. All procedures were performed by aseptic techniques. MIC was defined as thelowest concentration of the specific extract that inhibited the bacterial growth of the specific strain. Background color was subtracted from the readings (wells with extracts and without bacterial inoculum).

Samples (30 μL) from the clear wells (no visible growth) were plated onto BHI agar plates to determine MBC values (no colony growth on the plate). MBC was defined as the lowest concentrations at which 99.9% of the bacteria were killed. Three independent studies each in triplicates were performed to make statistically valid conclusions. Student t-test (Microsoft Office Excel) was used to determine the significant differences between the control and the extract / compound treatment ($P \leq 0.05$).

3.7. Time kill kinetics of extracts

Briefly, overnight grown bacterial cultures of *S. pyogenes* ATCC 19615 were standardized using the method described previously (see section 3.4.) yielding about 10^9 CFU/mL, and diluted (10-fold dilution) further up to approximately 5×10^4–5×10^5 CFU/mL using saline water and or BHI broth.

Bacteria was challenged with extracts / compound at ½ × MIC to 8 × MIC concentrations (Table 3) and incubated at 37°C for 24 hours. At various time points 100 μL samples were drawn and diluted (10-fold) with sterile saline water to yield colonies

within the range of quantification limits (30–300 CFU/mL). Viability of the bacterial cells were determined by spot plate method at the indicated time points (0-24 hours). A 30 µL sample in duplicates from different dilutions (usually up to the dilution factor of 0.000001) was plated onto the BHI agar plates for enumeration. Bacterial cells were enumerated after 24 hours of incubation at 37°C. Solvent controlwas included in the assay.BHI broth media with and without bacterial culture were considered as positive and negative bacterial growth controls, respectively.

Due to limited resources, only two highest concentrations of the solvent for each of the extract / compound were assayed. Solvent controls for different extract / compound were as follows; for cranberry aqueous and ethanolic extracts (4-8 mg (4 × MIC and 8 × MIC) of cranberry extracts per milliliter of sterile distilled water) 3.13-6.25% of H_2O, for sumac aqueous extract (2-4 mg (4 × MIC and 8 × MIC) of sumac extract per milliliter of sterilized distilled water) 1.56-3.13% of H_2O, for sumac ethanolic extract (1-2 mg (4 × MIC and 8 × MIC) of sumac extract per milliliter of sterilized distilled water) 0.78-1.56% of H_2O and for Penicillin G (8-16 ng (4 × MIC and 8 × MIC) of penicillin G per milliliter of sterilized distilled water) 0.01–0.02% of H_2O.Mean of triplicate counts was recorded and the corresponding bacterial density was calculated.

Bacterial density (CFU/mL) = (colony count/ (spotted volume × dilution factor))

3.8. Biofilm inhibition assays

3.8.1. Assessment of inhibitory effects of extracts on *S. pyogenes* biofilm formation by indirect viable cell count

The assay was conducted as described by Pettit et al. (*175*) with modifications. Biofilms were formed in the presence of extracts / compound. To do this, bacterial cells

(see preparation of bacterial cultures) were added in 96-well tissue culture plates (flat bottom with low evaporation lid), extracts at MBC and lower concentrations were added (sub-MIC was also tested) and the plate was incubated at 37°C for 24 hours (Table 3). The density of the bacterial culture was about 1×10^5 CFU/mL. Negative and positive controls were also designed for the assay (BHI broth with and without bacterial culture). As stated previously, due to limited resources only two solvent controls for the two highest concentrations of each of the extracts / compound were tested. Solvent controls were as follows; for cranberry aqueous and ethanolic extracts (8-16 mg/mL) 6.25-12.5% of H_2O, for sumac aqueous and ethanolic extracts (4-8 mg/mL) 3.13-6.25% of H_2O, and for penicillin G (8-16 ng/mL) 0.01-0.02% of H_2O.

After 24 hours of incubation period, planktonic bacteria were discarded, recovery media (BHI broth only) in the volume of 100 µL was added and serial dilutions (10-fold) of the viable biofilm bacteria were plated onto BHI agar plates, incubated for 24 hours at 37°C and enumerated for CFU/mL determination (Figure 3). Percent biofilm formation was calculated to compensate for differences in biofilm formation of *S. pyogenes* in the presence or absence of various concentrations of extracts / compounds.

Percent biofilm formation= [(log CFU mL^{-1} of treated well/ log CFU mL^{-1} of untreated well) × 100]

3.8.2. Assessment of inhibitory effects of extracts on *S. pyogenes* biofilm formation by metabolic activity measurement

The plate was prepared as described in the previous method (see 3.8.1). After 24-hour incubation, the planktonic cells were discarded and the recovery media (200 µL) was added. BHI broth with and without bacterial culture were considered as positive, and

negative controls respectively. Entire range of solvent controls for all the concentrations tested was included in the assay.

To measure the metabolic activity of the biofilms formed in presence of extracts / compound MTT at the concentration of 5 mg/mL (dissolved in normal sterile saline water, freshly prepared in dark) was added to each well in the volume of 20 µL. Addition of MTT followed by 2-4 hours incubation resulted in formation of insoluble purple formazan by viable bacterial cells. After the incubation time, 170 µL of the recovery media was discarded carefully not disrupting the purple color pigments. To dissolve the insoluble purple formazan, 50 µL of DMSO was added followed by vigorous pipetting. Purple color intensity is proportional to the number of viable cells in the well. Absorbance was read at 540 nm. Percent biofilm formation was calculated relative to positive control.

Percent biofilm formation = $(((A_{\text{treatment}} - A_{\text{negative control}}) / (A_{\text{positive control}} - A_{\text{negative control}})) \times 100)$

Where A is the absorbance reading at 540_{nm}

3.9. Biofilm eradication assays

3.9.1. Analyses of eradication effects of extracts on *S. pyogenes* biofilms by indirect cell count

The assay was conducted as described by Ogawa et al. (*176*) with slight modifications To conduct the assay, 24 hours static biofilms of *S. pyogenes* were formed in the absence of any extracts / compound. Bacterial culture with the approximate density of 5×10^4–1×10^5 CFU/mL was used to pre-form the biofilm. Free-floating bacteria were discarded and the pre-formed biofilm was challenged with various concentrations of

different extracts / compound (multiple of MBC was tested) for 3 hours. Positive, negative, and solvent controls were included in the assay. Solvent controls were as follows; 12.5-25% of H_2O for 16-32 mg/mL of cranberry aqueous and ethanolic extracts 6.25-12.50% of H_2O for 8-16 mg/mL of sumac aqueous and ethanolic extracts, 0.06-0.13% of H_2O for 64-128 ng/mL of penicillin G. Following 3 hours incubation, planktonic bacteria were discarded, recovery media (100 µL) was added and serial dilutions (10-fold) of the viable biofilm bacteria were plated onto BHI agar plates, incubated for 24 hours at 37°C and enumerated for CFU/mL determination.

3.9.2. Analyses of eradication effects of extracts on*S. pyogenes* biofilms by metabolic activity measurement

The preparation of the pre-formed biofilm was carried out as described in the previous method (see 3.9.1) (*176*). BHI broth with and without bacterial inoculum were considered positive, and negative controls. Solvent controls for the entire range of tested concentrations were included in the assay. After 3-hour exposure to the extracts / compounds, planktonic cells were discarded and the recovery media (200 µL) was added. MTT at the concentration of 5 mg/mL (dissolved in normal sterile saline water, in the dark) was prepared fresh and added to each well at the volume of 20 µL. Addition of MTT followed by 2-4 hours incubation resulted in formation of insoluble purple formazan by viable bacterial cells. After the sub-incubation time, 170 µL of the recovery media was discarded carefully not disrupting the purple color pigments. To dissolve the insoluble purple formazan, 50 µL of DMSO was added followed by vigorous pipetting. Purple color intensity is proportional to the number of viable cells in the well.

Absorbance was read at 540 nm. Percent biofilm eradication was calculated relative to positive control (0% eradication).

3.10. Adherence inhibition assays

3.10.1. Anti- adherent activity of selected extracts against attachment of *S. pyogenes* to uncoated surfaces

The assay was conducted as described by others (*97, 175, 177-181*) with slight modifications. The uncoated 96-well polystyrene microplate was incubated at 37 °C for one hour with the bacterial inoculum and different concentrations (equal or less than MBC) of the test compound(s). Approximate bacterial density was 5×10^5–1×10^6 CFU/mL. After one-hour treatment, loosely attached bacteria were discarded. Recovery media (100 µL) followed by vigorous pipetting was performed. Negative and positive controls were also designed for the assay (BHI broth with and without bacterial culture). As stated previously, due to limited resources only two solvent controls for the two highest concentrations of each of the extracts / compound were tested. Solvent controls were as follows; for cranberry aqueous extract (8-16 mg/mL) 6.25-12.5% of H2O, for sumac aqueous extract (4-8 mg/mL) 3.13-6.25% of H2O, and for penicillin G (8-16 ng/mL) 0.01-0.02% of H2O. Subsequently serial dilutions (10-fold) of the adherent bacteria were plated onto BHI agar plates for CFU/mL enumeration.

3.10.2. Anti- adherent activity of selected extracts against attachment of *S. pyogenes* to protein coated surfaces

The assay was conducted as described by others (*97, 177-181*) with slight modifications. Briefly, wells of polystyrene microplates were coated with fibronectin (in PBS, 10 µg/mL). To each well, 100 µL of the fibronectin solution was added and then the

plate was incubated at room temperature for 2 hours. Following incubation, the content was discarded and gently washed with 150 µL of PBS. To block the fibronectin-coated wells bovine serum albumin (BSA) (in PBS, 1%) in the volume of 120 µL was added and incubated at room temperature for one- hour (BSA is used for blocking the unoccupied spots and decreasing the non-specific binding. This is to ensure that any anti-adhesion attribute measured is not due to the lack of FN on binding sites of the well). Fibronectin-coated and BSA-blocked wells were washed with 150 µL of PBS, three times. Equal volumes of test compound at different concentrations (equal or less than MBC) and bacterial inoculum were mixed in another plate and incubated at room temperature for 10 minutes. To each of the protein coated wells 100 µL of the mixture was added. Approximate bacterial density was 5×10^5–1×10^6 CFU/mL. Loosely attached bacteria were discarded after 1-hour treatment. Recovery media (100 µL) was added to the wells followed by vigorous pipetting. Subsequently serial dilutions (10-fold) of the adherent bacteria were plated onto BHI agar plates for CFU/mL enumeration. Percent adherence of the bacteria to the proteins (BSA and fibronectin) relative to the initial inoculum was calculated. To ensure that the background adhesion has been taken into account, wells were only blocked with BSA (no Fn coating) and then were treated in the same manner to quantify the adherence of bacteria to BSA consequently measuring the sole adherence to fibronectin through fibronectin-binding proteins only versus non-specific binding through other adhesins.

3.10.3. Anti-adherent activity of selected extracts against attachment of *S. pyogenes* to epithelial cells

The assay was conducted according to the method previously described (*176, 182, 183*) with slight adaptation. HTEpiC cells (isolated from human normal tonsil tissue, catalog No. 3220) purchased from ScienCell Research Laboratories were maintained in accordance to the manufacturer's instructions. The cells were seeded in the wells of 96-well plates (cell density of 25,000 cells /well and the volume of 250 µL) and incubated for 24hours at 37°C, 5% CO_2 and 95% air. Cells were then washed with 250 µL of PBS, twice.

Equal volumes of test compound at different concentrations and bacterial inoculum were mixed and then incubated at room temperature for 10 minutes. Concentrations tested in were 2-4 mg/mL for cranberry aqueous extract and 8-16 ng/mL for penicillin G. Concentrations were chosen for the assay based on the results of cell cytotoxicity assay. Bacterial cultures were prepared as described earlier with slight modifications. Bacteria were diluted with antibiotic free cell culture media. Approximate density of bacterial cultures of *S. pyogenes* ATCC 19615 and clinical strain were 2.7×10^6–3.5×10^6 CFU/mL. To each well 200 µL of the mixture was added and incubated for 1 hour. The ratio of the tonsil cells to the bacteria was approximately 1:100. Weakly adhered bacteria were discarded and 100 µL of trypsin (1% in PBS) was added and incubated for 8-10 minutes at room temperature. To each well 100 µL of Triton X-100 (0.001% in PBS) was added and incubated at room temperature for another 2-4 minutes. BHI broth with and without bacterial culture were considered as positive, and negative controls. Solvent controls were as follows; 0.01–0.02% of H_2O for 8-16 ng/mL of penicillin and 1.56–3.13% of H_2O for 2-4 mg/mL of cranberry aqueous extract. Later serial dilutions (10-fold) of the adherent bacteria were plated onto BHI agar plates for

CFU/mL enumeration. Percent adherence of the bacterial cells to the tonsil cells relative to the positive control (control: 100%) was calculated.

3.14. Cell viability assay

Cells were seeded in the 96-well plate with the density of approximately 5×10^3 cells/well. Cranberry aqueous extracts at concentration ranging 2-16 mg/mL were added to the wells (in triplicates). The plate was incubated for 1 hour at 37°C. Following aspiration of the well content, recovery media in the volume of 100 µL was added. MTT in the concentration of 5 mg/mL dissolved in PBS was added to each well (10 µL). Plate was incubated for 2-4 hour at 37°C in dark. Subsequent to purple color development, absorbance was read. Percent cell viability was calculated by following formula;

$$\text{Percent cell viability} = (A_{\text{treated wells}} - A_{\text{blank}}/A_{\text{control wells}} - A_{\text{blank}}) \times 100$$

Where A is the absorbance read at 540_{nm}

3.15. Statistical analysis

Three independent studies each in triplicates were performed to make statistically valid conclusions. For MIC determination Student *t*-test was performed using Microsoft Excel to assess the significant differences between the solvent control and the extract / compound treatment ($P \leq 0.05$). Statistical significance of means (solvent control, positive control and the treatment) was assessed by one way analysis of variances (ANOVA); Tukey's multiple comparisons ($P < 0.05$). Correlation analysis was performed to compare the results obtained by plate count method and MTT assay. Data were tested for normality using Anderson-Darling Normality test ($P > 0.05$) prior to any analysis. All the analysis except Student *t*-test was conducted using Minitab v.16 software.

CHAPTER 4 RESULTS

4.1. Inhibitory activities of phytochemical-rich extracts of selected Atlantic Canada fruits against planktonic growth of *S. pyogenes*

The MIC which is defined as the lowest concentration of the extracts inhibiting the growth of *S. pyogenes* after overnight incubation, are shown in Table 3. To better evaluate the activity of the extracts against *S. pyogenes,* different strains of the bacterium were tested. MIC for all the tested extracts ranged from 0.25–8 mg/mL. Due to limited solubility of buckthorn extracts in water (16 mg/mL), MIC could not be detected.

Among aqueous extracts tested, both sumac and partridgeberry extracts have a MIC of 0.5 mg/mL, which were the most potent growth inhibitors against ATCC 19615, and sumac at the concentration of 0.25 mg/mL against ATCC 49399. Crowberry aqueous extract with the concentration of 0.5 mg/mL had the highest inhibitory effect against the clinical strain of *S. pyogenes*. Aqueous extracts of blueberry and haskap had the least inhibitory effect against ATCC 19615 and ATCC 49399 with concentrations of 8 and 4 mg/mL correspondingly. The least inhibitory effect against the clinical strain was recorded for mountain ash and blackcurrant at concentrations of 4 mg/mL.

Among the tested ethanolic extracts, sumac at concentration of 0.25 mg/mL had the greatest growth inhibitory activity against all strains. Crowberry along with haskap ethanolic extracts at the concentration of 4 mg/mL and blackcurrant, haskap, mountain ash at 2 mg/mL had the least inhibitory effects against ATCC 19615 and ATCC 49399 respectively. Blueberry ethanolic extract was the least effective one against the growth of the clinical strain at relatively high concentration of 8 mg/mL.

Overall MIC analysis of tested aqueous extracts against all three tested strains showed that inhibitory effect (except buckthorn with no recorded effect) in decreasing order were sumac > partridgeberry, crowberry, squash berry, cranberry > blackcurrant, mountain ash>haskap and >blueberry. Both aqueous and ethanolic extracts of all berries were equally potent inhibitors against the growth of test strains with exception of mountain ash, in which the ethanolic extract was more effective than the aqueous extract.

MBC for all the tested extracts against three strains ranged 4-64 mg/mL (Table3). MIC and MBC of crowberry extracts were 0.5-1 mg/mL and 32-64 mg/mL, respectively (Table 3). Except crowberry extracts, all other tested berry extracts had bactericidal effects. MBC value for blueberry extracts both aqueous and ethanolic against ATCC 49399 and clinical strain was higher than 64 mg/mL and could not be exactly determined due to poor solubility. Blueberry aqueous extract with values ranging from 64 and higher was least bactericidal extract among the tested materials.

Among aqueous extracts tested with regard to the MBC, sumac with the values ranging 4-8 mg/mL was most potent against three strains, ATCC 19615 being most susceptible strain. Sumac ethanolic extract with the value of 4 mg/mL is most effective in killing all three strains while blueberry ethanolic extract was least bactericidal with the concentrations of 64 mg/mL and higher.

Generally, susceptibility of *S. pyogenes* to the bactericidal activity of the extracts, aqueous and ethanolic, was not significantly different among different strains however, clinical strain seemed most susceptible, followed by 49399 and 19615.MBC numbers both aqueous and ethanolic in the decreasing order were sumac, squash berry, blackcurrant, cranberry, partridgeberry, haskap, mountain ash, and < crowberry,

blueberry. Both aqueous and ethanolic extracts of all berries were equally potent in killing the test strains and statistically no significant difference was observed.

Table 3: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of aqueous and ethanol extracts of selected Atlantic Canada fruits against *Streptococcus pyogenes* by micro-broth dilution method

	MIC (mg/mL)					
	Aqueous extracts			Ethanolic extracts		
	S. pyogenes strain					
Fruit source	19615	49399	Clinical	19615	49399	Clinical
Crowberry	1	1	0.50	4	1	2
Blackcurrant	4	1	4	1	2	1
Sumac berry	0.50	0.25	1	0.25	0.25	0.25
Squash berry	1	0.50	1	0.50	0.50	0.50
Cranberry	1	1	1	1	1	2
Wild Blueberry	8	4	8	1	1	8
Haskap	8	4	2	4	2	4
Partridgeberry	0.50	0.50	1	2	0.50	0.50
Buckthorn	>16	>16	>16	>16	>16	>16
Mountain ash	4	4	4	2	2	2
	MBC (mg/mL)					
	Aqueous extracts			Ethanolic extracts		
	S. pyogenes strain					
	19615	49399	Clinical	19615	49399	Clinical
Crow berry	64	64	32	64	64	32
Blackcurrant	16	16	16	16	16	8
Sumac berry	4	8	8	4	4	4
Squash berry	16	16	8	16	16	16
Cranberry	16	16	16	16	16	8
Blueberry	64	>64	>64	64	>64	>64
Haskap	16	32	16	16	32	16
Partridgeberry	16	16	16	16	16	8
Buckthorn	>16	>16	>16	>16	>16	>16
Mountain ash	16	16	32	16	16	32

MIC values for penicillin G (antibiotic control) are 2, 4, and 2 ng/mL for *S. pyogenes* ATCC 19615, ATCC 49399, and clinical strain respectively. Student *t*-test was used to determine the significant differences between the control and the extract / compound treatment ($P \leq 0.05$). MBC for penicillin G (antibiotic control) are 16, 64, and 16 ng/mL for *S. pyogenes* ATCC 19615, ATCC 49399, and clinical strain, respectively. Abbreviations: 19615; *S. pyogenes* ATCC 19615, 49399; *S. pyogenes* ATCC 49399, Clinical; a *S. pyogenes* clinical strain isolated from a patient

Killing rate of *S. pyogenes* ATCC 19615 by cranberry and sumac aqueous and ethanolic extracts was determined to assess the efficacy of selected extracts (Figure 3). Cranberry aqueous extract at concentration of 8 mg/mL (8 × MIC) following 24 hour exposure lowered the bacterial population to ~1.6-log CFU/mL below the initial inoculum (5.1-log CFU/mL) accounting for about 30% kill rate. At 24h of incubation the population in the control well relative to initial inoculum nearly doubled (~9.8-log CFU/mL) whereas in the 8 mg/mL cranberry aqueous extract treated well only 70% of the initial inoculum survived (~3.5-log CFU/mL). Unlike the comparatively sharp decrease at between the 8 to 24-hour incubation times with 8 mg/mL cranberry aqueous extract treatment, a rather smooth decline is observed for 6-8 hours time.

A similar trend was recorded for cranberry ethanolic extract however regrowth patterns for the two differ slightly. At concentration of 8 mg/mL (8 × MIC) after 24 hof incubation cranberry ethanolic extract lowered the bacterial population to ~1.8-log CFU/mL below the initial inoculum (~5.0-log CFU/mL) accounting for about a 35% kill rate. Except for 8 × MIC concentration (8 mg/mL) where there is smooth decline in the population from time 6 to 8 hours, a rather sharp increase is evident for other tested concentrations (0.5 × MIC to 4 × MIC).

Although minimum inhibitory and bactericidal concentrations for aqueous and ethanolic extracts of cranberry are relatively similar, time kill kinetics better elucidates how diverse their inhibitory activities against *S. pyogenes* could be. While at 24-hour exposure time, viable cells in 2 × MIC aqueous cranberry extract treated well (relative to initial inoculum) grew by 81%, ethanolic extracts led to 68% increase only.

At 2-hour time sumac aqueous extract at concentration of 8 × MIC (4 mg/mL) caused ~0.6-log CFU/mL drop and the number kept increasing to ~1.6-log CFU/mL (kill rate of 32%) at 8 hours to end with >3-log CFU/mL reduction relative to initial inoculum after 24 hours incubation. The logarithmic phase lingered at a concentration of 4 × MIC of sumac aqueous extract.

Sumac ethanolic extract at the highest concentration tested (2 mg/mL) at the 10 minute sampling time killed about 7% of the initial inoculum. The bacterial growth at 2 mg/mL was slowed down leading to kill rate of 1% relative to initial inoculum after 24 hours incubation. In other words, sumac ethanolic extracts completely reduced the reproducing capability of the strain within a 24h period.

Penicillin G at 16 ng/mL, and 6-8 hours exposure time decreased the population to ~1.0-1.2-log CFU/mL relative to initial inoculum (4.5-log CFU/mL) respectively and it reached the maxima at 24 hours where >3-log CFU/mL reduction was observed. Logarithmic phase was delayed at 4 × MIC. Kill rate of bacteria relative to initial inoculum at 8 hours time and the concentration of 8× MIC was 26%.

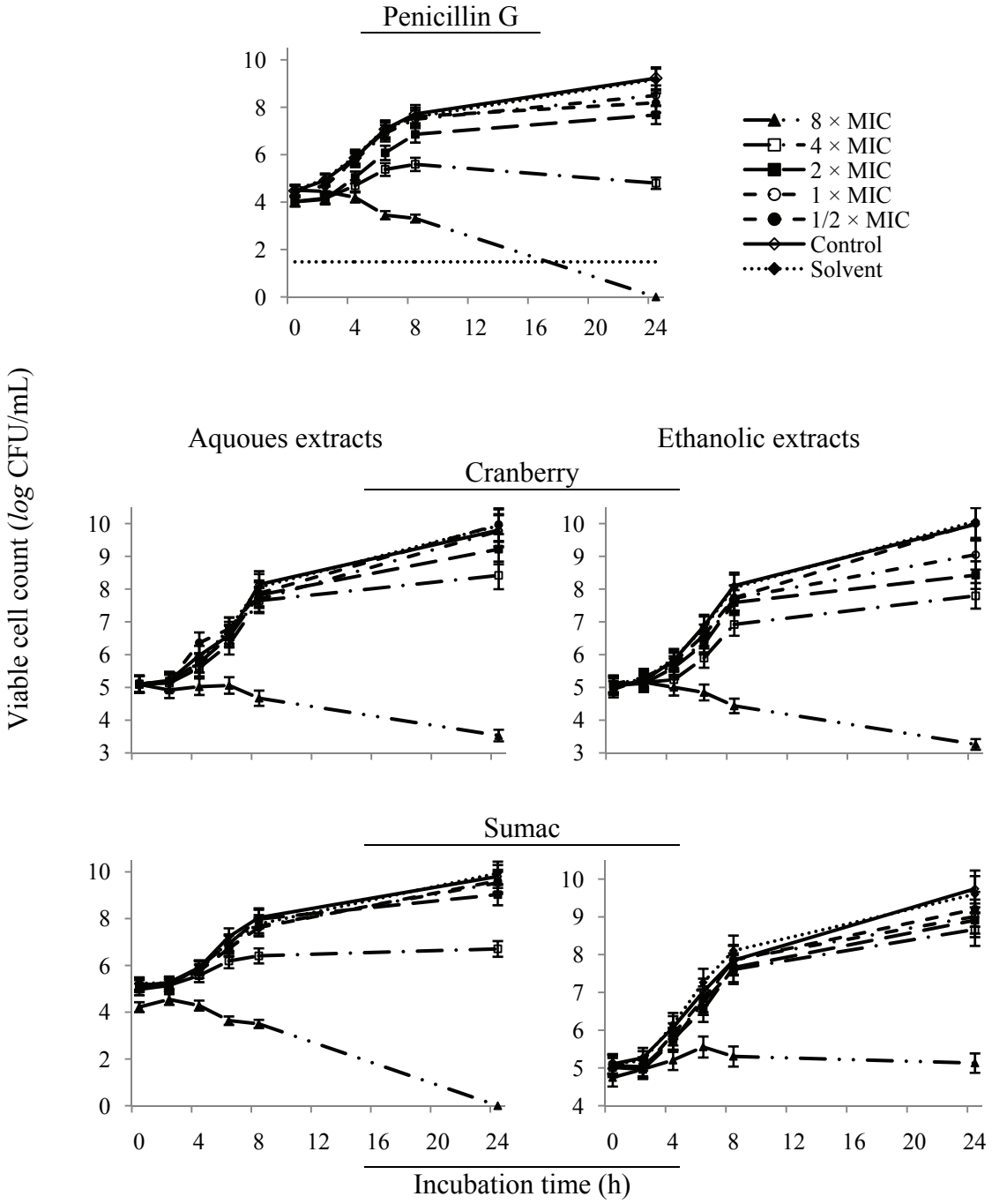


Figure 3: Time-kill kinetics for *S. pyogenes* ATCC 19615 in the presence of different extracts / compounds. Wells without extracts / compounds are considered control (positive control). Solvent (solvent control) means the highest concentration of distilled sterile water used to dissolve the extract (6.25% for cranberry aqueous and ethanolic extracts, 3.13% for sumac berry aqueous extract, 1.56% for sumac berry ethanolic extract, and 0.02% for penicillin G). The error bars represent the standard deviation of triplicate measurements. Limit of quantification is represented as dotted line (…….).

4.2. Dose-effect of selected phytochemicals-rich extracts of Atlantic Canada fruits on *S. pyogenes* biofilm formation

A range of concentrations of extracts from sub-MIC to multiples of MIC were examined by MTT assay and plate counts to better understand the dose effect of the extracts on the biofilm formation and to evaluate the correlation of two methods (Figure….). Overall, tested extracts could prevent the biofilm formation completely at concentrations higher than MIC (Figure6). A wide range of cranberry aqueous and ethanolic extract concentrations, 0.5-16 mg/mL, were tested and at high concentrations of 8-16 mg/mL viable cells were below detection limit and not quantifiable (only semi-quantitative measurement could be achieved by MTT assay). In the same way, sumac aqueous and ethanolic extracts were tested at concentrations of 0.13-8 mg/mL, though viable cells being undetectable at 2-8 mg/mL.

In general, MBIC against *S. pyogenes* ATCC 19615 and clinical ranged from 1 mg/mL to 4 mg/mL for the tested extracts both for MTT and plate count methods. The inhibition effects were largely dose-dependent for cranberry extracts. Sumac extract's inhibition effects were not as concentration-dependent as cranberry although at times insignificant. MBIC of sumac aqueous and ethanolic extracts, cranberry aqueous and ethanolic, as well penicillin G detected by MTT assay for ATCC 19615 were in the order of 2, 4, 4, 4 mg/mL and 4 ng/mL. MBIC obtained by viable cell counts for aqueous and ethanolic extracts of sumac, aqueous and ethanolic extracts of cranberry, and penicillin G against ATCC 19615 are 1, 1, 2, 4, mg/mL and 8 ng/mL, correspondingly. MBIC obtained from the two methods for clinical strain's biofilm inhibition by sumac aqueous

and ethanolic extracts, cranberry aqueous and ethanolic, as well penicillin G were 1-2, 1, 2, 2, mg/mL and 4 ng/mL respectively. Although two methods were in considerable agreement detecting the MBIC for clinical strain in this case but dose-effects could not be explained clearly by MTT assay. Wide ranges of cranberry aqueous extract concentrations, 0.5-16 mg/mL, were tested nevertheless at high concentrations of 8-16 mg/mL viable cells were not quantifiable.

In the plate count method, 2 mg/mL of cranberry aqueous extract notably inhibited the biofilm formation of ATCC 19615 ~17% (~2.5-log CFU/mL reduction relative to positive control). Although results of two methods followed a similar trend and were highly correlated (Table4), however 4 mg/mL was the MBIC accounting for 48% inhibition suggested by MTT assay (Figure 6). Metabolic activity measurement of cranberry aqueous extract against clinical strain shows rather different pattern meaning that at the presence of sub-MIC concentration biofilm formation was significantly elevated (relative to positive control). This pattern was absent in plate count method. Clinical strain's biofilm formation was significantly hindered by 2 mg/mL cranberry aqueous extract to ~15% (~1.1-log CFU/mL reduction) and ~33% for plate and MTT method respectively (Figure 6).

Cranberry ethanolic extract at concentration of 4 mg/mL lessened the ATCC 19615 biofilm formation significantly; 32% (~2.5-log CFU/mL reduction) and 43% for plate and MTT method respectively. At concentration of 2 mg/mL of cranberry ethanolic extract, biofilm formation of clinical isolate of *S. pyogenes* was depressed to ~10% (0.8-log CFU/mL reduction) and 19% for plate and MTT method respectively. Interestingly, similar to its counterpart, biofilm growth of clinical strain in the presence of cranberry

ethanolic extract, measured colorimetrically, was relatively higher, ~32%, at 0.5 mg/mL compare to other concentrations. Plate counts however show only insignificant clinical strain viable cell increase of about 3% (~0.2-log CFU/mL higher) relative to positive control for 0.5 mg/mL of cranberry ethanolic extract thus elucidating a significant difference between the results of MTT assay and plate count.

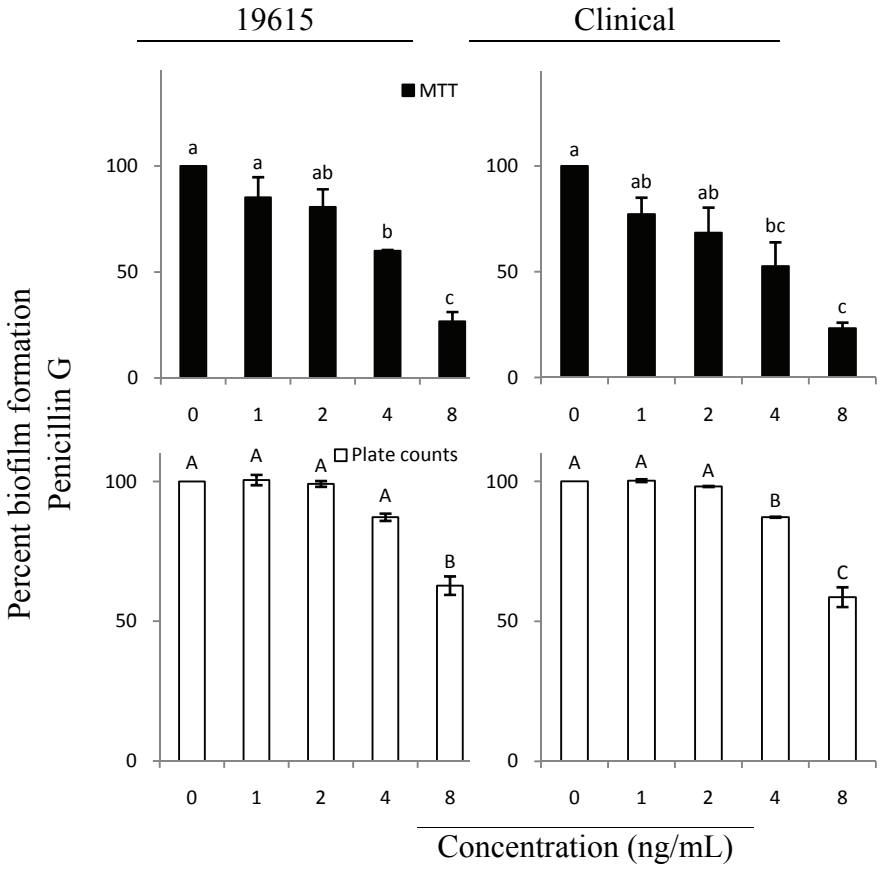


Figure 4: Inhibitory effects of penicillin G on *S. pyogenes* biofilm formation measured by quantitative and semi-quantitative methods. Data are expressed as percent biofilm formation relative to the positive control (control: 100%).The difference between solvent control and the positive control was insignificant. All panels represent the means of three independent experiments. A-C and a-c letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements.

Biofilm formation inhibitory effect of sumac aqueous extract against both the strains of *S. pyogenes,* measured by MTT assay, was evident at 2 mg/mL and on the other hand, plate counts suggest the concentration of 1 mg/mL with minimum significant biofilm diminishing effects. Another interesting observation is that unlike cranberry aqueous and ethanolic extract exhibiting dose dependent response, sumac aqueous extract exerts dose-independent effects whether significant or insignificant.

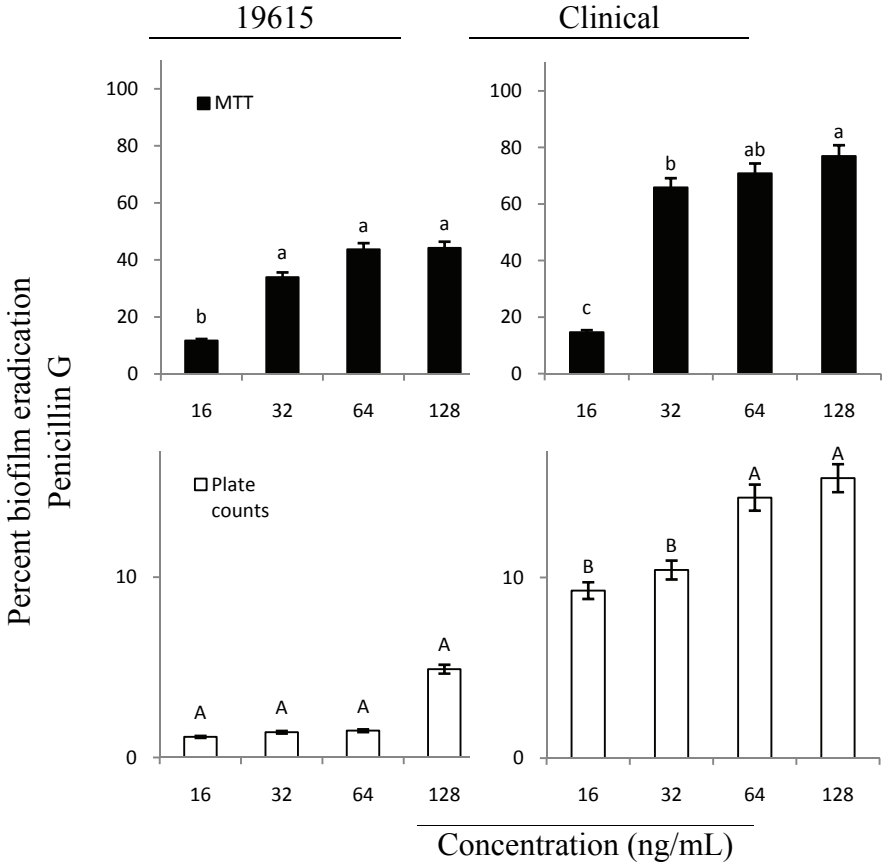


Figure 5: *S. pyogenes* biofilm eradication activity of penicillin G measured by quantitative and semi-quantitative methods. Percent biofilm-eradication activity of solvent control, 1 × MIC and ½ × MIC concentrations for all the tested compounds were not significantly different from positive control. All panels represent the means of three independent experiments. A-B and a-b letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, solvent and positive control. The error bars represent the standard deviation of triplicate measurements.

At 4 mg/mL concentration biofilm formation of ATCC 19615, even though neglectable, was higher (~2%) than the 2 mg/mL accounting for ~0.2-log CFU/mL higher viable biofilm cells hence showing no dose dependent activity. In plate count method, sumac aqueous extract lowers the viable biofilm cells of both strains 1.2-1.6-log CFU/mLat 1 mg/mL concentration while in MTT method the difference among control and treatment group become significant only at 2 mg/mL. Measurement of efficacy of sumac aqueous extract against *S. pyogenes* biofilm by MTT and plate count methods correlate strongly and weakly for 19615 and clinical strains correspondingly (Table 4).

As the most common beta lactam treatment of infections caused by *S. pyogenes* penicillin G was tested (Figure 4). Evidently confirmed by both methods, biofilm inhibitory effects are dose dependent however, concentration suggested as MBIC may differ. According to results obtained from MTT method, penicillin G at 4 ng/mL inhibits the biofilm formation of *S. pyogenes* ATCC 19615 and clinical strains to ~40%-50%. Plate counts suggest MBIC of *S. pyogenes* ATCC 19615 to be 8 ng/mL (1.6-log CFU/mLreduction relative to control) and the MBIC for clinical strain to be 4 ng/mL (~1.8-log CFU/mL reduction). For ethanolic extract of sumac, more or less similar inclination is observed. MTT method points 2-4 mg/mL of ethanolic extract of sumac as the MBIC for *S. pyogenes* ATCC 19615 while plate method suggests 1 mg/mL with the lowest significant inhibitory effects (1.8-log CFU/mL lower than control). At 1 mg/mL sumac ethanolic extract exhibits its least *S. pyogenes* clinical strain biofilm inhibitory effects confirmed by both the methods. Sumac extracts inhibit the biofilm formation of *S. pyogenes* clinical strain in dose-independent manner.

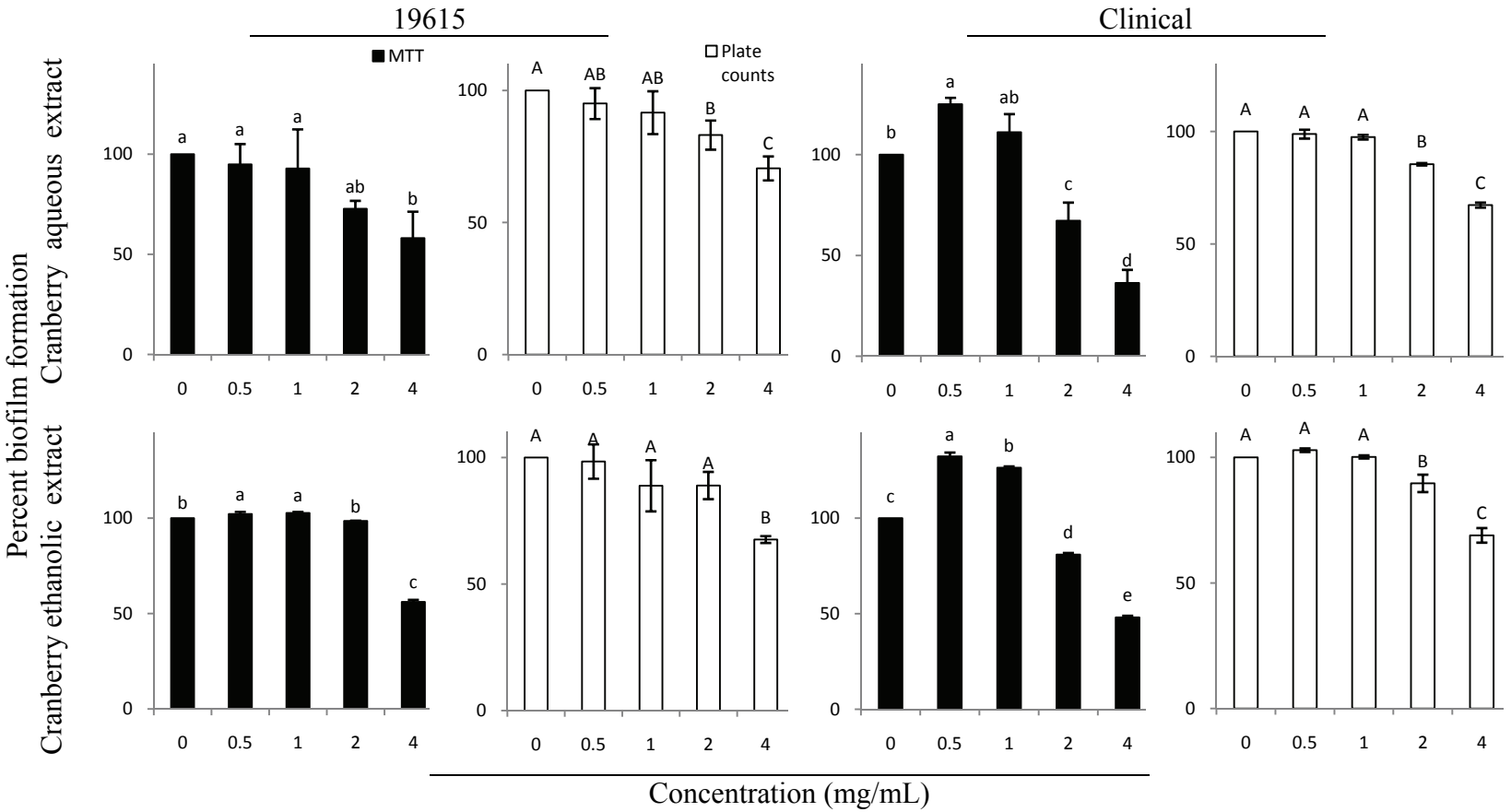


Figure 6-A: Inhibitory effects of cranberry extracts on *S. pyogenes* biofilm formation measured by quantitative and semi-quantitative methods. Data are expressed as percent biofilm formation relative to the positive control (control: 100%).The difference between solvent control and the positive control was insignificant. All panels represent the means of three independent experiments. A-C and a-e letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements. Abbreviations: 19615; *S. pyogenes* ATCC 19615, clinical; *S. pyogenes* clinical strain

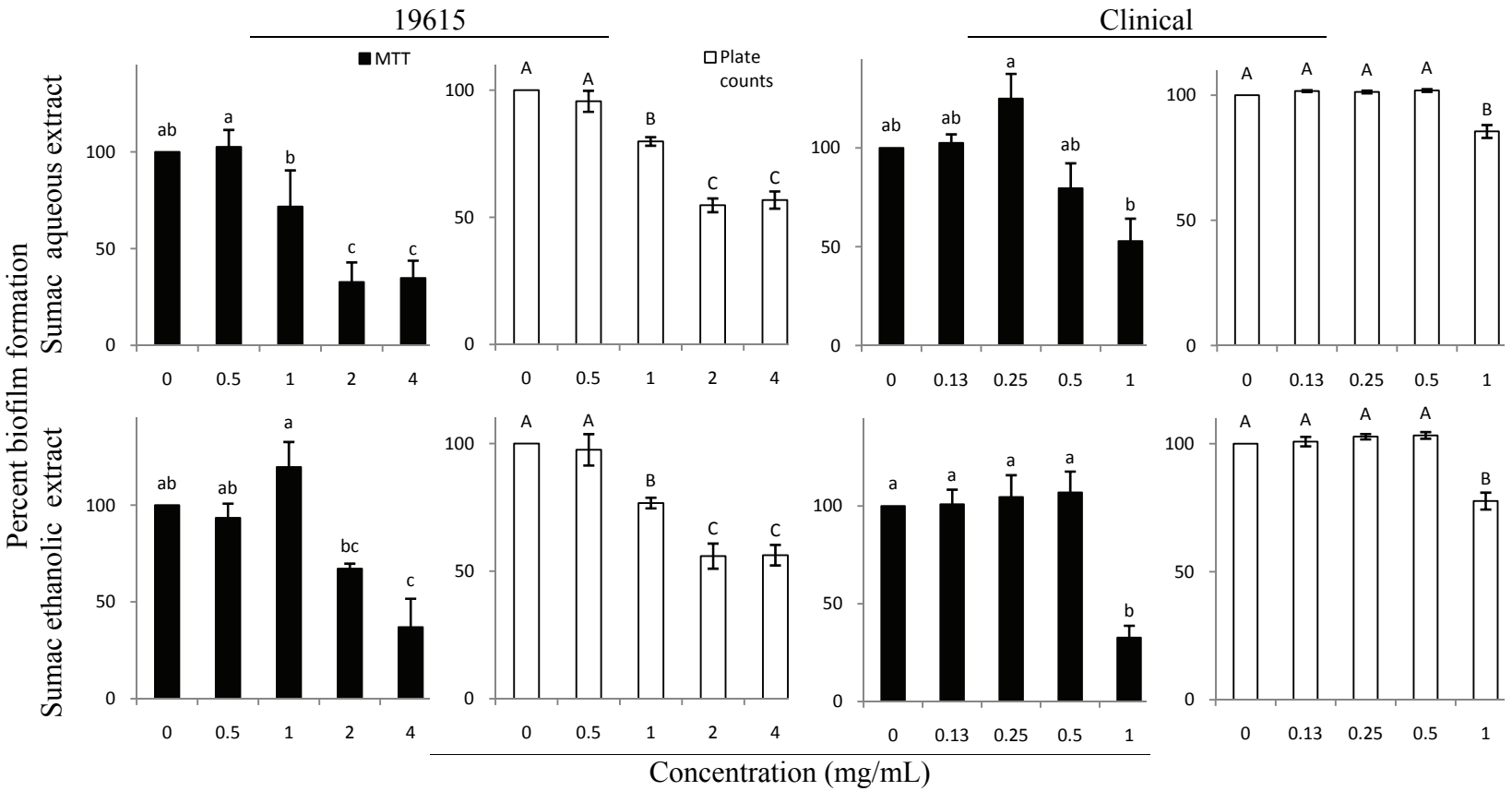


Figure 6-B: Inhibitory effects of sumac extracts on *S. pyogenes* biofilm formation measured by quantitative and semi-quantitative methods. Data are expressed as percent biofilm formation relative to the positive control (control: 100%).The difference between solvent control and the positive control was insignificant. All panels represent the means of three independent experiments. A-C and a-c letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements. Abbreviations: 19615; *S. pyogenes* ATCC 19615, clinical; *S. pyogenes* clinical strain

4.3. Dose-effect of selected phytochemicals-rich extracts of Atlantic Canada fruits on *S. pyogenes* pre-established biofilm

To better understand the dose effect of the extracts on eradication of pre-established the biofilms (24 hours old), a range of concentrations from sub-MIC to multiples of MIC extending to typically two times the MBC was examined by two methods of MTT assay and the plate count. Overall, tested extracts could eliminate the viable cells of pre-established biofilm to ~ 50% at two times the MBC if not completely (Figure7). The lowest concentration of the extracts that significantly (compare to control with 0% eradication) inhibited regrowth of the bacteria from the treated biofilm was defined as MBEC. In general, MBEC obtained by MTT and plate count methods for both strains (ATCC 19615 and the clinical) ranged 0.5-16 mg/mL for all the tested extracts. Moreover, it was evident that ~ 1-log CFU/mL more viable cells occupy the pre-established biofilms of ATCC 19615 than the clinical counterpart thus concluding that ATCC strain is a relatively stronger biofilm former than the clinical one.

A wide range of cranberry aqueous and ethanolic extract concentrations, 0.5-32 mg/mL were tested. Correspondingly, sumac aqueous and ethanolic extracts were tested at concentrations of 0.25-16 mg/mL, though viable cells were unquantifiable at 2-8 mg/mL (only semi-quantitative measurement by MTT assay could be made).MBEC of cranberry aqueous and ethanolic, sumac aqueous and ethanolic extracts as well penicillin G detected by MTT assay for ATCC 19615 were in the order of 4, 4, 2, 1 mg/mL and 32 ng/mL (Figure 5, 7). MBEC obtained by plate counts for aqueous and ethanolic extracts of cranberry, aqueous and ethanolic extracts of sumac and penicillin G against ATCC 19615 are 8, 8, 1, 0.5 mg/mL and >128 ng/mL, correspondingly. MBEC reordered by

MTT assay for clinical strain's biofilm eradication by cranberry aqueous and ethanolic, sumac aqueous and ethanolic extracts as well penicillin G were 8, 2, 16, 4 mg/mL and 16 ng/mL respectively. Similar results were suggested by plate counts for clinical strain's biofilm eradication except for cranberry ethanolic extract with the MBEC of 16 mg/mL. Eradiation effects were mostly dose dependent with some exceptions.

Cranberry aqueous extract at 8 mg/mL significantly eradicated the biofilm of ATCC 19615 (~0.8-log CFU/mLreduction compare to positive control with zero eradication). Yet MTT metabolic activity semi-quantitative measurement suggests 4 mg/mL to be effective enough to eradicate the biofilm of ATCC 19615 by ~37%. At 16 mg/mL (equaling MBC of cranberry aqueous extract) according to plate count and MTT methods eradication percentage of ATCC 19615 was 33% (2.7-log CFU/mL lower) and 95% respectively. In this study, we report MBEC of cranberry aqueous extract for the clinical strain to be 8 mg/mL nevertheless plate count confirms only ~10% eradication while MTT demonstrates ~62% eradication compare to positive controls. The response of both the test stains to cranberry aqueous extract is dose-dependent. At the highest concentration tested (32 mg/mL), viable bacteria of the eradicated *S. pyogenes* biofilm decreased to ~4.8-4.9-log CFU/mLillustrating ~36-42% eradiation after 3-hour treatment.Cranberry ethanolic extract displayed its eradication effects at 4 mg/mL against the biofilm of ATCC 19615 conducting the MTT method while at the same concentration viable bacterial count was only insignificantly different, ~6%, from the positive control. Minimum effective concentration of cranberry ethanolic extract against ATCC 19615 demonstrated by plate counts was 8 mg/mL (~0.9-log CFU/mL reduction). The same extract acted little differently on the clinical strain pre-formed biofilms.

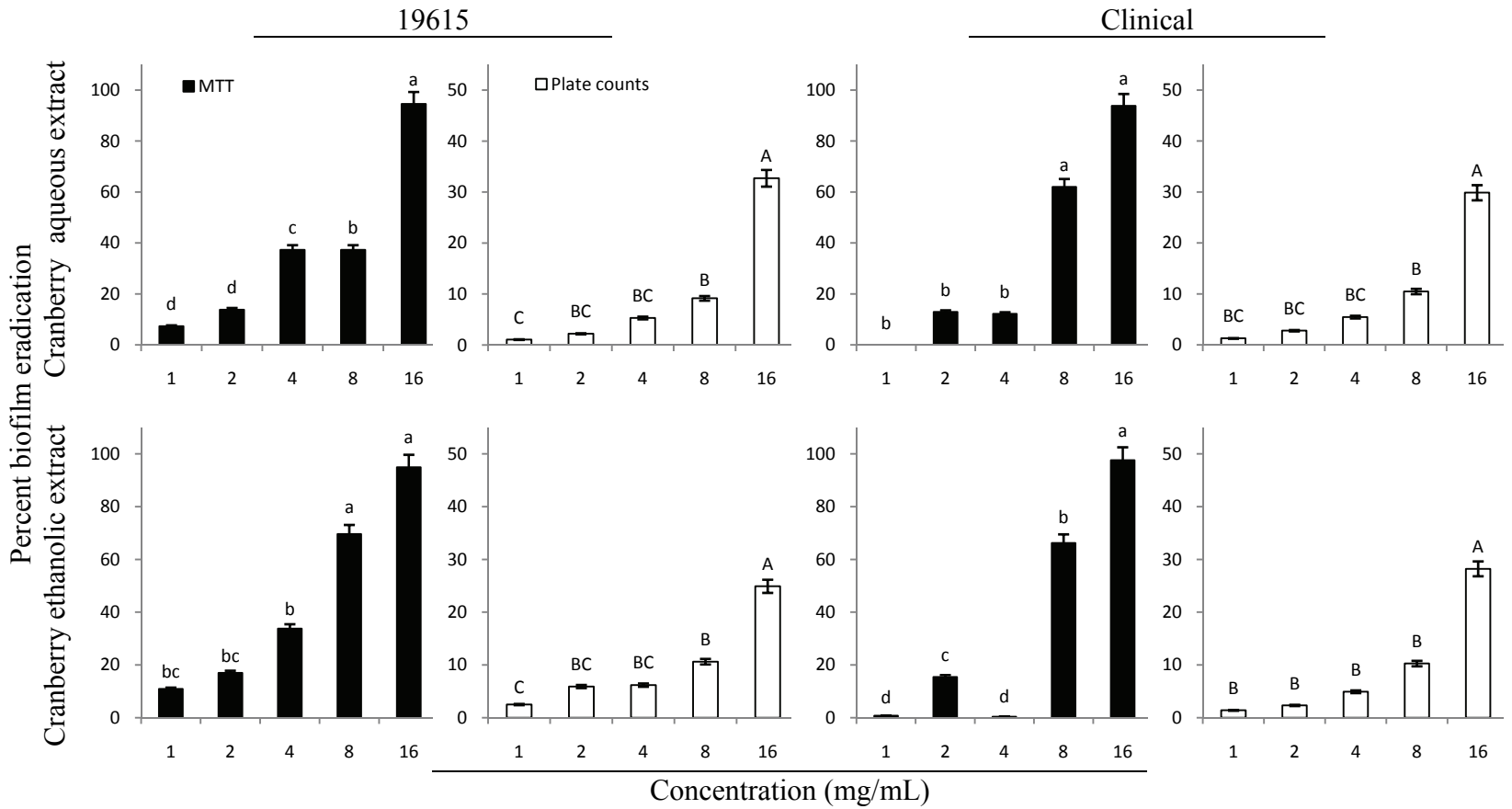


Figure 7-A: *S. pyogenes* biofilm eradication activity of cranberry extracts measured by quantitative and semi-quantitative methods. Percent biofilm-eradication activity of solvent control, 1 × MIC and ½ × MIC concentrations for all the tested compounds were not significantly different from positive control. All panels represent the means of three independent experiments. A-C and a-d letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements.

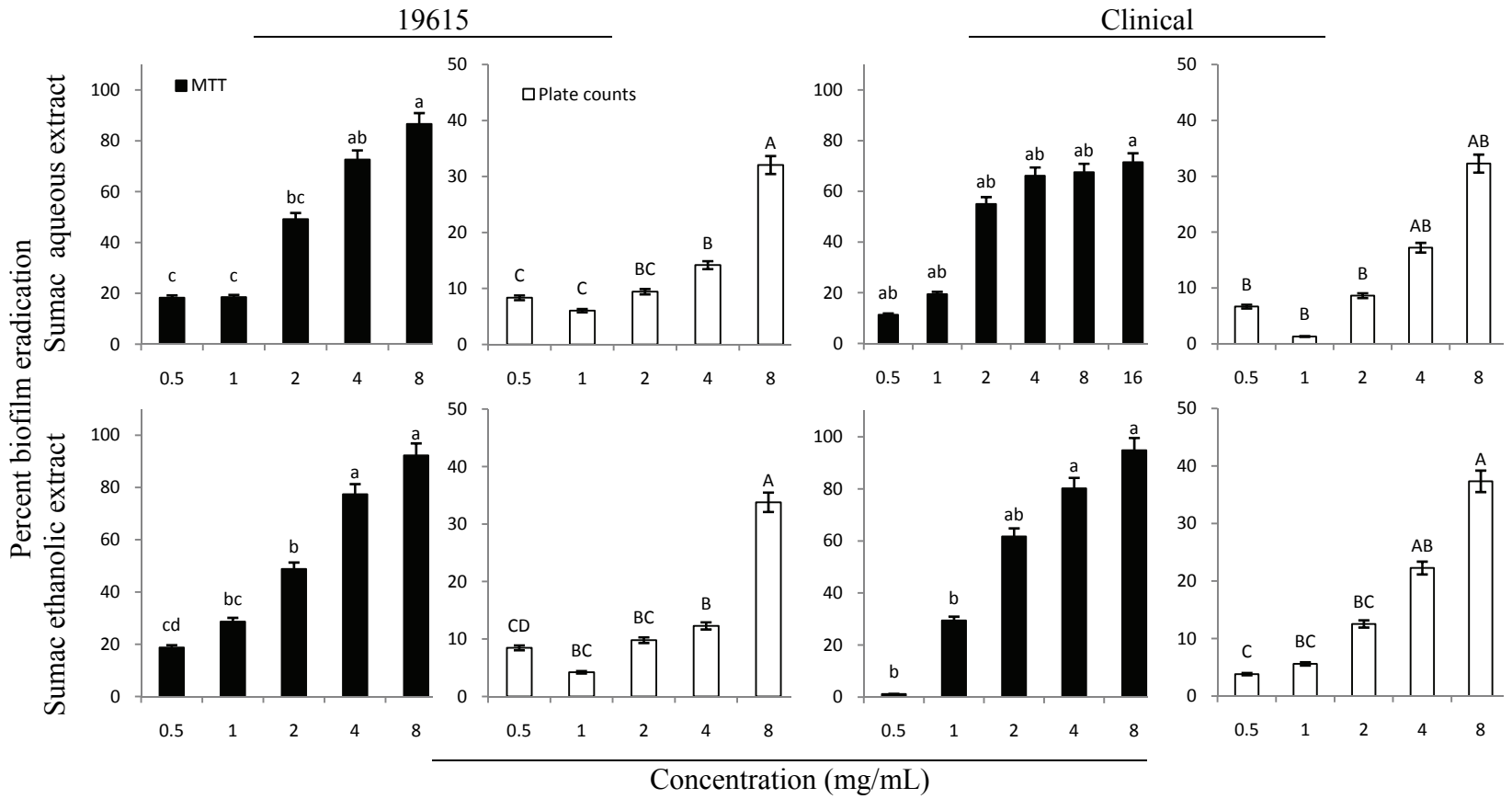


Figure 7-B: *S. pyogenes* biofilm eradication activity of sumac extracts measured by quantitative and semi-quantitative methods. Percent biofilm-eradication activity of solvent control, 1 × MIC and ½ × MIC concentrations for all the tested compounds were not significantly different from positive control. All panels represent the means of three independent experiments. A-D and a-d letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements.

Clinical strain biofilm was statistically significantly eradicated, ~15% by 2 mg/mL, and ~28% (2.1-log CFU/mL reduction) by 16 mg/mL cranberry ethanolic of extract respectively measured by MTT assay and plate counts. Ethanolic extract of cranberry had comparable effect to aqueous extract at the highest concentration tested, 32 mg/mL. Viable bacterial cells of the eradicated *S. pyogenes* biofilm decreased to ~4.3-4.5-log CFU/mL illustrating ~42-46% eradiation after 3-hour treatment.

Semi-quantitative measurement of percent eradiation effect of sumac aqueous extract against *S. pyogenes* biofilm was dose-dependent nevertheless plate counts were pointing at its relatively significant dose-independent effects. By viable cell quantification, 1 mg/mL exhibits smaller eradication effect (~2-4%) than the 0.5 mg/mL. While at 0.5 mg/mL sumac aqueous extract eliminated significant numbers of viable cells of ATCC 19615 biofilm, MTT assay unexpectedly reveals 2 mg/mL as MBEC. Sumac aqueous extract at 16 mg/mL showed significant eradication of clinical strain biofilm by both methods. At 16 mg/mL of sumac aqueous extract eradication could be reached up to ~42-47% for both the strains with the viable cell counts decreasing to ~4.3-4.4-log CFU/mL.

As suggested by MTT method, sumac ethanolic extract significantly eradicates the biofilm of ATCC 19615 by ~29% at concentration of 1 mg/mL in a dose-dependent manner whereas colony counts confirms 0.5 mg/mL with ~8% (0.7-log CFU/mL) eradication as MBEC in dose independent manner. Although rationally 1 mg/mL concentration should increase the eradication power but rather this falls to only to ~4% (0.4-log CFU/mL) eradication of ATCC 19615 biofilm. Clinical strain's biofilm is eradicated does-dependently at 4 mg/mL to significant level of ~80% and 22% by MTT

assay and plate counts, respectively. At the highest concentration with quantifiable counts (8 mg/mL), eradication was ~33-34% with 2.5-2.8-log CFU/mL drop in the viable cell numbers.

Table 4: Correlation of results obtained from plate count method and MTT assay

		Pearson's correlation coefficient (*r*) with *P*-value			
		S. pyogenes strain			
		19615	Clinical	19615	Clinical
Compound / Extract		Biofilm formation		Biofilm eradication	
Cranberry	Aqueous	0.988 (0.002)	0.948 (0.014)	0.977 (0.004)	0.937 (0.019)
	Ethanolic	0.916 (0.029)	0.936 (0.019)	0.926 (0.024)	0.917 (0.028)
Sumac	Aqueous	0.995 (0.000)	0.788 (0.113)	0.843 (0.073)	0.771 (0.127)
	Ethanolic	0.687 (0.200)	0.999 (0.000)	0.813 (0.094)	0.907 (0.034)
Antibiotic	Penicillin G	0.963 (0.008)	0.908 (0.033)	0.596 (0.289)	0.838 (0.076)

Correlation analysis was performed with Minitab v. 16.
Abbreviations: 19615; *S. pyogenes* ATCC 19615, clinical; *S. pyogenes* clinical strain

Viable bacterial cells of penicillin-eradicated *S. pyogenes* ATCC 19615 biofilm showed no effect up to eight times the MBC. Suggested by plate counts at 16 ng/mL clinical strain biofilm was ~9% eradicated. Semi-quantitative measures show eradication of biofilms from ~15-49% by 16-32 ng/mL of penicillin respectively for clinical and ATCC 19615 strain of *S. pyogenes* (Figure 5).

4.4. Dose-effect of selected phytochemicals-rich extracts of Atlantic Canada fruits on *S. pyogenes* adherence

Dose effect of aqueous extracts of cranberry and sumac against initial adhesion of *S. pyogenes* to uncoated polystyrene surface was tested at various concentrations ranging

from sub-MIC, multiples of MIC extending to MBC. Results are presented as log CFU/mL of adherent bacterial cells after 1-hour treatment (Table 5).

Cranberry aqueous extracts at concentrations of 0.5-16 mg/mL were tested however, the viable cells for wells treated with MBC concentration were not quantifiable. At 8 mg/mL, cranberry aqueous extract significantly reduced the adhesion of ATCC 19615 compare to the positive control ($P< 0.05$). The adherence was reduced to ~77% while non-treated bacteria adhered to the well surface up to ~86%. Solvent control (6.25% sterile distilled H_2O in BHI broth), although only moderately significant, led to more bacterial adhesion relative to positive control up to ~89%. Generally, cranberry aqueous extract had dose dependent anti-adhesion effect on attachment of ATCC 19615 to uncoated polystyrene surfaces. At the same concentration (8 mg/mL), cranberry aqueous extract did not had significant effect on the adhesion of clinical strain of *S. pyogenes* to uncoated polystyrene surfaces but the higher concentration (16 mg/mL) reduced the viable cell count to below the quantification limits. Dose-independent effects of cranberry aqueous extract against clinical strain were observed, yet were statistically insignificant ($P< 0.05$). Another interesting fact was that in the presence of solvent only adherence of clinical strain was elevated (~86%) to statistically significant level compare to positive control (~82%). Adherence inhibition activity of cranberry aqueous extract to uncoated polystyrene surfaces seems more outstanding, yet insignificant, towards ATCC 19615 than clinical strain.

A mixture of concentrations of sumac aqueous extracts was tested (0.25-8 mg/mL). At 1 mg/mL attachment of ATCC 19615 dropped to ~76%, compare to the positive control with 86% adhesion. Solvent (6.25% H_2O) had moderately positive and

significant effect (~3% adhesion increase) on the attachment of ATCC strain. Adherence of ATCC 19615 was inhibited in a statistically significant dose-dependent manner by sumac aqueous extract. Compare to the adhesion of ATCC strain, it took approximately double the concentration of sumac aqueous extract (2 mg/mL) to significantly reduce the adhesion of clinical strain to uncoated surface. Solvent effect on attachment of clinical strain was similar to that of ATCC strain. A similar dose-response trend was evident for the attachment of clinical strain. The highest concentration of sumac aqueous extract with quantifiable viable cells exhibited ~54% and 60% adherence of ATCC 19615 and clinical (4 mg/mL) correspondingly compare to the positive control of the assay. Bacterial counts fell below quantification limits for both strains at 8 mg/mL of sumac aqueous extract.

Penicillin considered the most common antibiotic therapy of the GAS infections was tested. No effect could be documented for penicillin G (0.5-16 ng/mL).

Inhibitory effects of aqueous extracts of cranberry and sumac on the adherence abilities of *S. pyogenes* to protein-coated surfaces, namely BSA and fibronectin were studied. Similar to the previous assays a range of concentrations from sub-MIC to MBC were included in the assay (Figure 8). Cranberry aqueous extract were examined at the concentrations of 0.5-16 mg/mL. At 8 mg/mL of cranberry aqueous extract adhesion of *S. pyogenes* to BSA coated wells were considerably reduced to ~75-80% compare to positive control with ~82-88% adherence for strains clinical and ATCC 19615 correspondingly.

Table 5: Inhibitory effects of cranberry and sumac aqueous extracts / compound on the adherence of *S. pyogenes* to uncoated surfaces after one-hour treatment

Conc. (mg/mL)	Adherent bacteria (log CFU/mL)				Conc. (ng/mL)	Penicillin G	
	Aqueous extracts						
	Cranberry		Sumac				
	19615	Clinical	19615	Clinical		19615	Clinical
0	4.93±0.35^{a}	4.60±0.02^{b}	4.93±0.35^{ab}	4.60±0.02^{ab}	0	4.57±0.13^{a}	4.80±0.13^{a}
0.25	NT	NT	4.59±0.20^{abc}	4.44±0.08^{ab}	0.25	NT	NT
0.5	4.87±0.42^{ab}	4.55±0.02^{b}	4.53±0.35^{bc}	4.33±0.12^{ab}	0.5	4.62±0.18^{a}	4.79±0.11^{a}
1	4.84±0.45^{ab}	4.62±0.01^{ab}	4.35±0.58^{c}	4.21±0.13^{bc}	1	4.59±0.14^{a}	4.80±0.14^{a}
2	4.81±0.34^{ab}	4.59±0.05^{b}	3.77±0.32^{d}	3.89±0.27^{c}	2	4.56±0.11^{a}	4.75±0.12^{a}
4	4.72±0.29^{bc}	4.51±0.06^{b}	3.30±0.23^{e}	3.32±0.33^{d}	4	4.55±0.08^{a}	4.71±0.16^{a}
8	4.42±0.17^{c}	4.47±0.07^{b}	BQL	BQL	8	4.54±0.09^{a}	4.69±0.17^{a}
16	BQL	BQL	NT	NT	16	4.46±0.05^{a}	4.63±0.10^{a}

Each data point represents log CFU/mL of adherent bacterial cells after 1-hour treatment. Viable adherent cell number of solvent control and positive control were not significantly different. a-e letters as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of triplicate measurements. Abbreviations: 19615; *S. pyogenes* ATCC 19615, clinical; *S. pyogenes* clinical strain, BQL; below lower limit of quantification, NT; not tested, Conc.; concentration

Dose effect of various concentrations of sumac aqueous extract from 0.25 to 8 mg/mL was examined. The adhesion of ATCC 19615 to BSA coated wells was significantly decreased from ~86% to ~73% after 1-hour treatment with 2 mg/mL of sumac aqueous extract. Adhesion was inhibited further to ~66% with 4 mg/mL of sumac aqueous extract and finally viable cell numbers drops below quantification limits with 8 mg/mL. In agreement with the inhibition of adherence to BSA, 2 mg/mL hinders the ability of ATCC 19615 to attach to fibronectin thus inhibition falls to ~1% compare to the control (~4%). Effect of sumac aqueous extract on attachment of ATCC 19615 to BSA and to fibronectin was dose-independent. Positive control of clinical strain adheres ~ 82% to BSA however, 2 mg/mL of sumac aqueous extract could bring this number down to ~70% and further to ~64% at 4 mg/mL concentration. Contradictory to this, sumac aqueous extract has insignificant effects on the adhesion of clinical strain to fibronectin and only at the concentration of 4 mg/mL percent adherence drops to ~2% compare to the control (adherence of ~6%). More or less effects of sumac aqueous extract on *S. pyogenes* clinical attachment to protein-coated surfaces are insignificantly dose-dependent.

Penicillin as the fighting agent of the GAS infections was checked at concentrations of 0.5-16 ng/mL. Interestingly at 8 ng/mL penicillin G caused a fall in percent adhesion of ATCC 19615 to BSA (~83%) compare to the control (~89%). The effect was dose dependent and at 16 ng/mL percent adhesion was reduced to ~81%.

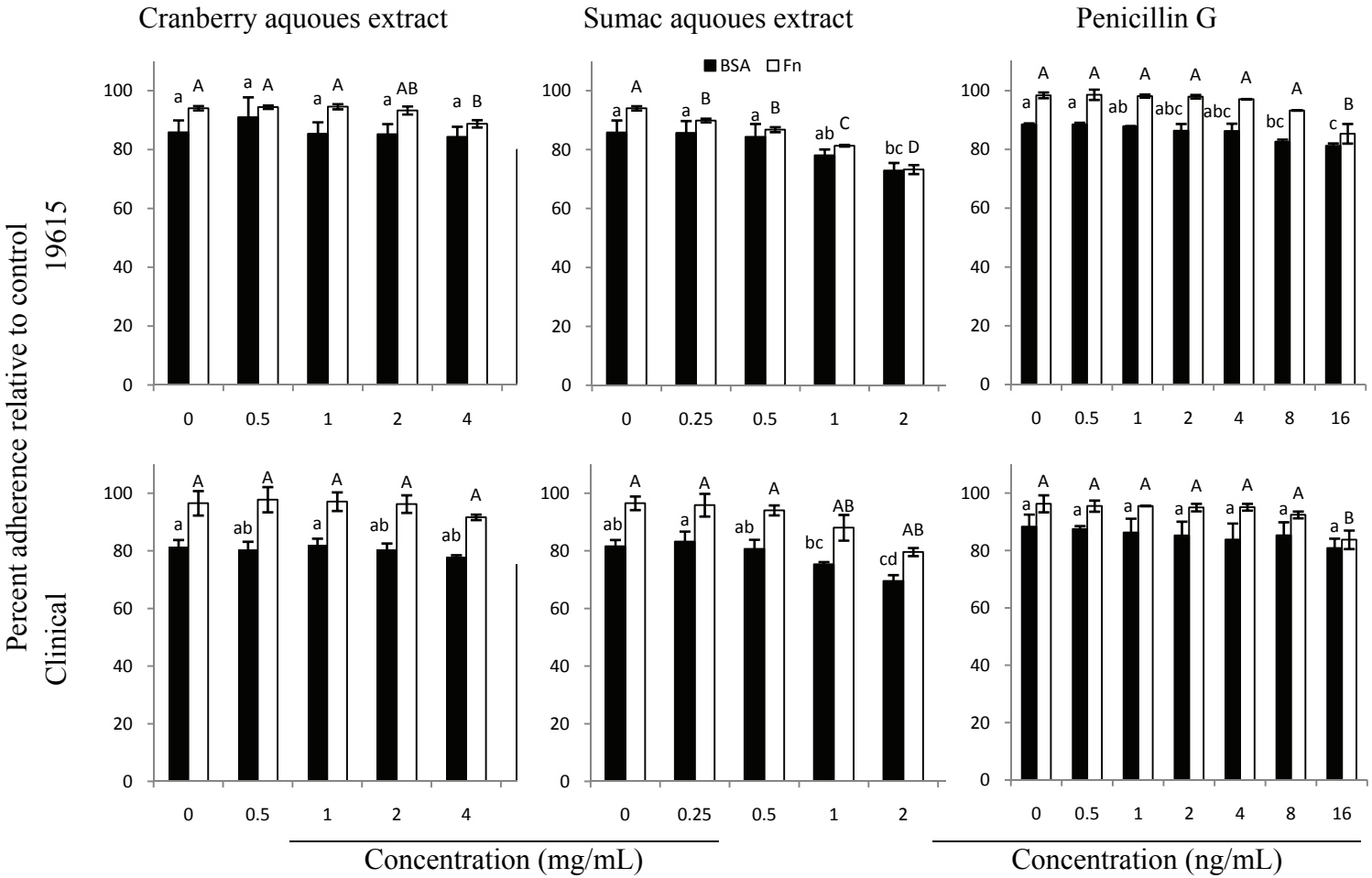


Figure 8: Inhibitory effects of cranberry and sumac aqueous extracts / compound on percent adherence of *S. pyogenes* to protein coated surfaces after 1-hour treatment. Data are presented as percent adherence relative to the initial inoculum. A-D and a-d letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. The error bars represent the standard deviation of at least two independent experiments. Abbreviation: BSA; bovine serum albumin, Fn: fibronectin

Conversely, penicillin G seemed to possess non-significant dose-independent effect on the attachment of ATCC 19615 to fibronectin thus reducing the percent adhesion from ~10% (control) to ~4% at concentration of 16 ng/mL. Comparable trend was noted for the effects of penicillin G on the adhesion attributes of clinical strain, though statistically not significant. Clinical strain attachment ability to BSA was reduced from ~88% to ~81% at 16 ng/mL. At equal concentration, fibronectin adherence capacity of clinical strain of *S. pyogenes* was cut from 8% to 3%.

Prior to initiation of the assay, cytotoxicity effects of the cranberry aqueous extract were examined by MTT assay (Figure 9). Analysis showed that cell viability percentage drops to ~20-27% at concentration of 8-16 mg/mL. At lower concentrations of 2 mg/mL and 4 mg/mL cell viability increased to 62 and 43% respectively.

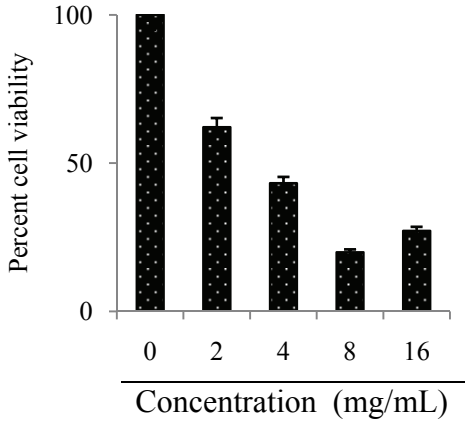


Figure 9: percent cell viability of tonsil (HTEpiC) cells after one-hour treatment with cranberry aqueous extract measured by MTT

Based on the results of cytotoxicity assay narrow range of concentrations 2-4 mg/mL was selected to the adherence inhibition test. At the concentrations tested cranberry aqueous extract did not affect the adherence of *S. pyogenes* to the tonsil cells

(Table6). Penicillin G at 16 ng/mL significantly reduced the adhesion of *S. pyogenes* ATCC 19615 and clinical to ~94% and ~93% (control: 100%), correspondingly.

Table 6: Inhibition effect of selected compounds on the adhesion of *S. pyogenes* to tonsil cells

Extract / compound		Percent adherence	
		S. pyogenes strain	
	Concentration	19615	Clinical
Penicillin G	8 ng/mL	99.1±0.7%[a]	97.2±1.4%[b]
	16 ng/mL	94.2±0.9%[b]	92.9±0.9%[c]
Cranberry aqueous extract	2 mg/mL	103.4±2.9%[a]	102.4±1.4%[a]
	4 mg/mL	101.3±1.8%[a]	101.5±1.5%[a]

Results are expressed as percent adherence of the bacteria to the HTEpiC cells relative to the control (control: 100%) ± standard deviation of triplicate measurements. Differences among solvent and positive controls were insignificant. a-c letters on bars as obtained by Tukey's multiple comparison test are used to indicate statistical significance ($P< 0.05$) of different concentration of compound, the solvent and positive control. Abbreviations:19615; *S. pyogenes* ATCC 19615, clinical; *S. pyogenes* clinical strain

CHAPTER 5 DISCUSSION

5.1. Dose-effect of phytochemicals-rich fruit extracts on planktonic growth of *S. pyogenes*

Fruits have repeatedly shown to be useful anti-infective agents against various microorganisms also pertinent to different kinds of berries (Table 2).The selected Atlantic Canada berries in this study are full of polyphenolic phytochemicals particularly flavonoids, anthocyanins, and phenolic acids. These polyphenols restrain the microbial growth in different ways. Among all microorganisms, *S. pyogenes* have also been tested for its susceptibility to natural antibacterial extracts or compounds (Figure 1). A few studies have examined the anti-streptococcal effect of phytochemicals against the ATCC test strain, ATCC 19615. Ethanolic extract of *Diospyros lotus* with high content of triterpenoids and tannins have MIC value of 5-10 mg/mL against *S. pyogenes* ATCC 19615 (*184*). Algae derived phlorotannin at 400 mg/L was bactericidal against *S. pyogenes* ATCC 19615 (*185*).

I have documented sumac extracts as the most potent phytochemical rich extracts against *S. pyogenes* ATCC 19615 and the clinical strain. All the cultivated berries excluding blueberry (MBC of ≥ 64 mg/mL) were relatively equally effective on the test strains (MBC of 8-32 mg/mL). Amongst wild grown berries, sumac berry extracts had relatively higher potency against test strains over others (MBC of 4-8 mg/mL). According to Smitran et al. (2015), isolates from GAS carrier adhere more than isolates from strep throat patients thus are more virulent. *S. pyogenes* ATCC 49399 have been isolated from a non-strep throat subject according to ATCC.

Blueberry and haskap aqueous extracts had the highest MIC values against ATCC 19615 and ATCC 49399. Mountain ash and blackcurrant extracts were least effective against clinical strain of GAS (Table 3). Blueberry wine pomace extracts with the MIC of 20 mg/mL inhibited the growth of *Salmonella* spp., *St. aureus*, and *E. coli* (*186*). Blueberry pomace extract at concentration of 400 µg/mL gallic acid equivalent also has bactericidal effect against the growth of *Pasteurella multocida,* the causative agent of cholera in poultry (*187*).Opposed to my results, phenolic fraction of haskap berries were effective against clinical strains of *S. aureus, P. aeruginosa, E. coli, S. agalactiae* at lower concentrations with the MIC ranging 125-500 µg/mL furthermore *S. agalactiae* being most sensitive and *P. aeruginosa* least sensitive (*156*). Similar to my results blackcurrant juice and methanolic extract were effective against wide range of microorganism (Gram-positive, Gram negative and fungi) at rather high concentrations (MIC / MBC of 62.5 to 500 mg/mL for juices and 3.125 to 500 mg/mL for extracts) (*155*). Comparable to my results mountain ash and blackcurrant extracts at relatively high concentrations of 20% have been effective growth inhibitors of Gram-positive and Gram-negative (*150*).

My results show that partridgeberry and cranberry exhibit similar effects on *S. pyogenes* growth. Cranberry, partridgeberry, and squash berry contain considerable amounts of flavonoids. Flavonoids can be effective antimicrobials *in vitro* and numerous studies have proven this fact (*41, 188, 189*). These compounds are produced by plants as defense chemicals to fight diverse microbial diseases therefore they could be effective growth inhibitors of pathogens (*190*). Flavonoids have been suggested to exert their

effect through complexing with extracellular proteins, soluble proteins, and bacterial cell walls (*191*).

My examination show the MIC and MBC values for aqueous and ethanolic extracts of cranberry in the range of 1-2 mg/mL and 8-16 mg/mL, respectively against all the tested strains (Table 3). Cranberry aqueous extract at 4 mg/mL inhibited the planktonic growth of *S. pyogenes* clinical, ATCC 19615 and ATCC 49399 by 56%, 58%, and 64% respectively. Cranberries contain large amounts of phenolic acids of which hydroxybenzoic is the most abundant (474–557 mg/100 g fresh weight) (*192*). Furthermore, phenolic acids have exhibited antimicrobial activities against a wide range of microbes (*41, 123, 193*). Phenolic acids possess greater antimicrobial effects against Gram-positive than Gram-negative. Phenolic acids, specifically protocatechuic, gentisic, ferulic, caffeic, vanillin, p-hydroxybenzoic acids and their alkyl esters demonstrated antibacterial and antifungal activities against representative microorganisms (*194*). Length of the alkyl chain (C_6 to C_{20}) may play a role in antimicrobial effect of phenolic acids (*193*). Increasing number of these chains can alter the cell membrane fluidity however the effects are dependent on the microorganism's cell-envelope structures (*195*). Polar hydroxyl group of phenolic acid could enter the molecular structure of the bacterial membrane by hydrogen bonds (*195*). The interesting fact that have been brought up in the same study was that Gram-negative bacteria are more affected by short chains (<C6) than longer ones (*195*). Fungi were inhibited by longer-chain alcohols than needed for Gram-negative inhibition thus longest chains were required to affect the Gram-positives (*195*).Commercial cranberry extract in a study showed remarkable antibacterial effect against Gram-positive bacteria (*Staphylococcus* species; *St. epidermidis* and *St. aureus*)

with MIC and MBC values in the range 0.16–5 mg/mL but ineffective against *E. coli* up to the tested concentration (20 mg/mL) (*136*).

Sumac extracts were most potent against *S. pyogenes*(Table 3). In a study by Nasar-Abbas et al. sumac (*Rhus coriaria*) extracts showed effect against Gram-positive and Gram-negative (*144*). MIC against Gram-positives like *Bacillus* species, and *L. monocytogenes* ranged 0.25-32% (w/v) and 0.67% (w/v) correspondingly (*144*). In general, Gram-negatives were more resistant with MIC ranging 0.42-0.67% (w/v) (*144*). Another study aimed at determining the antibacterial activity of *Rhus coriaria* reported the MIC for Gram-positive and Gram-negatives ranging 0.5-20 mg/mL (*145*). *Bacillus subtilis* and *Salmonella spp.* with MIC of 0.5 mg/mL and 20 mg/mL were the most sensitive and most resistant bacteria respectively (*145*).

Blueberry has anthocyanin content of 844 mg of cyanidin 3-glucoside equivalent/100 g dry weight and noticeably glycoside forms of malvidin (36%) are the most abundant anthocyanins (*196*). Anthocyanin's antimicrobial properties also have been repetitively investigated. Burdulis et al. studied bilberry and blueberry extracts (ultrasonic assisted methanolic extraction) for their antimicrobial activity (*197*). These berries are very well known for their high anthocyanin content. Bilberries also have high content of anthocyanin (~90%) with delphinidin 3-arabinoside being most abundant one accounting for 15.3% of the total anthocyanins (*197*). The extracts had inhibitory effects against Gram-positive and Gram-negative (*198*). American and European cranberry also have shown to be great antimicrobial candidate against Gram- positive and Gram-negative. American cranberry seem to cause morphological changes in the bacteria including deformation of bacteria, breakage and leakage of cell wall and membrane

(*198*). Further studies have shown that anthocyanin fraction of American cranberry at its native pH and the concentration of 14.8 mg/L reduced *E. coli* growth to below detectable level (*199*). Anthocyanin treated *E. coli* demonstrated localized collapse and abnormalities in the outer membrane and furthermore cytoplasm leaking (*199*).

Similarly in my research, crowberry aqueous extract was the most effective extract against clinical strain of *S. pyogenes* growth with the lowest MIC of 0.5 mg/mL. As reported by Halvorsen et al., total antioxidant capacity of crowberry fruits is superior to that of blueberry and raspberry fruits (*200*). Juice fractions of crowberry at 8.1 mg/g prevented the adhesion of *S. pneumoniae* to Calu-3 cells (respiratory epithelial cell) by 52% in no dose dependent manner (*201*).

At 5 × MIC concentration flavonoids derived from plants caused a rapid decline of ~2-log CFU/mL in viable cells of *St. aureus* after 15 minutes compared to initial inoculum (*202*). Similarly, 3-*O*-acyl-catechins at concentration of 32 mg/L caused 1.6-log CFU/mL reduction in the viable count of MRSA over 24 hours (*203*). In the same study, 3-*O*-acyl-catechins at 64 mg/L after 2 hour incubation produced 5-log CFU/mL reduction in MRSA viable cell numbers (*203*).

Wu et al. collected the ripened fruits of *Rhus hirta* (sumac) grown on Ontario, Canada and then subjected the ethanolic extract of the fruit to phytochemical profiling analysis (*204*). Analysis showed that the fruits consist of mainly gallic acid, caffeic acid, and ellagic acid in respective concentrations of 5.97, 8.35, and 3.18 mg/g dry weight of ethanolic extracts (*204*). MIC of ellagic acid and gallic acid *in vitro* analysis are 4 and 2 mg/mL respectively against *P. aeruginosa* (*205*). In time-kill studies, ½ × MIC concentration of gallic acid combined with ½ × MIC sulfamethoxazole and tetracycline

produced considerable decrease in the viable cell count of *P. aeruginosa* (4 to 5-log CFU/mL) between 8 and 24 hours (*205*). At ½ × MIC concentration of ellagic acid combined with ½ × MIC sulfamethoxazole similarly viable count over 24 hours had a reduction of 1-2 logCFU/mL (*205*). My results based on the time-kill assay of sumac ethanolic extract at 2 mg/mL against GAS ATCC 19615 suggest that at time of 6 hours bacterial growth significantly differs to that of positive control and this anti-infectivity effect continues over 24 hours period. In presence of 2 mg/mL of sumac ethanolic extract viable bacterial numbers of 24 hours grown bacteria are indifferent from that of initial inoculum thus meaning that the bacterial growth has been inhibited completely at half the MBC (Figure 1).

Experimentally sumac aqueous extract at 4 mg/mL drastically decreased the bacterial numbers to ~0.9-log CFU/mL below the initial inoculum in the 10 minutes sampling time and at 24-hours incubation time viable cell numbers dropped notably to below the quantification limits. Also at 8-hours time 2 mg/mL of sumac aqueous extract produced significant effects accounting for ~1.6-log CFU/mL difference compare to positive control. Viable bacterial numbers in presence of 2 mg/mL of sumac aqueous extract at all the time points were statistically indifferent from initial inoculum indicating complete inhibition of bacterial growth.

Penicillin of 16 ng/mL after 6-hours exposure caused ~3.6-log CFU/mL reduction compare to positive control and at 24 hour period viable ATCC 19615 bacterial numbers became lower than quantification limits. Similar to my finding, reported MIC of penicillin G for GAS varies depending on the strain, 0.003-0.12 µg/mL (*3, 101, 102*).

5.2. Dose-effect of phytochemicals-rich fruit extracts on biofilm formation of *S. pyogenes*

Phytochemicals have shown to inhibit the biofilm formation extensively (*126, 129, 132, 135*). Two terpenoids namely ursolic and oleanolic acids with the MBC of 256 and 1024 µg/mL respectively, were tested for their biofilm inhibition activities against *S. mutans* (*172*). Microtiter plate biofilm assay showed that ¼ × MIC to ½ × MIC concentrations of ursolic and oleanolic acids significantly inhibited the biofilm formation while 1/8 × MIC to 1/16 × MIC had only neglectable effects (*172*). Of the flavonoid family of compounds, catechin, and epigallocatechin gallate have shown to inhibit the biofilm formation of *P. aeruginosa* known to form strong biofilms at concentrations that did not kill the bacterium significantly (*206*).

Baldassarri et al. concluded that most *S. pyogenes* strains isolated from a variety of niches form biofilm to some extent however elsewhere was stated that *S. pyogenes* only is capable of forming microcolonies (*207*). I have shown that cranberry and sumac aqueous and ethanolic extracts could remarkably reduce and avert the biofilm formation when *S. pyogenes* biofilm is formed in the presence of the compounds at concentrations ranging 1-16 mg/mL (Figure 6) however the effects could be dose-dependent or dose-independent.

Biofilm formation activities of two strains of *S. pyogenes* (ATCC 19615 and clinical) were hindered by cranberry extracts in dose-dependent manner. Cranberry derived PAC exhibited dose dependent anti-biofilm activity against the Gram- negative bacteria tested, *P. aeruginosa* (*208*). Cranberry extracts at concentration of 2-4 mg/mL inhibited the ATCC 19615 biofilm establishment up to ~32% (2.5-log CFU/mL reduction

compare to untreated control). This is entirely in agreement with previous studies. High molecular weight element of commercial cranberry juice (~25% concentration) were effective in inhibiting the biofilm formations of different oral streptococci (*S. mutans*, *S. criceti*, *S. oralis* and *S. mitis*) at concentration of 100 to 500 µg/mL up to ~40% (*209*). Furthermore PAC of cranberry at 10 µg/mL reduced *P. aeruginosa* biofilm up to ~37% *(208)*. Cranberry have also proven to possess biofilm inhibition activity against *E. coli* and *P. gingivalis (209, 210)*. I did not observe notable variation in the potency of aqueous versus ethanolic extracts of cranberry in inhibiting the biofilm formation of *S. pyogenes*. At 0.5 mg/mL of cranberry aqueous and ethanolic extracts (sub-MIC), biofilm formation of GAS ATCC 19615 was inhibited by ~5% although statistically indifferent from the control while the same concentration had no anti-biofilm effect on the clinical strain. Very similar trend was observed with 100 µg/mL concentration of high molecular weight element of commercial cranberry juice against *S. sanguinis and S. sobrinus* where biofilm was inhibited by only 7-9% (*209*). Viable cells of *S. pyogenes* could not be quantified by plate count method at concentrations above 4 mg/mL indicating >3-log CFU/mL reduction compare to the control (~7.6-7.8-log CFU/mL).

The anti-biofilm effect of cranberry may be due to condensed tannins. Among the plentiful flavan-3-ols of cranberry, condensed tannins (or proanthocyanidins) are the most abundant component on weight basis (~85%) and these proanthocyanidins (PACs) are mostly made of (-)-epicatechin units (*192*). Methanolic extract of the stem bark of *Acacia mearnsii* known for high amounts of condensed tannins were shown to be effective against both Gram-positive and Gram-negative (*211*). Viable cell count (log CFU/mL) in presence of 1 × MIC and 2 × MIC of the extract decreased up to 2.2-log

CFU/mL and 1.4-log CFU/mL after 4 and 8 hours period respectively (*211*). Phlorotannin, a tannin derived from brown algae at two times the MBC (200 mg/L) killed *Vibrio parahaemolyticus* (a food born pathogen) after 0.5-2 hours of exposure (*185*). The flavonoids tested by Vikram et al. significantly inhibited the biofilm formation of *V. harveyi and E. coli* dose-dependently (*212*). Both of my strains seemed equally sensitive to the extracts although clinical strain of *S. pyogenes* was insignificantly more sensitive to the cranberry aqueous than to ethanolic extract.

Anti-biofilm activity may be related to high phenolic acid content of the *Rhus* species such as gallic, caffeic and ellagic acid (*204*). Gallic acid at 1-4 mg/mL concentration inhibited 15-70% of *S. mutans* biofilm establishment (*213*). Gallic acid, quercetin, and tannic acid all produced significant biofilm inhibition attributes against *S. mutans* however gallic acid was most potent (*214*). Ferulic and gallic acids both possess good anti-biofilm activity against the tested Gram- negatives (*215*). Ferulic acid hindered the biomass formation of the Gram- negative biofilms up to 40% (*215*). It has been suggested that both hydroxyl and carboxyl groups of the polyphenol are prerequisite for the anti-biofilm activity of the compounds (*214*).

Another specie, *Rhus glabra* consist of mainly methyl gallate which is also known for its antibacterial and anti-biofilm effects (*216*). Methyl gallate also at concentrations of 1-4 mg/mL rendered biofilm formation of *S. mutans* 80-99% (*213*). These compounds not only have shown anti-biofilm attributes towards Gram- positives but also have been effective against Gram- negatives. Gallic acid decreased the metabolic activity of biofilm formed by all the tested Gram- negatives to 88%-100% (*215*). Gallic acid produces inhibition activity against planktonic growth and biofilm establishment of fungi (*217*). At

the lowest concentration tested (0.156 mg/mL) a reduction of 2-log CFU/mL or more of planktonic cells was observed for all the Candida species examined (*217*). Though effective on planktonic growth of all species, gallic acid had minimal anti-biofilm effect at 5 mg/mL on some of the species (*C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*) (*217*). Gallic acid is antibacterial against *S. pyogenes* ATCC 19615 with the MIC and MBC of 100 µg/mL and >200 µg/mL respectively (*218*).

In overview, *S. pyogenes* ATCC 19615 biofilm was less susceptible to the extracts meaning that it could form stronger biofilm even in presence of extracts. Typically, higher concentrations of extracts are required to hinder the biofilm forming capabilities of ATCC 19615 than the clinical strain. On the other hand, although susceptible to lower concentrations, clinical strain shows more adaptation attributes to the environmental conditions than ATCC strain does.

Penicillin with the MBIC of generally 4-8 ng/mL inhibits the biofilm formation of *S. pyogenes* up to 41% (~1.9-log CFU/mL reduction compare to untreated). Biofilm establishment of GAS at MBC concentration of penicillin showed >3-log CFU/mL reduction however could not fully prevent the activity. This may be due to reduced sensitivity of *S. pyogenes* biofilm to penicillin treatment. Some scientist believe, biofilm formation or better put micro-colony establishments could not alone lead to penicillin treatment failure and that not every GAS strain biofilm is insensitive to the antibiotic (*105*). GAS antibiotic insensitivity could be caused by various biofilm attributes such as reduced growth rate within the biofilm lifestyle, establishment of three-dimensional structure, the EPS matrix, altered gene expressions, and the formation of resistant genes (*105*). In presence of sub-MIC concentration (1 ng/mL), insignificant difference compare

to control was observed. In the same study, it was also concluded that sub-MIC concentration of penicillin only induces the biofilm formation in susceptible strains (*207*). It was determined that penicillin susceptible GAS forms thicker biofilm than the macrolide resistant one (*207*).

5.3. Dose-effect of phytochemicals-rich fruit extracts on pre-established biofilm of *S. pyogenes*

Predicting the biofilm eradicating activity of the extracts based on planktonic cells susceptibility have been advised inappropriate (*219*). Phytochemicals have been capable of eradicating microbial biofilms generally in experimental setting. In a systematic exploration study of natural and synthetic flavonoids for their inhibition activity against the pre-established *St. aureus* biofilms, only 2% of compounds exhibited notable eradication activity up to 95% (*220*). The ten active compounds belonged to the classes of flavanones, chalcones, and flavans (*220*).

Grape phenolics with the total phenolic content up to 57 mg/g (milligrams gallic acid equivalents per Gram dry matter) could eradicate pre-established biofilms of *St. aureus* at concentration ranging 801-1950 mg/L after 20 hours treatment while no MBEC could be detected for gallic acid and ellagic acid up to the tested concentrations (>2500 mg/L) (*221*). Biofilm efficiency of my two strains differed rather significantly. *S. pyogenes* ATCC 19615 bacterial cells were more likely to change their lifestyle and form biofilms than the clinical strain of *S. pyogenes* after 24 hours static incubation. More bacterial cells of clinical strain of *S. pyogenes* were in planktonic form than ATCC 19615.

I have shown that the pre-formed biofilms of *S. pyogenes* could be eradicated at the MBEC of 8-16 mg/mL of cranberry extracts up to 28% relative to positive control (control: 0%). Eradication of *S. pyogenes* biofilms could be optimized by higher concentration of the cranberry extracts, 32 mg/mL, to approximately 42% (Figure 7). Results obtained from plate count propose dose-dependent effect of cranberry extracts on the eradication of *S. pyogenes* whilst MTT assay elucidates insignificant dose-independent activity eradicating the biofilm of *S. pyogenes* clinical strain. Both strains biofilm were insignificantly sensitive to the cranberry aqueous extracts.

In line with my results, commercial cranberry extracts produced weak eradication activity against *Staphylococcus* species with the MBEC of 5-10 mg/mL (*136*). Cranberry derived PACs at 10 μg/mL concentration have been proven to significantly eradicate the 24-hours biofilms (54.1%) of *P. aeruginosa* after 24-hour treatment (*208*).

Though plate count method showed dose-dependent eradication effects, observations collected from MTT assay were rather directed at dose-independent effects of cranberry extracts. The observation was more prominent for cranberry ethanolic extract effect on clinical strain where 2 mg/mL had eradicated ~15% more biofilm cells than 4 mg/mL.

Sumac extracts at 8 mg/mL disrupted *S. pyogenes* biofilm up to 37%. MBEC varied greatly among the strains from 0.5 to 16 mg/mL. This may be indicative of strain-dependent eradication effect of sumac extracts on *S. pyogenes* biofilms. Another interesting observation that was not evident for cranberry extracts was made for sumac extracts. A lower concentration of sumac ethanolic was required to minimally eradicate the *S. pyogenes* biofilm than the aqueous one. MBEC for ethanolic extract was two times,

and four times lower than aqueous one for GAS ATCC 19615 and clinical strain, correspondingly. *S. pyogenes* ATCC 19615 was less sensitive to sumac extracts than the clinical one and this may be related to the diverse biofilm efficiencies of strains. As stated earlier, ATCC strain has higher tendency to form mature biofilms than the clinical one thus *S. pyogenes* ATCC 19615 biofilm is more populated than the latter.

Caffeic acid one of the major constituents of *Rhus hirta* extracts, decreased the *St. aureus* 24 hour established biofilm biomass up to 60% whilst gallic acid another major component showed slight effect against MRSA but not the ATCC strain of the bacteria (*204, 222*). Similarly gallic and caffeic acid generally at the same concentration (1-4 mg/mL) lowered the metabolic activity of *St. aureus* 24 hour established biofilm although the effect was more prominent on the ATCC strain (*222*). Beside antibacterial effects, caffeic acid derivatives have also shown antifungal activity in eradicating the biofilm of *Candida albicans* (*223*). De Vita et al. demonstrated that caffeic acid derivatives at concentrations up to 256 µg/mL could eradicate 24-hours mature biofilms of *C. albicans* up to 50% (*223*). Authors conclude that while presence of methyl moiety plays a big role in the eradication activity of the caffeic acid and its derivatives, the two hydroxylic groups are not vital for the exhibited activities (*223*).

Sumac extracts at the highest concentration with quantifiable viable bacterial cells (8 mg/mL) disrupted the established biofilms of *S. pyogenes* ATCC 19615 and clinical ~2.8-log CFU/mL and 2.5-log CFU/mL (compare to untreated) respectively. *S. pyogenes* in biofilm like states could hide in their sanctuary thus enforcing difficulties in eradicating the biofilms by penicillin with the MBEC of >400 mg/L (*101*).

In a study, the 48-hour-old dynamic biofilms of *S. pyogenes* with M serotypes of M1, M2, M4, M6, M12, and M30 could only be eradicated at 400 to 800 mg/L of penicillin while the MIC ranged 0.04-0.08 mg/L (*101*). *S. pyogenes* ATCC 19615 biofilm could only be disrupted ~3% with 128 ng/mL of penicillin G but at the same concentration eradication of clinical strain biofilm rose to ~15%. MBEC of penicillin G determined by plate counts were >128 ng/mL and 16 ng/mL for ATCC and clinical strain. *S. pyogenes* ATCC 19615 pre-formed biofilm was significantly more resistant to penicillin G than the clinical strain. Generally biofilm of clinical strain of *S. pyogenes* was rather more difficult to eradicate than the ATCC strain by berry extracts as anticipated however conversely penicillin G was more potent on clinical pre-established biofilm than ATCC. This observation on penicillin G cannot be properly explained.

5.4. Dose-effect of selected phytochemicals-rich extracts of Atlantic Canada fruits on *S. pyogenes* adherence

GAS adhesion is generally mediated by capsule, pilus like structures and or elements of cell wall such as M protein, lipoteichoic acid, and fibronectin-binding protein(s) (*66, 67*). To date more than seventeen GAS specific adhesins have been identified (*5*). GAS adhesion to varying niches in the upper respiratory tract is initiated by receptor-specific adhesins (*5, 69*). Affinity of bacteria to attach to different substratum differs greatly among strains and is generally strain and tissue dependent(*224*). Some but not all GAS strains are capable of attaching to uncoated surface to some degree(*224*).

Presence of EPS produced by the bacteria on surface of the microorganism moreover promotes bacterial adhesion in comparison to the EPS-deficient specie with similar surface characteristics (*225*). Bacterial adhesion is promoted through polymeric

extension of polysaccharides (*225*). Pathogenesis of microbes can be prevented by anti-adhesive properties of phytochemicals of which polyphenols have been studied comprehensively (*45, 226*). Adhesion of *S. mutans* to the tooth surface, major cause of tooth decay responsible for $108 billion financial burden only in USA, was hindered after treatment with sub-MIC to MIC concentration of ursolic acid (MIC of 256 μg/mL), however higher concentration of 1024 μg/mL, completely inhibited the attachment process (*4, 172*). One of the major antimicrobial mechanism of action of phytochemicals is to bind to adhesins and or surface proteins consequently preventing its adhesion (*41*). Ursolic acid, known for its anti-adhesive activities, have been examined for its mode of action by transcriptome sequence analysis and the results suggest stimulation of motility genes where microbial cell is told to stay motile thus hindering its adhesion and biofilm formation (*172, 227*). Generally, cell surface attributes such as hydrophobicity, and surface charge of the bacteria are altered by phytochemicals to prevent or reduce the adhesion to abiotic and biotic surfaces (*228*).

To examine the anti-adhesive effects of phytochemicals on *S. pyogenes*, I have selected cranberry and sumac extracts which are rich in phytochemicals. Anti-adherence effect of cranberry juice and extract has been studied to some extent mostly against Gram-negative bacteria (*127, 169*). Two different strains (ATCC strain versus clinical isolate) were tested because adherence capabilities of GAS isolates differ from one to another. I have examined the anti-adhesion properties of the selected extracts against adherence of GAS to different substrata including uncoated, coated with fibronectin and BSA and tonsil cells to better test the adherence-inhibitory effects of phytochemicals. Isolates from asymptomatic GAS carriers are most likely to carry the gene responsible for

fibronectin binding protein F2 along with higher adherence capabilities than isolates from pharyngitis subjects (*224*). Level of invasiveness rather determines the degree of adhesiveness to laminin-coated surface in GAS and GBS (*224*). My observations confirm that both strains adhere equally to uncoated and BSA coated substrate. To the same extent, in equal experimental conditions more of *S. pyogenes* attached to fibronectin-coated surfaces (~8-15%). Some GAS strains are capable of binding to abiotic polystyrene surfaces whereas other strains need matrix or protein coated surfaces to adhere (*97, 224*).

In presence of 8 mg/mL of cranberry aqueous extract, *S. pyogenes* clinical and ATCC 19615 adhered 2-9% less, respectively, to the inert uncoated polystyrene surface than the untreated after 1-hour period (Table 5) whereas same concentration had no effect on the planktonic ATCC 19615 bacteria after 2-hour incubation (Figure 3). Cranberry contains considerable amounts of polyphenols particularly flavonoids. Polyphenols-rich tea extract at concentrations as low as 1-4 mg/mL prevented the attachment of *S. mutans* and *A. viscosus* (*229*). In line with Wang et al. findings (*188*), green tea polyphenols mainly (–)-epigallocatechin gallate (EGCg) at 250–500 μg/mL hindered the adherence of *P. gingivalis* to human buccal epithelial cells (*230*). Depending on the galloyl moiety, affectivity of (–)-epicatechin gallate (ECg) and (–)-gallocatechin gallate (GCg) were reduced against the adherence of *P. gingivalis* and subsequently (+)-catechin (C(+)), (–)-epicatechin (EC), (+)-gallocatechin (GC), and (–)-epigallocatechin (EGC) were least active thus indicative of importance of galloyl moiety (*230*).

Other flavonoids, particularly larger compounds, possess anti-adherence properties. *E. coli* attachment was decreased to human colon epithelial cells pretreated

with 54.8 μg/mL of apple flavonoid, phloretin (*189*). It has been suggested that polymeric flavonoids or other large molecule polyphenols may exhibit higher anti-adhesion effects against streptococci (*188*). Coffee high molecular weight fraction nearly completely, 91%, hindered the adhesion of *S. mutans* (*231*). Flavonols or PAC (of flavan-3-ol category) may be the reason for anti-adhesive attributes of cranberry (*232, 233*). A-type PAC of cranberry has shown effective anti-adhesion properties and is composed of monomer epicatechin, dimer epicatechin (epicatechin-(4 β→8, 2 β→O→7)-epicatechin), and trimer epicatechin (epicatechin-(4 β→8)-epicatechin)-(4 β→8, 2 β→O→7)-epicatechin) (*232, 234*). Flavonols have anti-adhesive attributes too and flavonols of cranberry mainly consist of myricetin-3- β-galactoside, myricetin-3-α-arabinofuranoside, quercetin-3- β-galactoside, quercetin-3- β-glucoside, quercetin-3- rhamnospyranoside, and quercetin-3-O-(6″-p-benzoyl)-β-galactoside (*233, 234*).

Cranberry aqueous extract imposed anti-adhesive attributes in dose-dependent manner against attachment of GAS ATCC 19615 to bare-substratum and rather dose-independent effects against clinical strain however its anti-adhesive effects were more outstanding towards GAS ATCC 19615 not clinical.

Effects of the extracts were dissimilar on the free-floating and adherent bacteria. Sumac aqueous extract exhibited relatively different anti-adherence effects than the cranberry aqueous extract. *S. pyogenes* clinical and ATCC19615 adhered 23-32% less, respectively, to the inert uncoated polystyrene surface than the untreated after 1-hour period in presence of 4 mg/mL of sumac aqueous extract (Table5) while same concentration killed only ~11% of the planktonic ATCC 19615 bacteria after 2-hour incubation (Figure 3).

Cranberry and sumac aqueous extract were equally effectual on the adherence of both strains to bare surface. Inhibitory effects of sumac and cranberry aqueous extracts on the attachment of GAS to uncoated polystyrene surface were rather dose-dependent. In solvent control wells adherence of *S. pyogenes* to uncoated polystyrene surface was promoted insignificantly. Clinical strain seemed to attach more to uncoated-substratum in nutritionally deprived environment than ATCC 19615 where I examined the solvent (sterile distilled water) effects on the attachment. Considering Šmitran et al. work, I may conclude that either my clinical strain of GAS under study is non-invasive or highly invasive.

Viable adherent bacterial cells could not be quantified at 8-16 mg/mL of sumac and cranberry aqueous extracts respectively thus reducing *S. pyogenes* adherence to uncoated polystyrene surface to ≤50%. Significantly, fewer of clinical strain of GAS attached to uncoated polystyrene surface than ATCC 19615. Šmitran et al. presented that noninvasive and highly invasive isolates of *S. pyogenes* adhered significantly more to uncoated plates than the low invasive strains from 71-98% (*224*).In my study, penicillin had little to no effect on the adherence of *S. pyogenes* strains to uncoated polystyrene substrate (Table 5).

For oral streptococci, the rate at which the nonspecific interactions between bacteria and BSA coatings strengthen was by twofold faster than specific interactions of the bacteria with salivary conditioning films (*70*). Adhesion of the *S. pyogenes* clinical and ATCC 19615 to BSA coated polystyrene was reduced by 7-8% (adherence of control: 82-88%) after 1-hour exposure to 8 mg/mL of cranberry aqueous extract (Figure 6). Irreversible adhesion caused by bond strengthening (measured by seconds) took a

faster rate for oral streptococci than Actinomyces (*70*). An explanation for this fact according to van der Mei et al. is that "specific interactions require a closer approach of interacting surfaces in order to face a substratum surface with its most favorable site with the removal of interfacial water and a more extensive rearrangement of surface structures" (*70*).

Cranberry aqueous extract at 8 mg/mL had higher adherence inhibition efficacy on fibronectin-binding proteins (13-16%) than on the nonspecific proteins (7-8% inhibition only). High molecular weight non-dialysable material extracted from cranberry juice (NDM) exhibit adhesion reduction activity in a dose-dependent manner at concentrations of 66–1330 μg/mL against attachment of *S. sobrinus* to hydroxyapatite beads coated with fructosyltransferase and glucosyltransferases (*235*). MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight) analysis have elucidated that at least one up to multiple A-type interflavan bonds exist in PACs of cranberry juice cocktail (*232*). *Myrothamnus flabellifolia* 50% ethanol extracts full of flavan-3-ols and oligomeric proanthocyanidins at 100 μg/mL decreased 50% of the adhesion of *P. gingivalis* (pre-treated for 30 minutes) to KB cells (ATCC® CCL-17™) by interacting with bacterial outer membrane proteins (*236*). Myricetin, a flavonol of cranberry, at 100 μg/mL, have experimentally shown to decrease the adhesin gene expression of *P. gingivalis* prerequisite for colonization significantly from 64% up to 93% after 8 hours treatment period (*233*).

Activity of 4 mg/mL of sumac aqueous extract on the adhesion of *S. pyogenes* clinical and ATCC 19615 to BSA compare to control was more perceptible with ~19-20% adherence rate reduction. Clearly, 4 mg/mL of sumac aqueous extract prevented less

of the adhesion of *S. pyogenes* to BSA (up to 20%) than to bare polystyrene surface (up to 32%) hence more effective on uncoated substrate. Most abundant phytochemicals found in sumac fruits according to literature are caffeic acid, gallic acid, ellagic acid and methyl gallate (*147, 204*). Green tea and oolong tea also contain substantial quantities of gallic acid and also epigallocatechin gallate and have shown to inhibit the attachment of *S. mutans* and other oral bacterial to collagen, tooth surfaces and gingival cell line (*188*). In the same study, fermented tea with high tannin content opposed to green tea and oolong tea had shown more activity towards attachment of *S. mutans* and other oral bacterial to collagen, tooth surfaces and gingival cell line (*188*). Catechins act by preventing the interactions between fibronectin of the host cells and bacteria through binding to the receptor (*188*).

Similar to cranberry aqueous extract, sumac aqueous extract although only relatively significant, inhibited more of fibronectin-binding adherence (25-32% for clinical and ATCC strain respectively) than it did on nonspecific adhesins to BSA (19-20%). *S. pyogenes* biofilm at 4 mg/mL of sumac aqueous extract was hindered 16-43% respectively for clinical and ATCC strain. In experimental settings, ionic strength of the medium and type of proteins would determine the degree of attachment of bacteria to the substratum (*225*). Bacterial adhesion to BSA coatings would differ depending on the ionic strength condition (*225*). BSA encourages bacterial adhesion at low ionic strength whilst high ionic strength would hinder the activity (*225*). This could be explained by that at high ionic strength BSA folds into a higher density core thus leaving fewer interaction sites accessible to attachment (*225*).

Noticeably sumac aqueous extract effectiveness on virulence of *S. pyogenes* is greater on clinical isolate than the ATCC strain whether eradicating the biofilm or preventing the adherence of the bacteria. Relatively the effects of sumac and cranberry aqueous extracts on the fibronectin-adherence are dose-independent. Fibronectin binding activity of the tested strains of *S. pyogenes* were equally sensitive to the inhibitory effects of extracts.

I have observed that fibronectin binding adhesins of the *S. pyogenes* are more susceptible to 16 ng/mL of penicillin (12-13% lesser adhesion relative to positive control) than the nonspecific proteins, which were only deactivated by ~8% (Figure 8). Inhibitory concentrations of penicillin stimulated loss of lipoteichoic acid consequently reducing the adherence of *S. sanguis* to host tissue surfaces (*237*).

Significant reduction in adhesion of GAS strains regardless of their degree of virulence to laminin were observed after overnight exposure of the bacteria to ½ × MIC of penicillin G (*224*). Penicillin seemed to enforce none dose-dependent effects on the fibronectin binding proteins of *S. pyogenes*. Comparable to my study, noninvasive, highly invasive and low invasive isolates of *S. pyogenes* adhered more to laminin-coated plates than to uncoated (*224*). Similar to analysis of dose-effects of extracts on the adhesion of GAS to uncoated surfaces, number of viable adherent bacterial cells (log CFU/mL) to BSA coated substrate decreased below quantification limits and could not be quantified at 8-16 mg/mL of sumac and cranberry aqueous extracts respectively hence resulting in >2-3-log CFU/mL reduction (compare to untreated).

Cranberry aqueous extract at 2-4 mg/mL concentrations did not influence the adherence of *S. pyogenes* to the tonsil cells (Table6). Higher concentrations of cranberry

aqueous extract could not be examined because of the cytotoxicity effects imposed on tonsil cells (Figure9). No literature have been found on the suitability of the tonsil cell line as substrate in adhesion studies of oral streptococci however some of the typical cell lines have been advised as inappropriate for such studies for example buccal epithelial cells due to occurrence of non-specific adhesion (*238*). Other studies have documented anti-adhesive effects of cranberry juice, extract and or its constituents on the adhesion of bacteria to host cells *in vitro* (*232*). In a comparative study, anti-adhesion activity of A-linked proanthocyanidins from cranberry juice cocktail and B-linked proanthocyanidins from commercial grape and apple juices, green tea and dark chocolate against attachment of P-fimbriated uropathogenic *E. coli* to uroepithelial cells were examined (*232*). In agreement with the previous research concluding that cranberry A-linked dimers possess stronger anti-adhesion activity than the B-linked dimmers, Howell et al. showed A-type PAC of cranberry at 60 µg/mL and B-type PAC of grape juice at 1200 µg/mL produced significant and insignificant inhibitory adhesion effects, respectively (*232*).

Numerous studies have pointed out the explicit interactions between *S. pyogenes* and different niches of the host nonetheless GAS adhesion mediated by specific molecules have not been completely understood (*5, 238*). This is due to diversity of the adhesins expressed on the GAS surface (*5*).

Penicillin G at 16 ng/mL significantly reduced the adhesion of *S. pyogenes* ATCC 19615 and clinical to tonsil cells. Others have observed similar trend. Sub-minimum inhibitory concentration of penicillin and rifampin alters the surface hydrophobicity of GAS thus reducing the adhesion to epithelial cells (*75-77*). Eickenberg et al. showed that penicillin G at sub-MIC concentration (27 µg/mL) could reduce the adherence ability of

E. coli to human buccal epithelial cells after 48 hours incubation (90% reduction) (*239*). In the same study penicillin G at sub-MIC concentration (1 μg/mL) had anti-adhesive effects against *S. pyogenes* attachment to same cells up to 70% within 3 hours period (*239*).

All taken together, no significant pattern of preferential attachment of both of test strains to either fibronectin or tonsil cells were observed suggesting that molecules implicated in virulence of *S. pyogenes* are niche specific and exceptionally distinctive adhesins are involved in the pathogenesis of the bacteria. Receptor specific adhesins diverge greatly based on the streptococcal strain, host cell and the tissue. No final statement can be made on whether number and location of A-type linkages in the oligomers, type of interflavan bonds and or degree of polymerization have any influence on anti-adhesion activity (*232*).

CHAPTER 6 CONCLUSION

6.1. Project summary

Results suggest that crude extracts of berries excluding common buckthorn possess antibacterial effect to some extent against the bacteria of study although at times with low potency. Extracts of dark colored berries namely crowberry and blueberry were least effective although the berries of the two crops are known for high antioxidant capacity due to their high anthocyanin content. The potency of the extracts against GAS even at the lowest value was incomparable to the potency of penicillin G. According to time-kill studies both cranberry and sumac aqueous and ethanolic extracts imposed dose and time dependent effects on growth of GAS ATCC 19615.

Selected extracts showed biofilm inhibitory activities against GAS and therefore utilization of these extracts can lower the biofilm formation to ≤50% at MBC concentrations. In general, cranberry extracts were less potent than sumac extracts against biofilm formation of GAS. The effects of aqueous and ethanolic extracts were rather equal against biofilm formation of GAS. Cranberry extracts were equally effective against biofilm formation of both GAS strains however sumac extract enforced more anti-biofilm effects on ATCC 19615 than on clinical strain. Cranberry extracts produced more dose-dependent effect against biofilm formation of both GAS strains than sumac extracts. Both types of extracts of both of the berries could eradicate to ≥50% the pre-established biofilms of *S. pyogenes* generally at MBC to 2 × MBC concentrations.

Suitability of the quantitative and semi-quantitative methods for biofilm studies was compared and I conclude that MTT assay could be very useful tool for determining the MBIC and MBEC of cranberry extracts in a timely manner cost effectively. On the

other hand, plate counts although labor intensive, time and cost inefficient are more appropriate for analyses of dose-effects.

Adhesion being the initial stage of infection has to be the most important target site for phytochemical origin antimicrobials. To decrease the attachment percent to bare and coated substratum to about ≤60%, GAS may be treated with ½ × MBC to MBC concentrations. Another noticeable pattern was that both berry extracts (cranberry and sumac aqueous extracts) were more potent on the prevention of adhesion of ATCC 19615 to fibronectin than on clinical strain. Ineffectively of cranberry aqueous extract against adhesion of GAS to HTEpiC cells may be due to many factors such as experimental conditions, and unsuitability of the cell-line for the assay.

In this study, I provide the results of a screening process conducted among fruits for identification of a potential antibacterial extract however as stated earlier to make clinical use of the data conducting various complementary assays shall be warranted. I also studied the anti-biofilm and anti-adhesion effects to further the understanding of phytochemicals mechanism of action. This and similar studies offer useful data for the soon to be post-antibiotic era that would definitely require comprehensive understanding of the natural antimicrobials target site for the development of new-antimicrobial agents for treatment of evolved and resistant microbes.

6.2. Future directions

New techniques such as flow cytometry shall be employed to analyze the physiological conditions of the cells in a mixed oral species biofilm pre and post treatment. Effect of the extracts on patterns of virulence gene expression in planktonic

and biofilm lifestyles of bacteria could also advance the knowledge of mechanism of action.

REFERENCES

1. Baron, S.; Davis, C. P., *Normal flora. In Medical Microbiology, 4th ed.; Baron, S., Eds.; University of Texas Medical Branch at Galveston: Texas, USA* **1996**.

2. Hayes, C. S.; Williamson Jr, H. A. R. O. L. D., Management of group A beta-hemolytic streptococcal pharyngitis. *American Family Physician* **2001,** *63*, 1557-1565.

3. Stevens, D. L., Tanner, M. H., Winship, J., Swarts, R., Ries, K. M., Schlievert, P. M., Kaplan, E., Severe group A streptococcal infections associated with a toxic shock-like syndrome and scarlet fever toxin A. *New England journal of medicine* **1989,** *321*, 1-7.

4. Frieden, T., Antibiotic resistance threats in the United States. *Centers for Disease Control and Prevention* **2013**, 11-93.

5. Mitchell, T. J., The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nature Reviews Microbiology* **2003,** *1*, 219-230.

6. Dajani, A.; Taubert, K.; Ferrieri, P.; Peter, G.; Shulman, S., Treatment of acute streptococcal pharyngitis and prevention of rheumatic fever: a statement for health professionals. *Pediatrics* **1995,** *96*, 758-764.

7. Bisno, A. L., Acute pharyngitis. *New England Journal of Medicine* **2001,** *344*, 205-211.

8. Neely, M. N.; Pfeifer, J. D.; Caparon, M., Streptococcus-zebrafish model of bacterial pathogenesis. *Infect Immun* **2002,** *70*, 3904-14.

9. Shulman, S. T.; Bisno, A. L.; Clegg, H. W.; Gerber, M. A.; Kaplan, E. L.; Lee, G.; Martin, J. M.; Van Beneden, C., Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases* **2012,** *55*, e86-e102.

10. Linder, J. A.; Bates, D. W.; Lee, G. M.; Finkelstein, J. A., Antibiotic treatment of children with sore throat. *JAMA : the journal of the American Medical Association* **2005,** *294*, 2315-22.

11. Zalzal, G. H.; Cotton, R. T., Pharyngitis and adenotonsillar disease. *Otolaryngology and head and neck surgery, 2nd edn. Mosby Year Book, St. Louis* **1993,** *1190*.

12. Carapetis, J. R.; Steer, A. C.; Mulholland, E. K.; Weber, M., The global burden of group A streptococcal diseases. *The Lancet Infectious Diseases* **2005,** *5*, 685-694.

13. Facklam, R. F.; Martin, D. R.; Marguerite, L.; Dwight, R. J.; Efstratiou, A.; Thompson, T. A.; Gowan, S.; Kriz, P.; Tyrrell, G. J.; Kaplan, E., Extension of the Lancefield classification for group A streptococci by addition of 22 new M protein gene sequence types from clinical isolates: emm103 to emm124. *Clinical Infectious Diseases* **2002,** *34*, 28-38.

14. Rodriguez-Iturbe, B.; Musser, J. M., The current state of poststreptococcal glomerulonephritis. *Journal of the American Society of Nephrology* **2008,** *19*, 1855-1864.

15. Cunningham, M. W., Pathogenesis of group A streptococcal infections. *Clinical microbiology reviews* **2000,** *13*, 470-511.

16. Chahine, E. B.; Sucher, A. J., Update on the Management of Streptococcal Pharyngitis. *US Pharm* **2013,** *38*, 51-56.

17. Courtney, H. S.; Hasty, D. L.; Dale, J. B., Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. *Annals of medicine* **2002,** *34*, 77-87.

18. Kreikemeyer, B.; McIver, K. S.; Podbielski, A., Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions. *Trends Microbiol* **2003,** *11*, 224-232.

19. Sherris, J. C.; Ray, C. G., *An Introduction to Infectious Diseases. In Medical Microbiology, 2nd ed.; Sherris, J.C., Eds.; Elsevier Science Publishing Co.: New York, USA* **1990**, pp. 149–169.

20. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P., Bacterial Biofilms: A Common Cause of Persistent Infections. *Science* **1999,** *284*, 1318-1322.

21. Manetti, A. G. O.; Zingaretti, C.; Falugi, F.; Capo, S.; Bombaci, M.; Bagnoli, F.; Gambellini, G.; Bensi, G.; Mora, M.; Edwards, A. M.; Musser, J. M.; Graviss, E. A.;

Telford, J. L.; Grandi, G.; Margarit, I., Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation. *Molecular Microbiology* **2007,** *64*, 968-983.

22. Akiyama, H.; Morizane, S.; Yamasaki, O.; Oono, T.; Iwatsuki, K., Assessment of Streptococcus pyogenes microcolony formation in infected skin by confocal laser scanning microscopy. *Journal of dermatological science* **2003,** *32*, 193-199.

23. Todar, K., *Todar's online textbook of bacteriology*. University of Wisconsin-Madison Department of Bacteriology: 2006.

24. Nobbs, A. H.; Lamont, R. J.; Jenkinson, H. F., Streptococcus adherence and colonization. *Microbiology and Molecular Biology Reviews* **2009,** *73*, 407-450.

25. Duarte, S.; Gregoire, S.; Singh, A. P.; Vorsa, N.; Schaich, K.; Bowen, W. H.; Koo, H., Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. *FEMS microbiology letters* **2006,** *257*, 50-6.

26. Gregoire, S.; Singh, A.; Vorsa, N.; Koo, H., Influence of cranberry phenolics on glucan synthesis by glucosyltransferases and Streptococcus mutans acidogenicity. *J Appl Microbiol* **2007,** *103*, 1960-1968.

27. He, J.; Chen, L.; Heber, D.; Shi, W.; Lu, Q. Y., Antibacterial Compounds from Glycyrrhiza u ralensis. *Journal of natural products* **2006,** *69*, 121-124.

28. Furiga, A., Lonvaud Funel, A., Dorignac, G., Badet, C., In vitro anti-bacterial and anti-adherence effects of natural polyphenolic compounds on oral bacteria. *J Appl Microbiol* **2008,** *105*, 1470-1476.

29. Almeida, L. S. B. D.; Murata, R. M.; Yatsuda, R.; Dos Santos, M. H.; Nagem, T. J.; Alencar, S. M. D.; Koo, H.; Rosalen, P. L., Antimicrobial activity of Rheedia brasiliensis and 7-epiclusianone against Streptococcus mutans. *Phytomedicine* **2008,** *15*, 886-891.

30. Mah, T. F. C.; O'Toole, G. A., Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **2001,** *9*, 34-39.

31. Bisno, A. L.; Gerber, M. A.; Gwaltney, J. M., Jr.; Kaplan, E. L.; Schwartz, R. H., Practice guidelines for the diagnosis and management of group A streptococcal

pharyngitis. Infectious Diseases Society of America. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **2002,** *35*, 113-25.

32. Schwartz, B.; Marcy, S. M.; Phillips, W. R.; Gerber, M. A.; Dowell, S. F., Pharyngitis—principles of judicious use of antimicrobial agents. *Pediatrics* **1998,** *101*, 171-174.

33. Pediatrics, A. A. o., *Red Book For PDA: 2003 Report Of The Committee On Infectious Diseases*. 2003.

34. Sun, H.; Xu, Y.; Sitkiewicz, I.; Ma, Y.; Wang, X.; Yestrepsky, B. D.; Huang, Y.; Lapadatescu, M. C.; Larsen, M. J.; Larsen, S. D.; Musser, J. M.; Ginsburg, D., Inhibitor of streptokinase gene expression improves survival after group A streptococcus infection in mice. *Proceedings of the National Academy of Sciences* **2012,** *109*, 3469-3474.

35. Dougherty, T., *Antibiotic Discovery and Development:.... Vol. 1*. Springer Science & Business Media: 2012.

36. Martin, J. M.; Green, M.; Barbadora, K. A.; Wald, E. R., Erythromycin-resistant group A streptococci in schoolchildren in Pittsburgh. *New England Journal of Medicine* **2002,** *346*, 1200-1206.

37. Marcy, S. M., Treatment options for streptococcal pharyngitis. *Clinical Pediatrics* **2007,** *46*, 36S-45S.

38. Choby, B. A., Diagnosis and treatment of streptococcal pharyngitis. *Am Fam Physician* **2009,** *79*, 383-90.

39. Benzie, I. F. F.; Wachtel-Galor, S., *Herbal Medicine: Biomolecular and Clinical Aspects, 2nd ed.; CRC Press: Florida, USA* **2011**, pp. 1-500.

40. Issa, A. Y.; Volate, S. R.; Wargovich, M. J., The role of phytochemicals in inhibition of cancer and inflammation: New directions and perspectives. *Journal of Food Composition and Analysis* **2006,** *19*, 405-419.

41. Cowan, M. M., Plant products as antimicrobial agents. *Clinical microbiology reviews* **1999,** *12*, 564-582.

42. Mohanasundari, C.; Natarajan, D.; Srinivasan, K.; Umamaheswari, S.; Ramachandran, A., Antibacterial properties of Passiflora foetida L.–a common exotic medicinal plant. *African Journal of biotechnology* **2007,** *6*.

43. Geyid, A.; Abebe, D.; Debella, A.; Makonnen, Z.; Aberra, F.; Teka, F.; Kebede, T.; Urga, K.; Yersaw, K.; Biza, T., Screening of some medicinal plants of Ethiopia for their anti-microbial properties and chemical profiles. *J Ethnopharmacol* **2005,** *97*, 421-427.

44. Fabio, A.; Cermelli, C.; Fabio, G.; Nicoletti, P.; Quaglio, P., Screening of the antibacterial effects of a variety of essential oils on microorganisms responsible for respiratory infections. *PTR Phytotherapy Research* **2007,** *21*, 374-377.

45. Janecki, A.; Kolodziej, H., Anti-adhesive activities of flavan-3-ols and proanthocyanidins in the interaction of group A-streptococci and human epithelial cells. *Molecules* **2010,** *15*, 7139-7152.

46. Green, A. E.; Rowlands, R. S.; Cooper, R. A.; Maddocks, S. E., The effect of the flavonol morin on adhesion and aggregation of Streptococcus pyogenes. *FEMS microbiology letters* **2012,** *333*, 54-58.

47. Bisno, A.; Brito, M.; Collins, C., Molecular basis of group A streptococcal virulence. *The Lancet Infectious Diseases* **2003,** *3*, 191-200.

48. Shi, E., Flesh-Eating Bacteria: Various Strains and Virulence. *(University of California, Davis, California, USA). Personal communication* **2009**, 1-8.

49. Mickelson, M., Chemically defined medium for growth of Streptococcus pyogenes. *Journal of bacteriology* **1964,** *88*, 158-164.

50. Gera, K.; McIver, K. S., Laboratory growth and maintenance of Streptococcus pyogenes (the Group A Streptococcus, GAS). *Current protocols in microbiology* **2012**, 9D. 2.1-9D. 2.13.

51. Futai, M.; Noumi, T.; Maeda, M., ATP synthase (H+-ATPase): results by combined biochemical and molecular biological approaches. *Annual review of biochemistry* **1989,** *58*, 111-36.

52. Bender, G. R.; Sutton, S. V.; Marquis, R. E., Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. *Infect Immun* **1986,** *53*, 331-8.

53. Kakinuma, Y., Inorganic cation transport and energy transduction in Enterococcus hirae and other streptococci. *Microbiology and molecular biology reviews : MMBR* **1998,** *62*, 1021-45.

54. Bierne, H.; Dramsi, S., Spatial positioning of cell wall-anchored virulence factors in Gram-positive bacteria. *Current opinion in microbiology* **2012,** *15*, 715-723.

55. Zapun, A.; Vernet, T.; Pinho, M. G., The different shapes of cocci. *FEMS microbiology reviews* **2008,** *32*, 345-360.

56. Ferretti, J. J.; McShan, W. M.; Ajdic, D.; Savic, D. J.; Savic, G.; Lyon, K.; Primeaux, C.; Sezate, S.; Suvorov, A. N.; Kenton, S., Complete genome sequence of an M1 strain of Streptococcus pyogenes. *Proceedings of the National Academy of Sciences* **2001,** *98*, 4658-4663.

57. Riddle, D. J.; Bessen, D. E.; Caparon, M. G., Variation in Streptococcus pyogenes NAD+ glycohydrolase is associated with tissue tropism. *Journal of bacteriology* **2010,** *192*, 3735-3746.

58. Kreikemeyer, B.; McIver, K. S.; Podbielski, A., Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions. *Trends Microbiol* **2003,** *11*, 224-232.

59. de Andrade Barboza, S.; Meygret, A.; Vincent, P.; Moullec, S.; Soriano, N.; Lagente, V.; Minet, J.; Kayal, S.; Faili, A., Complete genome sequence of noninvasive Streptococcus pyogenes M/emm28 strain STAB10015, isolated from a child with perianal dermatitis in French Brittany. *Genome announcements* **2015,** *3*, e00806-15.

60. Soriano, N.; Vincent, P.; Moullec, S.; Meygret, A.; Lagente, V.; Kayal, S.; Faili, A., Closed genome sequence of noninvasive Streptococcus pyogenes M/emm3 strain STAB902. *Genome announcements* **2014,** *2*, e00792-14.

61. Soriano, N.; Vincent, P.; Auger, G.; Cariou, M.-E.; Moullec, S.; Lagente, V.; Ygout, J.-F.; Kayal, S.; Faili, A., Full-length genome sequence of type M/emm83 group A Streptococcus pyogenes strain STAB1101, isolated from clustered cases in Brittany. *Genome announcements* **2015,** *3*, e01459-14.

62. Soriano, N.; Vincent, P.; Piau, C.; Moullec, S.; Gautier, P.; Lagente, V.; Faili, A.; Kayal, S., Complete Genome Sequence of Streptococcus pyogenes M/emm44 Strain STAB901, Isolated in a Clonal Outbreak in French Brittany. *Genome announcements* **2014,** *2*, e01174-14.

63. Suvorov, A. N.; Ferretti, J. J., Physical and genetic chromosomal map of an M type 1 strain of Streptococcus pyogenes. *Journal of bacteriology* **1996,** *178*, 5546-5549.

64. Bisno, A. L., Acute pharyngitis: etiology and diagnosis. *Pediatrics* **1996,** *97*, 949-954.

65. Wannamaker, L. W., Perplexity and precision in the diagnosis of streptococcal pharyngitis. *Archives of Pediatrics & Adolescent Medicine* **1972,** *124*, 352.

66. Starr, C. R.; Engleberg, N. C., Role of hyaluronidase in subcutaneous spread and growth of group A streptococcus. *Infection and Immunity* **2006,** *74*, 40-48.

67. Manetti, A. G.; Zingaretti, C.; Falugi, F.; Capo, S.; Bombaci, M.; Bagnoli, F.; Gambellini, G.; Bensi, G.; Mora, M.; Edwards, A. M., Streptococcus pyogenes pili promote pharyngeal cell adhesion and biofilm formation. *Molecular Microbiology* **2007,** *64*, 968-983.

68. Günther, E.; Ozegowski, J. H.; Köhler, W., Occurrence of Extracellular Hyaluronic Acid and Hyaluronatlyase in Streptococci of Groups A, B, C, and G. *Zentralblatt für Bakteriologie* **1996,** *285*, 64-73.

69. Hasty, D.; Ofek, I.; Courtney, H.; Doyle, R., Multiple adhesins of streptococci. *Infection and Immunity* **1992,** *60*, 2147.

70. van der Mei, H. C.; Rustema-Abbing, M.; de Vries, J.; Busscher, H. J., Bond strengthening in oral bacterial adhesion to salivary conditioning films. *Applied and environmental microbiology* **2008,** *74*, 5511-5515.

71. Moscoso, M.; García, E.; López, R., Biofilm formation by Streptococcus pneumoniae: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *Journal of bacteriology* **2006,** *188*, 7785-7795.

72. Doyle, R. J., Contribution of the hydrophobic effect to microbial infection. *Microbes and Infection* **2000,** *2*, 391-400.

73. Wadström, T.; Schmidt, K. H.; Kühnemund, O.; Havlícek, J.; Köhler, W., Comparative studies on surface hydrophobicity of streptococcal strains of groups A, B, C, D and G. *Journal of general microbiology* **1984,** *130*, 657-664.

74. Domenech, M.; García, E.; Moscoso, M., Biofilm formation in Streptococcus pneumoniae. *Microbial Biotechnology* **2012,** *5*, 455-465.

75. Lachica, R.; Zink, D., Plasmid-associated cell surface charge and hydrophobicity of Yersinia enterocolitica. *Infection and Immunity* **1984,** *44*, 540-543.

76. Tylewska, S.; Hjerten, S.; Wadström, T., Effect of subinhibitory concentrations of antibiotics on the adhesion of Streptococcus pyogenes to pharyngeal epithelial cells. *Antimicrobial agents and chemotherapy* **1981,** *20*, 563-566.

77. Miörner, H.; Johansson, G.; Kronvall, G., Lipoteichoic acid is the major cell wall component responsible for surface hydrophobicity of group A streptococci. *Infection and Immunity* **1983,** *39*, 336-343.

78. Wicken, A.; Knox, K. W., Lipoteichoic acids: a new class of bacterial antigen. *Science* **1975,** *187*, 1161-1167.

79. Fischer, W., Bacterial phosphoglycolipids and lipoteichoic acids. In *Glycolipids, Phosphoglycolipids, and Sulfoglycolipids*, Springer: 1990; pp 123-234.

80. Beachey, E. H.; Ofek, I., Epithelial cell binding of group A streptococci by lipoteichoic acid on fimbriae denuded of M protein. *The Journal of experimental medicine* **1976,** *143*, 759-771.

81. Manjula, B.; Acharya, A. S.; Mische, S.; Fairwell, T.; Fischetti, V., The complete amino acid sequence of a biologically active 197-residue fragment of M protein isolated from type 5 group A streptococci. *Journal of Biological Chemistry* **1984,** *259*, 3686-3693.

82. André, I.; Persson, J.; Blom, A.; Nilsson, H.; Drakenberg, T.; Lindahl, G.; Linse, S., Streptococcal M protein: structural studies of the hypervariable region, free and bound to human C4BP. *Biochemistry* **2006,** *45*, 4559-4568.

83. Fischetti, V. A., Streptococcal M protein: molecular design and biological behavior. *Clinical microbiology reviews* **1989,** *2*, 285.

84. Bessen, D.; Jones, K. F.; Fischetti, V. A., Evidence for two distinct classes of streptococcal M protein and their relationship to rheumatic fever. *The Journal of experimental medicine* **1989,** *169*, 269-283.

85. Bernish, B.; van de Rijn, I., Characterization of a two-component system in Streptococcus pyogenes which is involved in regulation of hyaluronic acid production. *Journal of Biological Chemistry* **1999,** *274*, 4786-4793.

86. Albertí, S.; Ashbaugh, C. D.; Wessels, M. R., Structure of the has operon promoter and regulation of hyaluronic acid capsule expression in group A Streptococcus. *Molecular Microbiology* **1998,** *28*, 343-353.

87. Stollerman, G. H.; Dale, J. B., The importance of the group a streptococcus capsule in the pathogenesis of human infections: a historical perspective. *Clinical Infectious Diseases* **2008,** *46*, 1038-1045.

88. Johnson, D. R.; Stevens, D. L.; Kaplan, E. L., Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. *Journal of Infectious Diseases* **1992,** *166*, 374-382.

89. Hall-Stoodley, L.; Costerton, J. W.; Stoodley, P., Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology* **2004,** *2*, 95-108.

90. Lemon, K.; Earl, A.; Vlamakis, H.; Aguilar, C.; Kolter, R., Biofilm development with an emphasis on Bacillus subtilis. In *Bacterial Biofilms*, Springer: 2008; pp 1-16.

91. Götz, F., Staphylococcus and biofilms. *Molecular Microbiology* **2002,** *43*, 1367-1378.

92. Davey, M. E.; O'toole, G. A., Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews* **2000,** *64*, 847-867.

93. O'Toole, G. A.; Kolter, R., Initiation of biofilm formation in Pseudomonas fluorescens WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. *Molecular Microbiology* **1998,** *28*, 449-461.

94. Lewis, K., Multidrug Tolerance of Biofilms and Persister Cells. In *Bacterial Biofilms*, Romeo, T., Ed. Springer Berlin Heidelberg: 2008; Vol. 322, pp 107-131.

95. Lewis, K., Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology* **2007,** *5*, 48-56.

96. Cvitkovitch, D. G.; Li, Y. H.; Ellen, R. P., Quorum sensing and biofilm formation in Streptococcal infections. *Journal of Clinical Investigation* **2003,** *112*, 1626.

97. Lembke, C.; Podbielski, A.; Hidalgo-Grass, C.; Jonas, L.; Hanski, E.; Kreikemeyer, B., Characterization of biofilm formation by clinically relevant serotypes of group A streptococci. *Applied and environmental microbiology* **2006,** *72*, 2864-2875.

98. Cho, K. H.; Caparon, M. G., Patterns of virulence gene expression differ between biofilm and tissue communities of Streptococcus pyogenes. *Molecular Microbiology* **2005,** *57*, 1545-1556.

99. Neu, H.; Gootz, T., Antimicrobial chemotherapy. **1996**.

100. Conley, J.; Olson, M. E.; Cook, L. S.; Ceri, H.; Phan, V.; Davies, H. D., Biofilm formation by group a streptococci: is there a relationship with treatment failure? *Journal of Clinical Microbiology* **2003,** *41*, 4043-4048.

101. Shen, Y.; Köller, T.; Kreikemeyer, B.; Nelson, D. C., Rapid degradation of Streptococcus pyogenes biofilms by PlyC, a bacteriophage-encoded endolysin. *Journal of Antimicrobial Chemotherapy* **2013**, dkt104.

102. Limsuwan, S.; Kayser, O.; Voravuthikunchai, S. P., Antibacterial activity of Rhodomyrtus tomentosa (Aiton) Hassk. leaf extract against clinical isolates of Streptococcus pyogenes. *Evidence-Based Complementary and Alternative Medicine* **2012,** *2012*.

103. Wannamaker, L. W.; Rammelkamp, C. H.; Denny, F. W.; Brink, W. R.; Houser, H. B.; Hahn, E. O.; Dingle, J. H., Prophylaxis of acute rheumatic fever: by treatment of the preceding streptococcal infection with various amounts of depot penicillin. *The American journal of medicine* **1951,** *10*, 673-695.

104. Pichichero, M. E., Margolis, P. A., A comparison of cephalosporins and penicillins in the treatment of group A beta-hemolytic streptococcal pharyngitis: a meta-analysis supporting the concept of microbial copathogenicity. *The Pediatric Infectious Disease Journal* **1991,** *10*, 275.

105. Kuhn, S. M.; Preiksaitis, J.; Tyrrel, G. J.; Jadavji, T.; Church, D.; Davies, H. D., Evaluation of potential factors contributing to microbiological treatment failure in Streptococcus pyogenes pharyngitis. *The Canadian Journal of Infectious Diseases* **2001,** *12*, 33.

106. Varaldo, P. E.; Debbia, E. A.; Nicoletti, G.; Pavesio, D.; Ripa, S.; Schito, G. C.; Tempera, G., Nationwide Survey in Italy of Treatment of Streptococcus pyogenes Pharyngitis in Children: Influence of Macrolide Resistance on Clinical and Microbiological Outcomes. *Clinical Infectious Diseases* **1999,** *29*, 869-873.

107. Passali, D.; Lauriello, M.; Passali, G.; Passàli, F.; Bellussi, L., Group A streptococcus and its antibiotic resistance. *Acta otorhinolaryngologica italica* **2007,** *27*, 27.

108. Molinari, G.; Talay, S. R.; Valentin-Weigand, P.; Rohde, M.; Chhatwal, G. S., The fibronectin-binding protein of Streptococcus pyogenes, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infection and Immunity* **1997,** *65*, 1357-1363.

109. Nyberg, P.; Sakai, T.; Cho, K. H.; Caparon, M. G.; Fässler, R.; Björck, L., Interactions with fibronectin attenuate the virulence of Streptococcus pyogenes. *The EMBO journal* **2004,** *23*, 2166-2174.

110. Moerman, D. E., An analysis of the food plants and drug plants of native North America. *Journal of ethnopharmacology* **1996,** *52*, 1-22.

111. Abachi, S.; Lee, S.; Rupasinghe, H., Molecular Mechanisms of Inhibition of Streptococcus Species by Phytochemicals. *Molecules* **2016,** *21*, 215.

112. Limsuwan, S.; Voravuthikunchai, S. P., Boesenbergia pandurata (Roxb.) Schltr., Eleutherine americana Merr. and Rhodomyrtus tomentosa (Aiton) Hassk. as antibiofilm producing and antiquorum sensing in Streptococcus pyogenes. *FEMS Immunology & Medical Microbiology* **2008,** *53*, 429-436.

113. Ogbulie, J.; Ogueke, C.; Nwanebu, F., Antibacterial properties of Uvaria chamae, Congronema latifolium, Garcinia kola, Vemonia amygdalina and Aframomium melegueta. *African Journal of biotechnology* **2007,** *6*.

114. Akoachere, J. T.; Ndip, R.; Chenwi, E.; Ndip, L.; Njock, T.; Anong, D., Antibacterial effects of Zingiber Officinale and Garcinia Kola on respiratory tract pathogens. *East African medical journal* **2002,** *79*, 588-592.

115. Farrukh, U.; Shareef, H.; Mahmud, S.; Ali, S. A.; Rizwani, G. H., Antibacterial activities of Coccinia grandis L. *Pak. J. Bot* **2008,** *40*, 1259-1262.

116. Salari, M.; Amine, G.; Shirazi, M.; Hafezi, R.; Mohammadypour, M., Antibacterial effects of Eucalyptus globulus leaf extract on pathogenic bacteria isolated from specimens of patients with respiratory tract disorders. *Clinical Microbiology and Infection* **2006,** *12*, 194-196.

117. Rashid, F.; Ahmed, R.; Mahmood, A.; Ahmad, Z.; Bibi, N.; Kazmi, S. U., Flavonoid glycosides fromPrunus armeniaca and the antibacterial activity of a crude extract. *Archives of pharmacal research* **2007,** *30*, 932-937.

118. Prachayasittikul, S.; Suphapong, S.; Worachartcheewan, A.; Lawung, R.; Ruchirawat, S.; Prachayasittikul, V., Bioactive metabolites from Spilanthes acmella Murr. *Molecules* **2009,** *14*, 850-867.

119. Ordoñez, A.; Gomez, J.; Cudmani, N.; Vattuone, M.; Isla, M., Antimicrobial activity of nine extracts of Sechium edule (Jacq.) Swartz. *Microbial ecology in health and disease* **2003,** *15*, 33-39.

120. Berahou, A. A., A.; Fdil, N.; Benharref, A.; Jana, M.; Gadhi, C., Antibacterial activity of Quercus ilex bark's extracts. *J Ethnopharmacol* **2007,** *112*, 426-429.

121. Cichewicz, R. H., Thorpe, P. A., The antimicrobial properties of chile peppers (Capsicum species) and their uses in Mayan medicine. *J Ethnopharmacol* **1996,** *52*, 61-70.

122. Fabio, A., C. Cermelli, G. Fabio, P. Nicoletti, and P. Quaglio, Screening of the antibacterial effects of a variety of essential oils on microorganisms responsible for respiratory infections. *Phytotherapy Research* **2007,** *4*, 374-377.

123. Fernández, M. A.; Garcia, M.; Saenz, M., Antibacterial activity of the phenolic acids fractions of Scrophularia frutescens and Scrophularia sambucifolia. *Journal of ethnopharmacology* **1996,** *53*, 11-14.

124. Reichling, J.; Neuner, A.; Sharaf, M.; Harkenthal, M.; Schnitzler, P., Antiviral activity of Rhus aromatica (fragrant sumac) extract against two types of herpes simplex viruses in cell culture. *Die Pharmazie-An International Journal of Pharmaceutical Sciences* **2009,** *64*, 538-541.

125. Ahmed, G. F.; Elkhatib, W. F.; Noreddin, A. M., Inhibition of Pseudomonas aeruginosa PAO1 adhesion to and invasion of A549 lung epithelial cells by natural extracts. *Journal of infection and public health* **2014,** *7*, 436-444.

126. Morán, A.; Gutiérrez, S.; Martínez-Blanco, H.; Ferrero, M.; Monteagudo-Mera, A.; Rodríguez-Aparicio, L., Non-toxic plant metabolites regulate Staphylococcus viability and biofilm formation: a natural therapeutic strategy useful in the treatment and prevention of skin infections. *Biofouling* **2014,** *30*, 1175-1182.

127. Nicolosi, D.; Tempera, G.; Genovese, C.; Furneri, P. M., Anti-adhesion activity of A2-type proanthocyanidins (a cranberry major component) on uropathogenic E. coli and P. mirabilis strains. *Antibiotics* **2014,** *3*, 143-154.

128. Chan, M.; Hidalgo, G.; Asadishad, B.; Almeida, S.; Muja, N.; Mohammadi, M. S.; Nazhat, S. N.; Tufenkji, N., Inhibition of bacterial motility and spreading via release of cranberry derived materials from silicone substrates. *Colloids and Surfaces B: Biointerfaces* **2013,** *110*, 275-280.

129. Rane, H. S.; Bernardo, S. M.; Howell, A. B.; Lee, S. A., Cranberry-derived proanthocyanidins prevent formation of Candida albicans biofilms in artificial urine through biofilm-and adherence-specific mechanisms. *Journal of Antimicrobial Chemotherapy* **2014,** *69*, 428-436.

130. McCall, J.; Hidalgo, G.; Asadishad, B.; Tufenkji, N., Cranberry impairs selected behaviors essential for virulence in Proteus mirabilis HI4320. *Canadian journal of microbiology* **2013,** *59*, 430-436.

131. Lacombe, A.; McGivney, C.; Tadepalli, S.; Sun, X.; Wu, V. C., The effect of American cranberry (Vaccinium macrocarpon) constituents on the growth inhibition, membrane integrity, and injury of Escherichia coli O157: H7 and Listeria monocytogenes in comparison to Lactobacillus rhamnosus. *Food microbiology* **2013,** *34*, 352-359.

132. Feng, G.; Klein, M. I.; Gregoire, S.; Singh, A. P.; Vorsa, N.; Koo, H., The specific degree-of-polymerization of A-type proanthocyanidin oligomers impacts Streptococcus mutans glucan-mediated adhesion and transcriptome responses within biofilms. *Biofouling* **2013,** *29*, 629-640.

133. Li, D.; Baert, L.; Xia, M.; Zhong, W.; Jiang, X.; Uyttendaele, M., Effects of a variety of food extracts and juices on the specific binding ability of norovirus GII. 4 P particles. *Journal of food protection* **2012,** *75*, 1350.

134. O'May, C.; Ciobanu, A.; Lam, H.; Tufenkji, N., Tannin derived materials can block swarming motility and enhance biofilm formation in Pseudomonas aeruginosa. *Biofouling* **2012,** *28*, 1063-1076.

135. Feldman, M.; Grenier, D., Cranberry proanthocyanidins act in synergy with licochalcone A to reduce Porphyromonas gingivalis growth and virulence properties, and to suppress cytokine secretion by macrophages. *Journal of applied microbiology* **2012,** *113*, 438-447.

136. LaPlante, K. L.; Sarkisian, S. A.; Woodmansee, S.; Rowley, D. C.; Seeram, N. P., Effects of cranberry extracts on growth and biofilm production of Escherichia coli and Staphylococcus species. *Phytotherapy Research* **2012,** *26*, 1371-1374.

137. Su, X.; Howell, A. B.; D'Souza, D. H., Antibacterial effects of plant-derived extracts on methicillin-resistant Staphylococcus aureus. *Foodborne pathogens and disease* **2012,** *9*, 573-578.

138. Sagdic, O.; Aksoy, A.; Ozkan, G., Evaluation of the antibacterial and antioxidant potentials of cranberry (gilaburu, Viburnum opulus L.) fruit extract. *Acta Alimentaria* **2006,** *35*, 487-492.

139. Česonienė, L.; Daubaras, R.; Kraujalytė, V.; Venskutonis, P. R.; Šarkinas, A., Antimicrobial activity of Viburnum opulus fruit juices and extracts. *Journal für Verbraucherschutz und Lebensmittelsicherheit* **2014,** *9*, 129-132.

140. Eryilmaz, M.; Ozbilgin, S.; Ergene, B.; Yilmaz, B. S.; Altun, M.; Saltan, G., Antimicrobial activity of Turkish Viurnum species. *Bangladesh Journal of Botany* **2014,** *42*, 355-360.

141. Turker, A. U.; Yildirim, A. B.; Karakas, F. P., Antibacterial and antitumor activities of some wild fruits grown in Turkey. *Biotechnology & Biotechnological Equipment* **2012,** *26*, 2765-2772.

142. Česonienė, L.; Daubaras, R.; Viškelis, P.; Šarkinas, A., Determination of the total phenolic and anthocyanin contents and antimicrobial activity of Viburnum opulus fruit juice. *Plant foods for human nutrition* **2012,** *67*, 256-261.

143. Cesoniene, L.; Jasutiene, I.; Sarkinas, A., Phenolics and anthocyanins in berries of European cranberry and their antimicrobial activity. *Medicina (Kaunas, Lithuania)* **2008,** *45*, 992-999.

144. Nasar-Abbas, S.; Halkman, A. K., Antimicrobial effect of water extract of sumac (Rhus coriaria L.) on the growth of some food borne bacteria including pathogens. *International journal of food microbiology* **2004,** *97*, 63-69.

145. Kossah, R.; Zhang, H.; Chen, W., Antimicrobial and antioxidant activities of Chinese sumac (Rhus typhina L.) fruit extract. *Food control* **2011,** *22*, 128-132.

146. Fazeli, M. R.; Amin, G.; Attari, M. M. A.; Ashtiani, H.; Jamalifar, H.; Samadi, N., Antimicrobial activities of Iranian sumac and avishan-e shirazi (Zataria multiflora) against some food-borne bacteria. *Food control* **2007,** *18*, 646-649.

147. Nasar Abbas, S.; Halkman, A. K.; Alhaq, M., Inhibition of some foodborne bacteria by alcohol extract of sumac (Rhus coriaria L.). *Journal of food safety* **2004,** *24*, 257-267.

148. Liepiņa, I.; Nikolajeva, V.; Jākobsone, I., Antimicrobial activity of extracts from fruits of Aronia melanocarpa and Sorbus aucuparia. **2013**.

149. Nohynek, L. J.; Alakomi, H.-L.; Kähkönen, M. P.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K.-M.; Puupponen-Pimiä, R. H., Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutrition and cancer* **2006,** *54*, 18-32.

150. Krisch, J.; Galgóczy, L.; Tölgyesi, M.; Papp, T.; Vágvölgyi, C., Effect of fruit juices and pomace extracts on the growth of Gram-positive and Gram-negative bacteria. *Acta Biologica Szegediensis* **2008,** *52*, 267-270.

151. Paudel, B.; Bhattarai, H. D.; Kim, I. C.; Lee, H.; Sofronov, R.; Ivanova, L.; Poryadina, L.; Yim, J. H., Estimation of antioxidant, antimicrobial activity and brine shrimp toxicity of plants collected from Oymyakon region of the Republic of Sakha (Yakutia), Russia. *Biological research* **2014,** *47*.

152. Matsuura, H.; Saxena, G.; Farmer, S.; Hancock, R.; Towers, G., Antibacterial and antifungal compounds from Empetrum nigrum. *Planta medica* **1995,** *61*.

153. Ikuta, K.; Hashimoto, K.; Kaneko, H.; Mori, S.; Ohashi, K.; Suzutani, T., Anti-viral and anti-bacterial activities of an extract of blackcurrants (Ribes nigrum L.). *Microbiology and immunology* **2012,** *56*, 805-809.

154. Krisch, J.; Ördögh, L.; Galgóczy, L.; Papp, T.; Vágvölgyi, C., Anticandidal effect of berry juices and extracts from Ribes species. *Open Life Sciences* **2009,** *4*, 86-89.

155. Miladinović, B.; Kostić, M.; Šavikin, K.; Đorđević, B.; Mihajilov-Krstev, T.; Živanović, S.; Kitić, D., Chemical profile and antioxidative and antimicrobial activity of juices and extracts of 4 black currants varieties (Ribes nigrum L.). *Journal of food science* **2014,** *79*, C301-C309.

156. Palíková, I.; Heinrich, J.; Bednář, P.; Marhol, P.; Křen, V. r.; Cvak, L.; Valentová, K. i.; Růžička, F.; Holá, V.; Kolář, M., Constituents and antimicrobial properties of blue honeysuckle: a novel source for phenolic antioxidants. *Journal of Agricultural and Food Chemistry* **2008,** *56*, 11883-11889.

157. Raudsepp, P.; Anton, D.; Roasto, M.; Meremäe, K.; Pedastsaar, P.; Mäesaar, M.; Raal, A.; Laikoja, K.; Püssa, T., The antioxidative and antimicrobial properties of the blue honeysuckle (Lonicera caerulea L.), Siberian rhubarb (Rheum rhaponticum L.) and some other plants, compared to ascorbic acid and sodium nitrite. *Food control* **2013,** *31*, 129-135.

158. Khan, A.; Nazir, S.; Tahir, K.; Khan, Z. U. H.; Wu, N.; Yasmeen, R.; Ali, I., Antimicrobial assay of methanolic crude of Lonicera lanceolata. *African Journal of Microbiology Research* **2014,** *8*, 2534-2539.

159. Tahir, K.; Nazir, S.; Khan, A.; Khan, Z. U. H.; Islam, R. U.; Ammar, M.; Rehman, A. U., Antimicrobial and enzyme inhibitory assay of constituents of Lonicera lanceolata. *African Journal of Pharmacy and Pharmacology* **2014,** *8*, 529-534.

160. Ho, K.; Tsai, C.; Huang, J.; Chen, C.; Lin, T.; Lin, C., Antimicrobial activity of tannin components from Vaccinium vitis-idaea L. *Journal of Pharmacy and Pharmacology* **2001,** *53*, 187-191.

161. Cheng, H. Y.; Lin, T. C.; Yang, C. M.; Shieh, D. E.; Lin, C. C., In vitro anti-HSV-2 activity and mechanism of action of proanthocyanidin A-1 from Vaccinium vitis-idaea. *Journal of the Science of Food and Agriculture* **2005,** *85*, 10-15.

162. Grange, J.; Davey, R., Detection of antituberculous activity in plant extracts. *Journal of applied bacteriology* **1990,** *68*, 587-591.

163. Locatelli, M.; Epifano, F.; Genovese, S.; Carlucci, G.; Koncić, M.; Kosalec, I.; Kremer, D., Anthraquinone profile, antioxidant and antimicrobial properties of bark extracts of Rhamnus catharticus and R. orbiculatus. *Natural product communications* **2011,** *6*, 1275-1280.

164. Biswas, D.; Wideman, N. E.; O'Bryan, C. A.; Muthaiyan, A.; Lingbeck, J. M.; Crandall, P. G.; Ricke, S. C., Pasteurized blueberry (Vaccinium corymbosum) juice inhibits growth of bacterial pathogens in milk but allows survival of probiotic bacteria. *Journal of food safety* **2012,** *32*, 204-209.

165. Park, Y. J.; Biswas, R.; Phillips, R. D.; Chen, J., Antibacterial activities of blueberry and muscadine phenolic extracts. *Journal of food science* **2011,** *76*, M101-M105.

166. Shen, X.; Sun, X.; Xie, Q.; Liu, H.; Zhao, Y.; Pan, Y.; Hwang, C.-A.; Wu, V. C., Antimicrobial effect of blueberry (Vaccinium corymbosum L.) extracts against the growth of Listeria monocytogenes and Salmonella Enteritidis. *Food control* **2014,** *35*, 159-165.

167. Lacombe, A.; Wu, V. C.; White, J.; Tadepalli, S.; Andre, E. E., The antimicrobial properties of the lowbush blueberry (Vaccinium angustifolium) fractional components against foodborne pathogens and the conservation of probiotic Lactobacillus rhamnosus. *Food microbiology* **2012,** *30*, 124-131.

168. Schmidt, B. M.; Howell, A. B.; McEniry, B.; Knight, C. T.; Seigler, D.; Erdman, J. W.; Lila, M. A., Effective separation of potent antiproliferation and antiadhesion components from wild blueberry (Vaccinium angustifolium Ait.) fruits. *Journal of Agricultural and Food Chemistry* **2004,** *52*, 6433-6442.

169. Ofek, I.; Goldhar, J.; Zafriri, D.; Lis, H.; Adar, R.; Sharon, N., Anti-Escherichia coli adhesin activity of cranberry and blueberry juices. *New England Journal of Medicine* **1991,** *324*, 1599.

170. Zhang, C. In *Effects of Blueberries on the Biofilm Formation of Vibrio parahaemolyticus*, 2015 Annual Meeting (July 25-28, 2015), 2015; Iafp: 2015.

171. Zimmer, K. R.; Blum-Silva, C. H.; Souza, A. L. K.; WulffSchuch, M.; Reginatto, F. H.; Pereira, C. M. P.; Macedo, A. J.; Lencina, C. L., The antibiofilm effect of Blueberry Fruit cultivars against Staphylococcus epidermidis and Pseudomonas aeruginosa. *Journal of medicinal food* **2014,** *17*, 324-331.

172. Zhou, L.; Ding, Y.; Chen, W.; Zhang, P.; Chen, Y.; Lv, X., The in vitro study of ursolic acid and oleanolic acid inhibiting cariogenic microorganisms as well as biofilm. **2013,** *Oral diseases 19*, 494-500.

173. Pegg, R. B., Rybarczyk, A., Amarowicz, R., Chromatographic separation of tannin fractions from a bearberry-leaf (Arctostaphylos uva-ursi l. sprengel) extract by SE-HPLC- A short report. *Polish Journal of Food & Nutrition Sciences* **2008,** *58*.

174. Gunathilake, K.; Rupasinghe, H. V., Inhibition of Human Low-Density Lipoprotein Oxidation In Vitro by Ginger Extracts. *Journal of medicinal food* **2014,** *17.4*, 424-31.

175. Pettit, R. K.; Weber, C. A.; Kean, M. J.; Hoffmann, H.; Pettit, G. R.; Tan, R.; Franks, K. S.; Horton, M. L., Microplate Alamar blue assay for Staphylococcus epidermidis biofilm susceptibility testing. *Antimicrob Agents Chemother* **2005,** *49*, 2612-2617.

176. Ogawa, T.; Terao, Y.; Okuni, H.; Ninomiya, K.; Sakata, H.; Ikebe, K.; Maeda, Y.; Kawabata, S., Biofilm formation or internalization into epithelial cells enable Streptococcus pyogenes to evade antibiotic eradication in patients with pharyngitis. *Microbial pathogenesis* **2011,** *51*, 58-68.

177. Anderson, E. L.; Cole, J. N.; Olson, J.; Ryba, B.; Ghosh, P.; Nizet, V., The fibrinogen-binding M1 protein reduces pharyngeal cell adherence and colonization phenotypes of M1T1 Group A Streptococcus. *Journal of Biological Chemistry* **2014,** *289*, 3539-3546.

178. Labrecque, J.; Bodet, C.; Chandad, F.; Grenier, D., Effects of a high-molecular-weight cranberry fraction on growth, biofilm formation and adherence of Porphyromonas gingivalis. *Journal of Antimicrobial Chemotherapy* **2006,** *58*, 439-443.

179. Okada, N.; Watarai, M.; Ozeri, V.; Hanski, E.; Caparon, M.; Sasakawa, C., A Matrix Form of Fibronectin Mediates Enhanced Binding ofStreptococcus pyogenes to Host Tissue. *Journal of Biological Chemistry* **1997,** *272*, 26978-26984.

180. Terao, Y.; Kawabata, S.; Nakata, M.; Nakagawa, I.; Hamada, S., Molecular Characterization of a Novel Fibronectin-binding Protein of Streptococcus pyogenesStrains Isolated from Toxic Shock-like Syndrome Patients. *Journal of Biological Chemistry* **2002,** *277*, 47428-47435.

181. Kreikemeyer, B., Nakata, M., Oehmcke, S., Gschwendtner, C., Normann, J., Podbielski, A. , Streptococcus pyogenes Collagen Type I-binding Cpa Surface Protein Expression profile, binding characteristics, biological functions, and potential clinical impact. *Journal of Biological Chemistry* **2005,** *280*, 33228-33239.

182. Becherelli, M.; Manetti, A. G.; Buccato, S.; Viciani, E.; Ciucchi, L.; Mollica, G.; Grandi, G.; Margarit, I., The ancillary protein 1 of Streptococcus pyogenes FCT-1 pili mediates cell adhesion and biofilm formation through heterophilic as well as homophilic interactions. *Molecular Microbiology* **2012,** *83*, 1035-1047.

183. Franklin, L.; Nobbs, A. H.; Bricio-Moreno, L.; Wright, C. J.; Maddocks, S. E.; Sahota, J. S.; Ralph, J.; O'Connor, M.; Jenkinson, H. F.; Kadioglu, A., The Agi/II family adhesin AspA is required for respiratory infection by Streptococcus pyogenes. *PLoS One* **2013,** *8*, e62433.

184. Ahmad, M. S.; Gul, H.; Ahmad, M.; Sarwar, N.; Abbasi, S. K.; Gulfraz, M., Phytochemical analysis and biological activities of Diospyros lotus L. fruit extracts. *International Journal of Pharmaceutical Chemistry* **2014,** *4*, 155-159.

185. Nagayama, K.; Iwamura, Y.; Shibata, T.; Hirayama, I.; Nakamura, T., Bactericidal activity of phlorotannins from the brown alga Ecklonia kurome. *Journal of Antimicrobial Chemotherapy* **2002,** *50*, 889-893.

186. Liu, C.; Liu, A.; Ma, Y.; Huang, K.; Li, Y.; Zhang, H. In *Study on Antibacterial Activity of Anthocyanins from Blueberry Wine Pomace*, 2015 International Power, Electronics and Materials Engineering Conference, 2015; Atlantis Press: 2015.

187. Salaheen, S.; Almario, J.; Biswas, D., Inhibition of growth and alteration of host cell interactions of Pasteurella multocida with natural byproducts. *Poultry science* **2014,** *93*, 1375-1382.

188. Wang, Y.; Chung, F. F.; Lee, S. M.; Dykes, G. A., Inhibition of attachment of oral bacteria to immortalized human gingival fibroblasts (HGF-1) by tea extracts and tea components. *BMC research notes* **2013,** *6*, 143.

189. Lee, J.-H.; Regmi, S. C.; Kim, J.-A.; Cho, M. H.; Yun, H.; Lee, C.-S.; Lee, J., Apple flavonoid phloretin inhibits Escherichia coli O157: H7 biofilm formation and ameliorates colon inflammation in rats. *Infection and Immunity* **2011,** *79*, 4819-4827.

190. Dixon, R.; Dey, P.; Lamb, C., Phytoalexins: enzymology and molecular biology. *Advances in enzymology and related areas of molecular biology* **1983,** *55*, 1-136.

191. Tsuchiya, H.; Sato, M.; Miyazaki, T.; Fujiwara, S.; Tanigaki, S.; Ohyama, M.; Tanaka, T.; Iinuma, M., Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant Staphylococcus aureus. *Journal of ethnopharmacology* **1996,** *50*, 27-34.

192. Blumberg, J. B.; Camesano, T. A.; Cassidy, A.; Kris-Etherton, P.; Howell, A.; Manach, C.; Ostertag, L. M.; Sies, H.; Skulas-Ray, A.; Vita, J. A., Cranberries and their bioactive constituents in human health. *Advances in Nutrition: An International Review Journal* **2013,** *4*, 618-632.

193. Patra, A. K., *Dietary Phytochemicals and Microbes*. Springer Netherlands: 2012.

194. Merkl, R.; Hradkova, I.; Filip, V.; ŠMIdRkal, J., Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech J Food Sci* **2010,** *28*, 275-279.

195. Kubo, I.; Muroi, H.; Kubo, A., Structural functions of antimicrobial long-chain alcohols and phenols. *Bioorganic & medicinal chemistry* **1995,** *3*, 873-880.

196. Lee, S. G.; Nam, T.-G.; Kim, D.-O.; Lee, J.; Koo, S. I.; Chun, O. K., Anthocyanin compositions and contents influence the antioxidant capacities of berries. *The FASEB Journal* **2012,** *26*, lb318.

197. Burdulis, D.; Sarkinas, A.; Jasutiene, I.; Stackevicene, E.; Nikolajevas, L.; Janulis, V., Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (Vaccinium myrtillus L.) and blueberry (Vaccinium corymbosum L.) fruits. *Acta poloniae pharmaceutica* **2008,** *66*, 399-408.

198. Cisowska, A.; Wojnicz, D.; Hendrich, A. B., Anthocyanins as antimicrobial agents of natural plant origin. *Natural product communications* **2011,** *6*, 149-156.

199. Lacombe, A.; Wu, V. C.; Tyler, S.; Edwards, K., Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against

Escherichia coli O157: H7. *International journal of food microbiology* **2010,** *139*, 102-107.

200. Hyun, T. K.; Kim, H. C.; Ko, Y. J.; Kim, J. S., Antioxidant, α-glucosidase inhibitory and anti-inflammatory effects of aerial parts extract from Korean crowberry (Empetrum nigrum var. japonicum). *Saudi Journal of Biological Sciences* **2015**.

201. Huttunen, S.; Toivanen, M.; Arkko, S.; Ruponen, M.; Tikkanen-Kaukanen, C., Inhibition activity of wild berry juice fractions against Streptococcus pneumoniae binding to human bronchial cells. *Phytotherapy Research* **2011,** *25*, 122-127.

202. Paul Dzoyem, J.; Hamamoto, H.; Ngameni, B.; Tchaleu Ngadjui, B.; Sekimizu, K., Antimicrobial action mechanism of flavonoids from Dorstenia species. *Drug discoveries & therapeutics* **2013,** *7*, 66-72.

203. Stapleton, P. D.; Shah, S.; Hamilton-Miller, J. M.; Hara, Y.; Nagaoka, Y.; Kumagai, A.; Uesato, S.; Taylor, P. W., Anti-Staphylococcus aureus activity and oxacillin resistance modulating capacity of 3-O-acyl-catechins. *International journal of antimicrobial agents* **2004,** *24*, 374-380.

204. Wu, T., McCallum, J. L., Wang, S., Liu, R., Zhu, H.,Tsao, R. , Evaluation of antioxidant activities and chemical characterisation of staghorn sumac fruit (Rhus hirta L.). *Food chemistry* **2013,** *138*, 1333-1340.

205. Jayaraman, P.; Sakharkar, M. K.; Lim, C. S.; Tang, T. H.; Sakharkar, K. R., Activity and interactions of antibiotic and phytochemical combinations against Pseudomonas aeruginosa in vitro. *International journal of biological sciences* **2010,** *6*, 556.

206. Jagani, S.; Chelikani, R.; Kim, D.-S., Effects of phenol and natural phenolic compounds on biofilm formation by Pseudomonas aeruginosa. *Biofouling* **2009,** *25*, 321-324.

207. Baldassarri, L.; Recchia, S.; Creti, R.; Imperi, M.; Pataracchia, M.; Orefici, G., P608 Effect of antibiotics at subMIC concentration on biofilm formation by Streptococcus pyogenes. *International journal of antimicrobial agents* **2007,** *29, Supplement 2*, S142.

208. Ulrey, R. K.; Barksdale, S. M.; Zhou, W.; van Hoek, M. L., Cranberry proanthocyanidins have anti-biofilm properties against Pseudomonas aeruginosa. *BMC complementary and alternative medicine* **2014,** *14*, 499.

209. Yamanaka, A.; Kouchi, T.; Kasai, K.; Kato, T.; Ishihara, K.; Okuda, K., Inhibitory effect of cranberry polyphenol on biofilm formation and cysteine proteases of Porphyromonas gingivalis. *Journal of periodontal research* **2007,** *42*, 589-592.

210. Ahuja, S.; Kaack, B.; Roberts, J., Loss of fimbrial adhesion with the addition of Vaccinum macrocarpon to the growth medium of P-fimbriated Escherchia coli. *The Journal of urology* **1998,** *159*, 559-562.

211. Olajuyigbe, O. O.; Afolayan, A. J., In vitro antibacterial and time-kill assessment of crude methanolic stem bark extract of Acacia mearnsii De Wild against bacteria in shigellosis. *Molecules* **2012,** *17*, 2103-2118.

212. Vikram, A.; Jayaprakasha, G.; Jesudhasan, P.; Pillai, S.; Patil, B., Suppression of bacterial cell–cell signalling, biofilm formation and type III secretion system by citrus flavonoids. *Journal of applied microbiology* **2010,** *109*, 515-527.

213. Kang, M. S.; Oh, J. S.; Kang, I. C.; Hong, S. J.; Choi, C. H., Inhibitory effect of methyl gallate and gallic acid on oral bacteria. *The Journal of Microbiology* **2008,** *46*, 744-750.

214. Sendamangalam, V.; Choi, O. K.; Kim, D.; Seo, Y., The anti-biofouling effect of polyphenols against Streptococcus mutans. *Biofouling* **2011,** *27*, 13-19.

215. Borges, A.; Saavedra, M. J.; Simões, M., The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling* **2012,** *28*, 755-767.

216. Saxena, G.; McCutcheon, A.; Farmer, S.; Towers, G.; Hancock, R., Antimicrobial constituents of Rhus glabra. *Journal of ethnopharmacology* **1994,** *42*, 95-99.

217. Alves, C. T.; Ferreira, I. C.; Barros, L.; Silva, S.; Azeredo, J.; Henriques, M., Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against Candida species. *Future microbiology* **2014,** *9*, 139-146.

218. Gehrke, I. T.; Neto, A. T.; Pedroso, M.; Mostardeiro, C. P.; Da Cruz, I. B.; Silva, U. F.; Ilha, V.; Dalcol, I. I.; Morel, A. F., Antimicrobial activity of Schinus lentiscifolius (Anacardiaceae). *Journal of ethnopharmacology* **2013,** *148*, 486-491.

219. Monte, J.; Abreu, A. C.; Borges, A.; Simões, L. C.; Simões, M., Antimicrobial activity of selected phytochemicals against Escherichia coli and Staphylococcus aureus and their biofilms. *Pathogens* **2014,** *3*, 473-498.

220. Manner, S.; Skogman, M.; Goeres, D.; Vuorela, P.; Fallarero, A., Systematic exploration of natural and synthetic flavonoids for the inhibition of Staphylococcus aureus biofilms. *International journal of molecular sciences* **2013,** *14*, 19434-19451.

221. Xu, C.; Yagiz, Y.; Hsu, W.-Y.; Simonne, A.; Lu, J.; Marshall, M. R., Antioxidant, antibacterial, and antibiofilm properties of polyphenols from muscadine grape (Vitis rotundifolia Michx.) pomace against selected foodborne pathogens. *Journal of Agricultural and Food Chemistry* **2014,** *62*, 6640-6649.

222. Luís, Â., Silva, F., Sousa, S., Duarte, A., Domingues, F., Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. *Biofouling* **2014,** *30*, 69-79.

223. De Vita, D.; Friggeri, L.; D'Auria, F. D.; Pandolfi, F.; Piccoli, F.; Panella, S.; Palamara, A. T.; Simonetti, G.; Scipione, L.; Di Santo, R., Activity of caffeic acid derivatives against Candida albicans biofilm. *Bioorganic & medicinal chemistry letters* **2014,** *24*, 1502-1505.

224. Šmitran, A.; Vuković, D.; Gajić, I.; Marinković, J.; Ranin, L., Effects of penicillin and erythromycin on adherence of invasive and noninvasive isolates of Streptococcus pyogenes to laminin. *Memórias do Instituto Oswaldo Cruz* **2015**, 00-00.

225. Zeng, H., *Polymer adhesion, friction, and lubrication*. John Wiley & Sons: 2013.

226. Furiga, A.; Lonvaud Funel, A.; Dorignac, G.; Badet, C., In vitro anti-bacterial and anti-adherence effects of natural polyphenolic compounds on oral bacteria. *JAM Journal of Applied Microbiology* **2008,** *105*, 1470-1476.

227. Ren, D.; Zuo, R.; Barrios, A. F. G.; Bedzyk, L. A.; Eldridge, G. R.; Pasmore, M. E.; Wood, T. K., Differential gene expression for investigation of Escherichia coli biofilm inhibition by plant extract ursolic acid. *Applied and environmental microbiology* **2005,** *71*, 4022-4034.

228. Goulter, R.; Gentle, I.; Dykes, G., Issues in determining factors influencing bacterial attachment: a review using the attachment of Escherichia coli to abiotic surfaces as an example. *Letters in applied microbiology* **2009,** *49*, 1-7.

229. Xiao, Y., Liu, T., Zhan, L., Zhou, X, The effects of tea polyphenols on the adherence of cariogenic bacterium to the collagen in vitro. *West China journal of stomatology* **2000,** *18*, 340-342.

230. Sakanaka, S.; Aizawa, M.; Kim, M.; Yamamoto, T., Inhibitory effects of green tea polyphenols on growth and cellular adherence of an oral bacterium, Porphyromonas gingivalis. *Bioscience, biotechnology, and biochemistry* **1996,** *60*, 745-749.

231. Stauder, M.; Papetti, A.; Mascherpa, D.; Schito, A. M.; Gazzani, G.; Pruzzo, C.; Daglia, M., Antiadhesion and antibiofilm activities of high molecular weight coffee components against Streptococcus mutans. *Journal of Agricultural and Food Chemistry* **2010,** *58*, 11662-11666.

232. Howell, A. B.; Reed, J. D.; Krueger, C. G.; Winterbottom, R.; Cunningham, D. G.; Leahy, M., A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. *Phytochemistry* **2005,** *66*, 2281-2291.

233. Grenier, D.; Chen, H.; Lagha, A. B.; Fournier-Larente, J.; Morin, M.-P., Dual Action of Myricetin on Porphyromonas gingivalis and the Inflammatory Response of Host Cells: A Promising Therapeutic Molecule for Periodontal Diseases. *PLoS One* **2015,** *10*, e0131758.

234. Singh, A. P.; Wilson, T.; Kalk, A. J.; Cheong, J.; Vorsa, N., Isolation of specific cranberry flavonoids for biological activity assessment. *Food chemistry* **2009,** *116*, 963-968.

235. Steinberg, D.; Feldman, M.; Ofek, I.; Weiss, E. I., Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. *Journal of Antimicrobial Chemotherapy* **2004,** *54*, 86-89.

236. Löhr, G.; Beikler, T.; Podbielski, A.; Standar, K.; Redanz, S.; Hensel, A., Polyphenols from Myrothamnus flabellifolia Welw. inhibit in vitro adhesion of Porphyromonas gingivalis and exert anti-inflammatory cytoprotective effects in KB cells. *Journal of clinical periodontology* **2011,** *38*, 457-469.

237. Lowy, F. D.; Chang, D. S.; Neuhaus, E. G.; Horne, D. S.; Tomasz, A.; Steigbigel, N. H., Effect of penicillin on the adherence of Streptococcus sanguis in vitro and in the rabbit model of endocarditis. *Journal of Clinical Investigation* **1983,** *71*, 668.

238. Sethman, C. R.; Doyle, R. J.; Cowan, M. M., Flow cytometric evaluation of adhesion of Streptococcus pyogenes to epithelial cells. *Journal of microbiological methods* **2002,** *51*, 35-42.

239. Eickenberg, H. U.; Hahn, H.; Opferkuch, W., *The influence of antibiotics on the host-parasite relationship*. Springer Science & Business Media: 2012.