EXPLORATION OF PHYTOCHEMICAL-RICH EXTRACTS OF SELECTED CANADIAN PLANTS AGAINST STREPTOCOCCAL PHARYNGITIS

By

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Dedicated
To my inspiring Thaththa, Amma, Akka, Malliya and Buddika
for being the
pillows, role models, catapults,
cheerleading squad and sounding boards
I have needed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Streptococcal pharyngitis is a significant health issue worldwide. Antibiotics and non-steroidal anti-inflammatory drugs are often prescribed as treatments. However, discovery of specific phytochemicals as novel antimicrobials agents or analgesic natural health product could help in overcoming the therapeutic challenges. In this study, 32 phytochemical extracts were investigated for anti-streptococcal and anti-inflammatory activity *in vitro*. Ethanol extract of licorice roots, sage leaves and echinacea flowers and essential oils of sage leaves and oregano flowering shoots showed significant inhibition of growth and biofilm formation of *Streptococcus pyogenes*. Furthermore, aqueous extracts of ginger rhizome, clove buds, echinacea flowers, thyme flowering shoots and ethanolic extracts of danshen roots showed greater *in vitro* anti-inflammatory activity on human tonsil epithelial cells. Therefore, considering both antimicrobial and analgesic abilities, extracts of oregano, sage, licorice, echinacea and thyme may have the potential to be used as natural health products, in the management of streptococcal pharyngitis.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAPH	2-azobis (2-amidinopropane) dihydrochloride
AE	Aqueous extracts
AI-2	Autoinducer 2
ANOVA	Analysis of variance
AP	Activation protein
ATCC	American type culture collection
BHI	Brain heart infusion
CFU	Colony forming unit
CGM	complete growth medium
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CV	Crystal violet
DMMB	1,9-dimethyl methylene blue
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DPPH	1,1-diphenyl-2-picrylhydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EE	Ethanol extracts
ELISA	Enzyme linked immune-sorbent assay
ENA-78	Epithelial cell-derived neutrophil activating protein 78
EO	Essential oil
EPS	Extracellular polymeric substances
F	Flowers
FB	Flower buds
FBS	Fetal bovine serum
FDA	Fluorescein-diacetate
FID	Flame ionizing detector
FRAP	Ferric reducing/antioxidant power
FS	Flowering shoots
GAE	Gallic acid equivalents

GAS	Group A streptococcus
GC	Gas liquid chromatography
GCP-2	Granulocyte chemotactic protein
hBD-2	Human β-defensin-2
HDMS	Hexamethyldisilazane
hr	hour
HRP	Horseradish peroxidase
HTonEpiC	Human tonsil epithelial cells
IB	Inner bark
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kDa	Kilo Dalton
L	Leaves
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinase
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm Inhibitory concentration
MIC	Minimum inhibitory concentration
min	Minutes
mRNA	Messenger ribonucleic acid
MTS	3- (4,5-dimethylthiazol-2-yl) -5- (3 – carboxymethoxyphenyl) -2- (4-sulfophenyl) - 2H – tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
Na_2SO_4	Sodium sulfate
NF-κB	Nuclear factor kappa B
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
ORAC	Oxygen radical absorbance capacity
P/S solution	Penicillin/streptomycin solution
PBS	Phosphate buffered saline
PG	Prostaglandin

PGN	Peptidoglycan
PLL	Poly-L-lysine
PMS	Phenazine methosulfate
R	Roots
Rh	Rhizome
S	Stem
s	Second
SEM	Scanning electron microscopy
TE	Trolox equivalents
TEpiCGS	Tonsil epithelial cell growth supplement
TEpiCM	Tonsil epithelial cell medium
TLR	Toll-like receptor
TMB	3, 3', 5, 5'- Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
TNS	Trypsin neutralization solution
TP	Total phenol
US$	United state dollars

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CHAPTER 1. INTRODUCTION

1.1 INTRODUCTION

Pharyngitis, an inflammation of the mucous membrane and underlying structures of the pharynx (Langlois and Andreae, 2011), is a very common disease and accounts for approximately 5% of medical visits. Pharyngitis is an be found in all age groups, however, is most common among school-aged children and adolescents (Shaikh et al., 2010), and mostly occurs during the winter and spring (Cirilli, 2013). It is caused by infectious agents such as viruses and bacteria, as well as non-infectious factors, such as excessive alcohol intake, allergies, throat injury, pollen and smoking (Renner et al., 2012). Viruses are the most common cause accounting about 70% of cases and around 15-36% of the incidence is caused by bacterial infection (Choby, 2009; Cooper et al., 2001).

S. pyogenes, called group A streptococcus (GAS), is the most important bacterial cause of pharyngitis. GAS, a Gram-positive coccus, is responsible for about 600 million cases of streptococcal pharyngitis worldwide on an annual basis (Carapetis et al., 2005). Approximately 7.3 million outpatient physician visits are attributable to pharyngitis which occurs each year among children in the United States (Pfoh et al., 2008). In the USA, the widespread prevalence of this disease results in considerable costs in diagnosis and management (Bisno et al., 1997). The societal cost (medicinal and non-medicinal) per case is over US$ 200, with almost one half being attributable to nonmedical costs (time costs, child care, non-prescription drugs, transportation, throat lozenges, throat sprays, vapor rubs and herbal teas) (Bisno et al., 1997; Pfoh et al., 2008). Although, the cost per case may be lower than that for other infections, GAS pharyngitis occurs frequently, resulting in an estimated national economic burden of US$ 224 to US$ 539 million annually in the United

States alone (Pfoh et al., 2008). In addition to illness resulting from direct infection, GAS can triggers the life-threatening post-infectious complications of these conditions, such as stroke, rheumatic heart disease, post-streptococcal glomerulonephritis and streptococcal toxic shock syndrome (Wessels, 2011). There are at least 517,000 deaths globally each year due to severe *S. pyogenes* infections and rheumatic fever disease alone causes 233,000 deaths (Shea et al., 2011). Thus, infections caused by GAS are a major public health concern in the United States, Canada and throughout the world (Shea et al., 2011).

Infection is initiated by adhesion of the bacteria to human epithelial cells (Terao, 2012). GAS surface components produce several specific adherence factors, mainly lipoteichoic acid, M protein, and certain membrane proteins that influence host-pathogen interactions (Bisno et al., 2003; Passali et al., 2007). Expression of these adhesion molecules promotes bacterial adherence to, and entry into, human cells (Passali et al., 2007). After that, colonization and development of biofilm occurs. Biofilms are structural complexes and dynamic communities of cells that are attached to a substrate and encased in extracellular polymeric substances containing polysaccharides, proteins, and extracellular microbial DNA (Bueno, 2014). GAS may grow continuously in size, proliferate and detach as individual cells from the biofilm after maturation (Post et al., 2004). Bacterial quorum sensing allows them to communicate and coordinate the gene expression (Waters and Bassler, 2005). Hereafter, they invade the pharyngeal tissue and cause a localized inflammatory reaction of the throat. Inflammation is a cascade of biochemical events progressing to the inflammatory response, involving the local vascular system, the immune system, and the injured tissue (White, 1999). Various pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-15, IL-17, IL-18 and tumor

necrosis factor-α (TNF-α) and chemokines, including IL-8, IL-17, IL-23 and granulocyte chemotactic protein (GCP-2), trigger inflammatory responses (Rock et al., 2010; White, 1999; Wojdasiewicz et al., 2014). After initial invasion of human tissues, it is reported that *S. pyogenes* can spread rapidly to various organs (Terao, 2012). Non-steroidal anti-inflammatory drugs (NSAIDs) are usually prescribed for the symptomatic relief of inflammation. Examples of NSAIDs include aspirin, ibuprofen, diclofenac, etodolac, nemesulide and fenoprofen (Cremonesi and Cavalieri, 2015). They inhibit different steps in the inflammatory process, including the activity of cyclooxygenase-1 (COX-1), COX-2, 5-lipoxygenase, an enzyme responsible for the production of leukotrienes (Cremonesi and Cavalieri, 2015; Ricciotti and FitzGerald, 2011). Effectiveness of these NSAIDs in management of GAS pharyngitis is well-established; however, some unexpected adverse events, such as shortness of breath and abdominal pain, direct clinicians and consumers to look for alternative treatments (Kim et al., 2013).

GAS pharyngitis requires antibiotic therapy (Bisno et al., 2002). The bacteriologic and clinical efficacy on pathogens, impact of antibiotics on normal flora, patients' allergies, and frequency of administration, duration of therapy, potential side effects, compliance and cost should be a criteria for antibiotic selection (Dajani et al., 1995; Wilcox, 2009). Despite over five decades of exposure to penicillin, *S. pyogenes* remains fully susceptible and continues to be the drug of first choice because of its proven efficacy and safety, narrow spectrum, and low cost (Dajani et al., 1995; Shulman et al., 2012). A dose of 125 mg to 250 mg of phenoxymethyl penicillin (penicillin V) administered three times daily for ten days has been recommended for oral treatment by the American Heart Association (Bisno et al., 1997).

However, antibiotic treatment failure has been reported in clinical case of streptococcal pharyngitis (Kuhn et al., 2001). Several explanations have been advanced for these treatment challenges, such as coexistence of oropharyngeal beta-lactamase producing bacteria, biofilm formation, interference by aerobic and anaerobic commensals and development of their resistance (Passali et al., 2007). Therefore, physicians need to make a clinical decision about whether the pharyngitis is attributable to GAS, when prescribing antibiotics. A wide variety of antibiotics have been shown to be effective against GAS (Bisno et al., 1997). Because the signs and symptoms of GAS pharyngitis overlap extensively with nonbacterial pharyngitis, it is difficult to make a diagnosis based solely on clinical findings (Bisno et al., 1997). No single element of the patient's history or physical examination reliably confirms or excludes GAS pharyngitis (Bisno et al., 1997). Despite many recommendations, physician management remains inconsistent, with most physicians using clinical suspicion as a reason to prescribe antibiotics for any type of pharyngitis (Bisno et al., 1997; Hayes and Williamson, 2001).

Although the resistance of GAS to penicillin has never been documented, bacteriologic and clinical treatment failures have increased in recent years as with all antibiotics (Brook, 2001). About 10-15% of patients are allergic to penicillin (Bass, 1991; Kuhn et al., 2001). Therefore, alternative treatments must be used in those patients with penicillin allergy, undesired side effects or compliance issues (Pichichero and Casey, 2007; Pichichero et al., 2000). However, with the development of allergy among those who have received penicillin and who have had treatment failures to other antibiotics, interest has been raised for discovering plant-based antimicrobial agents (Cowan, 1999; Wessels, 2011). On the other hand, identification of plant extracts or phytochemicals with anti-

streptococcal and anti-inflammatory properties are also of interest in developing antiseptic lozenges that can relieve pain from streptococcal pharyngitis.

The research hypothesis of this study is that specific phytochemical-rich herbal extracts can supress the planktonic growth and biofilm formation of *S. pyogenes* and also reduce the production of pro-inflammatory cytokines associated with inflammation condition *in vitro*, under the experimental conditions. Therefore, this study was conducted with the aim of screening phytochemical-rich extracts prepared from 12 selected herbal plants with the long-term objective of incorporating the selected extracts into dehydrated honey lozenges (Honibe^{TM}).

1.2. RESEARCH OBJECTIVES

1.2.1. Overall objective

To identify specific phytochemical-rich extracts with anti-streptococcal, anti-biofilm formation and anti-inflammatory activities from 12 herbal plants and to evaluate the feasibility of incorporation of selected efficacious extracts into dehydrated honey lozenges (Honibe^{TM}).

1.2.2. Specific objectives

1) To prepare phytochemical-rich extracts from the twelve selected herbal plants;
2) To identify extracts with inhibitory effects against *S. pyogenes* and anti-biofilm formation, to characterize the extracts for their phytochemical profiles and evaluate other characteristics such as antioxidant capacity; and
3) To identify extracts with anti-inflammatory properties using human tonsil epithelial cell (HTonEpiC) model system.

CHAPTER 2. LITERATURE REVIEW

2.1. PHARYNGITIS

Pharyngitis (sore throat) is an inflammatory syndrome of the mucous membrane and underlying structures of the pharynx (Langlois and Andreae, 2011). It is among the most common clinical manifestation and accounts for 1.3% of outpatient visits (15 million) to health care providers in the United States in 2006 (Hing et al., 2008; Wessels, 2011). Pharyngitis can be found in all age groups, but is most common among school-aged children and adolescents (Chiappini et al., 2011). The peak incidence in temperate climates occurs during the late autumn, late winter and early spring (Chiappini et al., 2011; Cirilli, 2013). The infection can be caused by infectious agents, such as viruses, bacteria and fungi, such as *Candida albicans* (immune-compromised) (Renner et al., 2012). Although these causes sometimes overlap, the majority of pharyngitis cases are of a viral etiology cause by viruses, such as Rhinovirus, Adenovirus, Epstein-barr virus, Influenza A and B viruses and Coronavirus (Shulman, 1989). However, approximately 5 to 10% of all cases of pharyngitis are a result of bacterial infection (Cooper et al., 2001). Group A beta-hemolytic streptococcus (GAS), the most common bacterial etiology, accounts for 15 to 37% of cases of acute pharyngitis in children and 5 to 20% in adults (Bisno et al., 2002; Choby, 2009). Other bacteria that occasionally cause pharyngitis include groups C and G streptococci, *Neisseria gonorrhoeae, Mycoplasma pneumoniae, Chlamydophila pneumoniae, Arcanobacterium haemolyticum, Yersinia enterocolitica, and Corynebacterium diphtheriae* (Bisno et al., 2002; Renner et al., 2012; Shulman, 1989).

Non-infectious factors, including airborne pollutants (e.g., household cleaners, automobile exhaust), allergies, smoking, exposing to second-hand smoke, excessive alcohol intake, seasonal allergies, chemical irritations by occupational or hazard-associated irritants, which can damage the epithelial lining of the throat, increase the risk of bacterial infection (Renner et al., 2012). People with seasonal allergies to pollen often experience pharyngitis as a result of postnasal drip (Renner et al., 2012). Other than these factors, some other illnesses, such as gastro-esophageal reflux disease and laryngo-pharyngeal reflux and postnasal drainage can also cause pharyngitis. Frequent heartburn encourages throat irritation if the digestive acids rise as high as the throat (Renner et al., 2012). Those who are immune-compromised from HIV/AIDS, diabetes, cancer treatment, or corticosteroid medication use are also at higher risk (Renner et al., 2012).

2.2. STREPTOCOCCAL PHARYNGITIS

Streptococcal pharyngitis, also called strep throat or Group A *Streptococcus* (GAS) pharyngitis, is the most common bacterial cause of pharyngitis. GAS pharyngitis is observed in people of any age, though the prevalence of infection is higher in children (Cirilli, 2013), presumably because of the combination of multiple exposures (in school or daycare) and low immunity. Severe pharyngitis is mostly diagnosed in 5 to 15 year old children, accounting for 37%, whereas 5-15% in adults and 24% in infants less than five years of age (Shaikh et al., 2010; Shulman et al., 2012). However, it is rare in children younger than three years (Chiappini et al., 2011). GAS bacteria, specifically *S. pyogenes* is responsible for over 700 million mild cases of throat infection and more than 650,000 severe invasive infections worldwide, per year (Carapetis et al., 2005). In general, GAS causes 37% of cases of acute pharyngitis in children older than 5 years, whereas 5% of

total cases are Group C *Streptococcus*, 1% of total cases are *C. pneumoniae*, *M. pneumoniae* (1%) and anaerobic species (1%) (Regoli et al., 2011). Furthermore, there are at least 517,000 deaths each year due to severe complications associated with GAS diseases (Carapetis et al., 2005).

Several behavioral or conditional factors can increase the risk of developing GAS pharyngitis. *S. pyogenes* is highly communicable and can cause disease in healthy people of all ages who do not have type-specific immunity against the specific serotype responsible for infection. Furthermore, it is transmitted from human-to-human via direct contact, droplet spread from people with pharyngeal colonisation by coughing and sneezing or carriage and contaminated fomites (Milne et al., 2011). Although salivary and respiratory droplets and nasal discharge from pharyngeal patients by coughing and sneezing are rapid ways of spreading the infection (Choby, 2009; Shulman, 1989; Stevens, 2000), carriers who have chronic asymptomatic pharyngeal and nasopharyngeal colonization are not usually at higher risk of spreading disease because they have a relatively small reservoir of organisms (Stevens, 2000).

2.3. ETIOLOGY

S. pyogenes is a Gram-positive, non-motile and non-spore forming coccui that grows in chains or in pairs of cells (Bisno et al., 2003). *S. pyogenes* is an important extracellular bacterial pathogen where they often colonize as the part of the normal human flora. It is estimated that between 5-15% of normal individuals harbor *S. pyogenes*, or *S. agalactiae,* usually asymptomatically in the nasopharynx (Cunningham, 2000). These organism cause infection when the host defenses are compromised, when the bacteria is able to exert its virulence, or when it is introduced to vulnerable tissues (Bisno et al., 2003). Most often, *S.*

pyogenes causes only mild to moderate infections, such as streptococcal pharyngitis, impetigo (infection of the superficial layers of the skin), cellulitis (infection of the deep layers of the skin) and scarlet fever (skin rash). However, *S. pyogenes* can cause intoxications that include toxic shock syndrome, myositis and necrotizing fasciitis, or immune-mediated post-streptococcal sequelae, such as rheumatic fever and acute glomerulonephritis, following acute infections (Martin and Green, 2006).

Beta-hemolytic streptococci are classified into several groups based on the specific polysaccharide antigen (Lancefield antigen) found on the cell walls of bacteria (Cunningham, 2000). *S. pyogenes* is the most common species referred to as a group A beta-hemolytic streptococci. Group A polysaccharide is a polymer of N-acetylglucosamine linked to a rhamnose backbone (Cunningham, 2000). Group A streptococci were also serologically separated into *emm* types (M-serotypes) based on the cell surface M protein (Metzgar and Zampolli, 2011). The M protein is a two polypeptide chains that form an alpha-helical coiled-coil arrangement, anchored in the cell membrane and spanning the cell wall (Metzgar and Zampolli, 2011). Currently, more than 100 M-serotypes have been identified by this cell wall M protein, which is associated with their virulence (Metzgar and Zampolli, 2011). GAS capsule has peptidoglycan (PGN) and lipoteichoic acids (LTA), fibronectin-binding proteins (e.g. Protein F1 and F2), fibrinogen-binding protein and collagen binding proteins (Bisno et al., 2003). The PGNs are anchored to the GAS cell wall and consist with N-acetyl glucosamine and N-acetyl muramic acid. These cell surface components of *S. pyogenes* accounts for many of the bacterium's determinants of virulence.

2.4. PATHOPHYSIOLOGY OF GAS PHARYNGITIS

Acute diseases occur primarily in the respiratory tract, bloodstream or the skin (Martin and Green, 2006). *S. pyogenes* is able to colonize, rapidly multiply and spread in the host by escaping phagocytosis and misleading the immune system. *S. pyogenes* produces a wide array of virulence factors that act against the host in several ways. For example M protein, protein F and LTA assist in adherence of bacteria to the epithelium while capsule hyaluronic acid masks the presence of bacteria and strengthen by M protein, inhibits phagocytosis. *S. pyogenes* invades host cells, using enzymes, such as streptokinase, hyaluronidase and streptolysins. As well, exotoxins in some instances, lead to systemic toxic shock syndrome (Todar, 2011). Developmental stages of streptococcal pharyngitis include adherence to the mucosal surface of the pharynx, colonization, development of biofilm, release of chemical signals, expression of virulence genes, proliferation of GAS in pharynx, invasion of the host tissue and inflammation (Stevens, 2000).

2.4.1. Adhesion to and invasion of host tissues

GAS infection is initiated by adhesion of *S. pyogenes* to the epithelial cells, including those in the oral and nasal cavities and/or skin (Terao, 2012). Then, this host-pathogen interaction avoids numerous electrostatic and mechanical forces, such as mucous and salivary fluid flow mechanisms and exfoliation of the epithelium that tend to dislodge bacteria (Bisno et al., 2003; Cunningham, 2000). GAS uses multiple and complex different strategies for adherence by expressing specific adherence factors, mainly LTA, M protein, protein F, fibrinogen-binding protein, collagen binding proteins and hyaluronic acid. However, these factors differ from strain-to-strain, depending on the specific genetic background of the

strain, the site of tissue preferences for infection, and local environmental factors (Bisno et al., 2003).

LTA serves as the first step of adhesion by binding to specific fibronectin receptors on human buccal or tonsillar epithelial cells and provides strong adherence (Courtney et al., 1992). This hydrophobic interaction of LTA is strengthened by M protein which allows the infection to persist in the oropharyngeal cavity (Beachey and Ofek, 1976). It was found that LTA accounted for approximately 60% of adhesion to epithelial cells and other adhesions, such as M protein (Beachey and Ofek, 1976; Courtney et al., 1992), protein F (Hanski and Caparon, 1992), glyceraldehyde-3-phosphate dehydrogenase, 70-kDa galactose-binding protein, were involved for the remaining 40% (Cunningham, 2000). Furthermore, several extracellular host cell proteins also been involved in attachment or adherence to GAS, including fibronectin, fibrinogen, collagen, vitronectin, a fucosylated glycoprotein and hyaluronate-binding receptor on keratinocytes (Cunningham, 2000).

2.4.2. Colonization and development of biofilm

The next developmental stage is colonization in the upper respiratory tract (the portal of entry), resulting from a failure in the innate defences. Hyaluronic acid, a compound of the capsule of *S. pyogenes,* is chemically similar to those of human connective tissues (cardiac, skeletal, and smooth muscles and neuronal tissues. As a result of this molecular mimicry, it allows the GAS to go unrecognized as antigen by the host prevents opsonized phagocytosis by neutrophils or macrophages (Cunningham, 2000). The M proteins are considered as the main virulence factors of GAS which are responsible and clearly associated with both colonization and resistance to phagocytosis (Tart et al., 2007). These M protein can bind with a variety of host proteins to prevent activation of the alternate

complement pathway and thus evade phagocytosis by macrophages and destruction by leucocytes (Cusick et al., 2012). Furthermore, antibodies produced by the host due to antigen M protein, are also reacting against other human protein , such as cardiac myosin, tropomyosin, vimentin, laminin, and several heart valve proteins (Bisno et al., 2002). Furthermore, due to this cross-reaction with myosin leading to heart damage and finally in to rheumatic fever caused by *S. pyogenes* infections (Cusick et al., 2012).

Biofilm formation is an important protective mode, contributing to nasopharyngeal colonization and persistence. A biofilm is a complex multicellular community surrounded by a mature matrix and adherent to an inert or living surface (Stoodley et al., 2002). This matrix is composed of polysaccharides, nucleic acids, and proteins known as extracellular polymeric substances (EPS) (Post et al., 2004; Steinberger and Holden, 2005). The EPS is important for the biofilm since it allows GAS to survive and proliferate in a hostile environment (Lewis, 2001; Stoodley et al., 2002). Moreover, this matrix provides sufficient nutrients for growth, structural stability and protection from toxic compounds and reagents in the host environment, including those produced by the immune responses of the host (Lewis, 2001; Manetti et al., 2007; Steinberger and Holden, 2005). Biofilm formation and development is a dynamic process (Post et al., 2004) and schematic diagram of development stages of biofilm is presented in Figure 2.1. Initial adhesion, attachment of bacteria to a surface, proliferation and production of EPS, maturation and detachment are the five stage of biofilm development (Post et al., 2004). After initial adhesion of planktonic cells, biofilm grow continuously in size, GAS proliferate in pharynx and detach as individual cells from the bulk after maturation (Hall-Stoodley et al., 2004; Post et al., 2004). These detached bacteria can regain their planktonic status and freely move through

the surrounding area to a new surface, where a new biofilm can be formed (Hall-Stoodley et al., 2004; Steinberger and Holden, 2005).

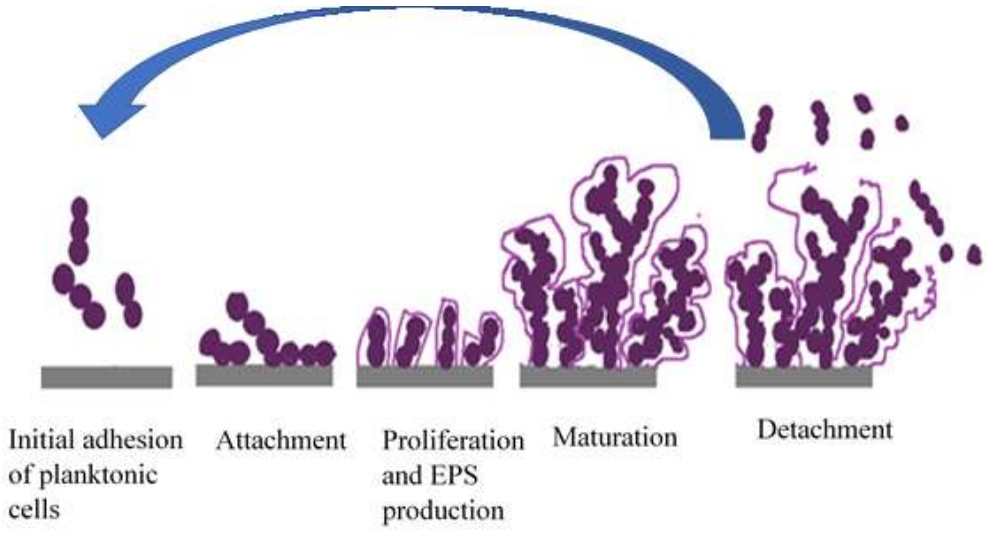

Figure 2.1: Development stages of *S. pyogenes* biofilm. Adapted from (Harper et al., 2014)

2.4.3. Release of chemical signals and expression of virulence genes

S. pyogenes starts to proliferate and form a society when host environmental conditions are favorable (Tart et al., 2007). Cell-cell communication is accomplished through the exchange of extracellular signalling molecules termed auto-inducers (Waters and Bassler, 2005). Generally, auto-inducers are secreted peptides that are processed from larger propeptides, such as autoinducer-2 (AI-2) (Marouni and Sela, 2003). When these auto-inducers reach a critical threshold concentration as a function of cell density, a signal transduction cascade is triggered. This triggering provides the basis for alterations in gene expression (Bassler, 1999). The control of gene expression, in response to cell density, is also known as "quorum sensing," which communicates not only within bacterial societies, but also involves interactions among neighboring bacterial species and therefore, it alters

particular behaviors of the entire community (Bassler, 1999; Tart et al., 2007; Waters and Bassler, 2005).

2.4.4. Intracellular Invasion and Spread through Tissues

GAS has the potential to invade human epithelia and several proteins are involved in streptococcal epithelial cell invasion (Cywes and Wessels, 2001). For example, it was reported that both M1 protein and protein F1 are considered invasins. It was determined that the M1 serotypes of GAS display by high frequency intracellular invasion of tonsil epithelial cells (Dombek et al., 1999). After the intracellular invasion, *S. pyogenes* spreads rapidly to various organs through the blood stream (Terao, 2012). Hyaluronidase, DNases, streptokinase, streptolysins and the streptococcal pyrogenic exotoxin B are extracellular products which distribute factors which promote liquefaction of pus and help the bacteria invade tissue planes (Bisno et al., 2003). Furthermore, hyaluronidase enzymatically degrades hyaluronic acid, which is present in the connective tissue and DNases are responsible for the degradation of DNA (Bisno et al., 2003). Streptococci invade the pharyngeal tissue and causes a localized inflammatory reaction in the throat and tonsils.

2.5. POST STREPTOCOCCAL SEQUELAE

GAS pharyngitis may cause suppurative complications, such as otitis media, retropharyngeal cellulitis, peritonsillar abscess, retropharyngeal abscess, and cervical adenitis. Non-suppurative complications, such as acute rheumatic fever and acute glomerulonephritis may also results (Regoli et al., 2011). Post-streptococcal glomerulonephritis (PSGN) is another complication of streptococcal infection, which develops approximately within 10 days following streptococcal pharyngitis. Studies have reported that PSGN is caused by the deposition of antigen-antibody-complement

complexes on the basement membrane of kidney glomeruli and leads to inflammation of the kidney (Eison et al., 2011).

2.6. CURRENT TREATMENTS AND PREVENTION

Treatment goals of patients with streptococcal pharyngitis, include eradicating GAS from the throat, decreasing the risk of bacterial transmission, to abating of clinical signs and symptoms and preventing some of the suppurative and non-suppurative complications (Hayes and Williamson, 2001; Rafei and Lichenstein, 2006). Streptococcal pharyngitis is self-limited and resolves within a few days, even without treatment. However, the risk of complications is there until it is treated. Antibiotics are often prescribed for streptococcal pharyngitis. Patients are no longer contagious within 24 hours of starting appropriate antibiotic treatments (Chiappini et al., 2011). Commonly recommended antibiotic therapies for streptococcal pharyngitis are shown in Table 2.1. Antibiotic selection requires consideration of patients' allergies, bacteriological and clinical efficacy, frequency of administration, duration of therapy, potential side effects, compliance and cost (Choby, 2009).

The Infectious Diseases Society of America, Canadian Paediatric Society and World Health Organization recommends a 10-day course of oral antibiotics, such as penicillin V (250 mg, 2 or 3 times a day for children, and 3 or 4 times a day for adults) or a cephalosporin (Wagner and Mathiason, 2008). Oral penicillin still remains the first drug of choice in most clinical situations, although the more expensive cephalosporins and, perhaps, amoxicillin-clavulanate potassium, provide superior bacteriological and clinical cure rates (Brook and Foote, 1997). Beta lactamase enzyme production by normal oral bacteria is one of the most common mechanisms of bacterial resistance. This enzyme can

cleave the beta lactam ring of the antibiotics like amoxicillin which belongs to the beta lactam antibiotic class. Therefore, clavulanate potassium, a form of clavulanic acid is usually prescribed with amoxicillin to inactivate beta lactamase enzyme (Salvo et al., 2009).

Despite the genetic diversity of GAS and the heavy exposure of penicillin over five decades, they have other desirable features, such as low cost, a low incidence of side effects, effectiveness and no documentation of resistance in GAS. Group beta-lactam antibiotics, like penicillin, demonstrate bactericidal activity by inhibiting the synthesis of bacterial cell wall compounds, such as PGN (Brook, 2001). During the cell wall synthesis, a single peptidoglycan chain is cross-linked to other peptidoglycan chains through DD-transpeptidase enzyme, also called a penicillin binding protein (Sun et al., 2014). In the class beta-lactams, such as penicillin, contain a characteristic four-membered beta-lactam ring can bind to the DD-transpeptidase, and cause inhibition of its cross-linking activity. Therefore, by preventing this final transpeptidation step of the synthesis of the peptidoglycan in cell wall, penicillin can influence to the cell growth and replication of GAS. Furthermore, bacterial cells without cell wall are become highly vulnerable to the hostile environment in the host and eventually die (Navarre and Schneewind, 1999). First-generation oral cephalosporin and erythromycin are recommended for patients with penicillin allergy, with clindamycin, azithromycin, or clarithromycin as an alternative treatment option (Wessels, 2011). Macrolides inhibits protein synthesis by binding to 23S ribosomal target sites in GAS (Zhanel et al., 2001).

Table 2.1: Recommended antibiotic therapies for streptococcal pharyngitis treatment. Modified from (Martin and Green, 2006) and (Hayes and Williamson, 2001).

Class of antibiotics	Antibiotic	Dose/dosage	Dosing frequency	Duration of therapy
Beta-Lactams	Penicillin VK	≤ 27 kg : 250 mg > 27 kg : 500 mg,	2 to 3 times	10 days
	Penicillin G benzathine	≤ 27 kg : single dose of 600,000 units Intramuscular > 27 kg : single dose of 1.2 million units Intramuscular	Single injection	One dose
	Amoxicillin	≤ 27 kg : 250 mg > 27 kg : 500 mg	2-3 times	10 days
Cephalosporin	Cephalexin	Child: 25 to 50 mg/kg/day Adult: 500 mg	2-4 divided doses 2 times daily	10 days
	Cefadroxil	Child: 30 mg/kg per day Adult: 1 g	2 divided doses Once daily	10 days
	Cefaclor	30 mg/kg/day	4 divided doses	10 days
	Cefuroxime axetil	15 mg/kg	2 divided doses	10 days
	Cefixime	8 mg/kg	single dose	10 days
	Cefdinir	14 mg/kg	single dose	5 days
Macrolides	Azithromycin	Child: 12 mg/ kg Adult: 500 mg on day 1; 250 mg on days 2 through 5	Once daily Once daily	5 days
	Erythromycin ethylsuccinate	Child: 40 mg/kg/day > 27 kg : 400 mg/kg	2-4 divided doses 4 times daily	10 days
	Erythromycin esotlate	Child: 20 to 40 mg per kg per day Adult: not recommended	2-4 divided doses -	10 days -

Prevention of the attachment and growth of *S. pyogenes* biofilms would preferably be more appropriate than treating existing biofilms. Prevention of streptococcal pharyngitis can be categorized as primordial (improved social health determinants), primary (vaccination) and secondary (antimicrobial prophylaxis-monthly benzathine penicillin) strategies (Seckeler and Hoke, 2011). For example, it is reported that formation of biofilm can be prevented by early aggressive antibiotic prophylaxis, before bacterial cells irreversibly attach to the throat surfaces (Taraszkiewicz et al., 2013).

2.7. CHALLENGES IN CURRENT TREATMENTS

One of the major challenges associated with treatments for GAS pharyngitis is antibiotic failure, resulting from epidemiologic, clinical, and microbiological factors. Although, GAS is always susceptible *in vitro* to penicillin, bacteriological and clinical failure rates with penicillin therapy have been steadily increasing over time. Presently, this failure accounts for approximately 10-15% of patients treated with penicillin (Brook, 1985, 2001; Pichichero et al., 2000; Pichichero and Margolis, 1991). Many studies have suggested several potential explanations for treatment failures, such as co-pathogenicity of β-lactamase-producing normal pharyngeal flora, tolerance to penicillin, GAS co-aggregation, bacterial interference, intracellular localization, poor patient compliance (inappropriate dose, duration of therapy, or choice of antibiotic), recurrent exposure to GAS, non-infectious carrier stage and reacquisition from close contact or fomite (Brook, 2001; Pichichero and Casey, 2007; Pichichero et al., 2000). However, there is no evidence that *S. pyogenes* has become resistant to penicillin. Beta-lactamase enzyme producers are not generally found among normal oropharyngeal bacteria in individuals who have not recently been exposed to antibiotics (Brook, 2001). However, due to repeated penicillin

administration, over 75% of the tonsils of patients that were surgically removed because of recurrent infection, were reported to have beta-lactamase producing strains (Brook, 1985). It was determined that beta-lactamase producing bacteria play an important protective role against GAS by inactivating penicillin (Brook, 2001; Brook and Gober, 1995).

When severe infections are complicated by large bacterial loads, the effectiveness of penicillin may be less in the stationary phase of bacterial growth, because they target cell wall synthesis (Brook, 2001). Penicillin tolerance is also another explanation for treatment failure. Repeated exposure to sub-lethal concentrations of antibiotic causes resistance to eradication of GAS in the throat. However, previous studies proved that the contribution of penicillin tolerance to treatment failure plays a minimum role. It accounts for less than 0.1% among GAS isolates collected from pharyngitis patients, after 10 days of penicillin therapy (Orrling et al., 1996; Vega et al., 2013). Although current guidelines recommend alternative antibiotics, such as first-generation cephalosporins and macrolide for the patients with penicillin allergy, there are issues in using those even with non-allergy patients (Choby, 2009). Resistance to macrolides, such as erythromycin, clarithromycin, and azithromycin, has been reported among GAS isolates in certain areas of the United States, Canada and Europe (Katz et al., 2003; Richter et al., 2005).

The GAS biofilm supports a streptococcal pharyngitis that is very hard to be eliminated by conventional antibiotic therapy. The matrix of the biofilm confers a protection against antibiotics and it has environmental promoters that contribute to drug resistance development (up to 1000-fold decrease in susceptibility) in comparison with planktonic cultures (Bueno, 2014; Post et al., 2004). Even though the matrix may not be effective at inhibiting the penetration of antibiotics, it may retard the rate of penetration

which induce the expression of genes within the biofilm that arbitrates tolerance. Bacterial cells which are in the slow-growing stage of the biofilm matrix, may reduce biofilm susceptibility to antibiotics. Because, these slow-growing bacterial cells are normally located deeply in the biofilm structure, thus experience of nutrient stress (limitation). Bacterial cells can become dormant and later become active and continue the biofilm formation (Taraszkiewicz et al., 2013). Therefore, scientists and physicians are looking forward to identify better alternatives for control GAS biofilm formation or to increase the efficiency of antibiotics against growth inhibition, disruption, or eradication of GAS biofilm.

Noncompliance by patients is another challenge in antibiotic treatments. Patients are tempted to discontinue the antibiotic course, if they get symptomatic relief without completing the recommended duration of therapy. Furthermore, recurrent infection with the same serotype, following initial treatment, may be associated with milder symptoms (career stage); these individuals are contagious to others in their environment and are themselves susceptible to acute rheumatic fever (Pichichero and Casey, 2007; Pichichero et al., 2000). Chronic carriers are at low risk of transmitting disease or developing invasive GAS infections, and there is generally no need to treat carriers (Choby, 2009). Some studies show that demographic and clinical factors, such as age of the patient, are also associated with treatment failure. Younger children, aged 1-8 years, are more likely to have GAS treatment failures and significantly more common recurrences than older children, aged 13 to 19 years (Pichichero et al., 1998). Furthermore, higher rates of microbiological failure are shown by carrier stage patients who are under 15 years old.

2.8. GAS PHARYNGITIS AND INFLAMMATION

2.8.1. Inflammation

Inflammation is a part of the complex biological response of vascular tissues, to harmful stimuli, such as pathogens, damaged cells, or irritants. It is a protective physiological attempt of the host to remove those detrimental stimuli and to initiate the healing process (Dong et al., 2014). A cascade of biochemical events propagates and develops the inflammatory response, involving the local vascular system, the immune system and various cells within the injured tissue. Typical signs of inflammation include redness, swelling, pain, heat and loss of function (Ricciotti and FitzGerald, 2011).

2.8.2. Chemical mediators of inflammation

There are chemical mediators released from cells during inflammation, such as vasoactive amines (histamine and serotonin), cytokines, prostaglandin E2 (PGE2) and kinins (bradykinin) (White, 1999). They originate from blood plasma, white blood cells, platelets, endothelial cells lining the blood vessels, and other types of damaged tissue cells. Prostaglandins, a group of chemical mediators, are synthesized from arachidonic acid by cyclooxygenase (COX) and lipoxygenase enzyme activity (Hla and Neilson, 1992; Ricciotti and FitzGerald, 2011; White, 1999). They promote vascular permeability and increase the efficacy of substances, which aggregate platelets during blood clotting and which are associated with the pain and fever of inflammation (Ramani et al., 2015).

The group of inflammatory cytokines are the most important group of compounds participating in the pathogenesis. Toll-like receptors (TLR) regulate the inducible expression of these different molecules that are involved in inflammatory immune response

(White, 1999). Two types of cytokines, inflammatory cytokines and anti-inflammatory cytokines are categorized based of their biological effect (Wojdasiewicz et al., 2014).

2.8.2.1. Pro-inflammatory cytokines and chemokines

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-2 receptor, enzymes, such as COX-2 and pro-inflammatory chemokines, such as IL-6R, IL-12, IL-23, granulocyte chemotactic protien-2 (GCP-2), and growth-regulated oncogene-alpha have vasoactive and chemotactic properties (Schett, 2011; Takeda and Akira, 2004; White, 1999). They promote inflammation in response to tissue injury and infection by producing pro-inflammatory mediators through activation of nuclear factor-kappa B (NFκB) ligand (Ramani et al., 2015). Chemokines (chemotactic cytokines/chemoattractive cytokine) are a specific type of cytokine which work as attractants of different types of blood leukocytes (neutrophils, monocytes, lymphocytes, and eosinophils) to sites of infection and inflammation (Baggiolini, 1998). Chemokines play a major role in host defense mechanisms and are functionally categorized into four groups (CC, XC, CXC, and CX_3C), based on the position of conserved cysteine residues on their molecules (Rollins, 1997).

2.8.2.2. Anti-inflammatory cytokines

Anti-inflammatory cytokines, such as IL-4, IL-10, IL-11, and IL-13 (Opal and DePalo, 2000), help in down-regulating the inflammatory response by giving negative feedback which suppresses the activity of pro-inflammatory cytokines (Schett, 2011). Inhibition of the synthesis of inflammatory cytokines results in obstructing apoptosis of chondrocytes, decreasing secretion of metalloproteinases and the level of PG2, while increasing proteoglycan synthesis (Wojdasiewicz et al., 2014).

2.8.3. Inflammation vs GAS pharyngitis

Epithelial cells of the upper respiratory tract are the first line barrier, protecting the host from various bacterial pathogens. TLRs are one class of cell receptors involved in the recognition of the presence of microbial pathogens (Takeda and Akira, 2004; Takeda et al., 2003). Currently, there are more than 10 TLRs identified which recognize multiple pathogen-associated molecular patterns (PAMPs), including LTA, PGN, dsRNA and unmethylated bacterial CpG DNA (Schwandner et al., 1999; Takeda et al., 2003; Tanaka et al., 2003). LTA and PGN are predominant components of the outer cell wall of GAS and they elicit most of the clinical manifestation of streptococcal pharyngitis infection (Schwandner et al., 1999). LTA maintains the structure which bridges and connects the carbohydrate network of bacterial cell walls and also mediates GAS adhesion to tonsil epithelial cells (Cunningham, 2000). LTA and PGN are recognized by TLR2 (Takeda et al., 2003). Genomic DNA, rich in unmethylated CpG DNA from bacteria, are recognized by TLR9 with myeloid differentiation factor 88 (MyD88) signaling adaptor and transcription factor NFκB (Kumar et al., 2009). Finally, this cellular recognition, after the invasion of *S. pyogenes,* is mediated by TLRs. This leads to signaling events, resulting in the coordinated activation of transcription factors that induce the expression of pro-inflammatory cytokines, chemokines, and other inflammatory mediators (Schwandner et al., 1999; Takeda et al., 2003).

Several *in vitro* studies have emphasized the importance of MyD88 signaling adaptor which regulates TLR-mediated pathways in the inflammatory response to *S. pyogenes* (Gratz et al., 2008; Loof et al., 2010; Takeda and Akira, 2004). MyD88 is the central signaling adaptor molecule that mediates cellular stimulation from most of TLRs,

except for TLR3 (Gratz et al., 2008). In some cases, excessive TLR activation may cause detrimental inflammation that can induce tissue damage. Mice studies have shown that MyD88 signaling adaptor targets effector cells at the site of streptococcal infection and prevents extravasation of cells, thereby determining and maintaining the quality of the inflammatory response (Gratz et al., 2008; Loof et al., 2010).

2.8.4. Signs and symptoms of GAS pharyngitis

The signs and symptoms of GAS pharyngitis extensively overlap with viral pharyngitis. Therefore, diagnosis of streptococcal pharyngitis based solely on clinical findings, such as patient's history or physical examination, is difficult. (Choby, 2009). Common symptoms of GAS pharyngitis are sudden onset of sore throat, fever, headache, pain on swallowing and rarely abdominal pain, nausea and vomiting (Choby, 2009; Langlois and Andreae, 2011). The incubation period, the time elapsed between exposure to a pathogenic organism and when symptoms and signs are first apparent, is 2 to 5 days, and communicability of the infection is the highest during the acute phase of the illness, usually 7-10 days. Fever usually resolves within 3 to 5 days and streptococcal pharyngitis is self-limited, usually within one week (Chiappini et al., 2011). Other than those, pharyngeal erythema, uvular inflammation, anterior cervical lymphadenopathy and tonsillar exudates are common physical examination criteria (signs) (Choby, 2009; Cirilli, 2013; Shulman, 1989). Palatal petechiae and rash are highly specific, but uncommon (Choby, 2009).

2.8.5. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used for over a century for the management of conditions involving inflammation of soft tissue, such as the upper and lower respiratory tract (Cremonesi and Cavalieri, 2015). Aspirin, ibuprofen, piroxicam, ketoprofen, naproxen, etodolac, mefenamic acid, diclofenac, niflumic acid, fentiazac, feprazone and flurbiprofen nabumetone, sulindac, tolmetin, ketorolac, celecoxib and selective COX-2 inhibitors are examples of the most common NSAIDs (Davis and Brogden, 1994; Thomas et al., 2000). The primary mechanism of action of NSAIDs is the inhibition of COX, an enzyme responsible for the synthesis of pro-inflammatory prostaglandins (PG) (Cremonesi and Cavalieri, 2015; Ricciotti and FitzGerald, 2011). PG2 synthesis starts with the release of arachidonic acid from cell membranes and then, COX enzyme converts the arachidonic acid into PGs (Simmons et al., 2004). PGs is important in promoting inflammation and relevant consequences, such as pain, and fever. The COX has two distinct membrane-anchored enzymes, COX-1 and COX-2 (Goldmann et al., 2010; Radi, 2009). Both enzymes produce PGs; however, COX-1 is constitutively expressed in most tissues and produces PGs that activate platelets and protect the stomach and intestinal lining (Dubois et al., 1998; Simmons et al., 2004). COX-2 is an inducible enzyme that is expressed upon stimulation with pathogenic bacteria or bacterial wall constituents, such as PGN and LTA or cytokines (Goldmann et al., 2010; Simmons et al., 2004). However, NSAIDs block COX enzymes and reduce production of prostaglandins (Ricciotti and FitzGerald, 2011). Therefore, inflammation, pain, and fever are reduced. Since the prostaglandins that protect the stomach and promote blood clotting are also reduced, NSAIDs that block both COX-1 and COX-2 can cause ulcers in the stomach and intestines

(Radi, 2009). Table 2.3 summarized findings of several studies which were carried out to assess the efficacy of using NSAIDs for treating pain or fever. Symptom relief from analgesics, such as paracetamol (acetaminophen) and NSAIDs seems to be highly effective (Thomas et al., 2000).

Nausea, vomiting, diarrhea, constipation, decreased appetite, rash, dizziness, headache, and drowsiness are some of reported side effects with NSAIDs. Some individuals are allergic to NSAIDs and may develop shortness of breath when an NSAID is taken. The risk of side effects are not significantly higher, but it is difficult to conclude that they are not different from a placebo in terms of side effects, according to the previous studies (Davis and Brogden, 1994; Kim et al., 2013).

Table 2.2: Alternative treatments to antibiotics for sore throat.

NSAIDs name	Treatment	Findings (% Relative effect compare to the control)	Reference
Aspirin	800 mg, single dose	reducing throat pain by 55% at 1 h	(Schachtel et al., 1991)
Ibuprofen	400 mg, single dose	reducing throat pain by 32% to 80% after 2-4 hr, and 70% at 6 hr (adult), reducing throat pain by 25% in children after 2 hr	(Schachtel et al., 1988)
Nimesulide	200 mg, twice daily	has shown superior antipyretic potency to indomethacin, ibuprofen, aspirin and paracetamol in rats with yeast-induced fever	(Davis and Brogden, 1994)
Niflumic acid	1000 mg daily for 4-5 day	reducing throat pain by 17% after 2 days, 33% after 4 days	(Sauvage et al., 1990)
Tiaprofenic acid	for 5 days plus antibiotics	reducing throat pain by 14% after 2 days and 93% after 5 days in children	(Benarrosh and Ulmann, 1989)
Morniflumate suppositories	400 mg twice daily for 4 days plus antibiotics	34% reduction in pharyngeal pain after 4 days	(Manach and Ditisheim, 1990)

2.9. METHODS FOR EXTRACTION AND ASSESSMENT OF BIOACTIVITY OF HERBAL PLANTS

2.9.1. Bioactive phytochemicals

Plants are rich in a wide variety of secondary metabolites (phytochemicals), such as tannins, terpenoids, alkaloids, and flavonoids (Cowan, 1999). Chemical structures of some polyphenols commonly find in some medicinal plants are shown in Figure 2.2. Phytochemicals have different biological properties, including anti-microbial (Lai and Roy, 2004; Sfeir et al., 2013), anti-biofilm formation (Darsini et al., 2015; Mutalib et al., 2015), anti-inflammatory (Fawole et al., 2010; Hamalainen et al., 2007), anti-oxidant (Carlsen et al., 2010; Fawole et al., 2010; Saboo et al., 2014), anti-cancer (Li-Weber, 2009; Saboo et al., 2014; Valdiani et al., 2014), which promote human health and help to reduce the risk of chronic disease. For centuries traditional healers have been using medicinal plants to prevent or cure infectious conditions. Table 2.3 summarized major phytochemicals and therapeutic uses of selected herbal plant used in Canadian traditional medicine. Although, those phytochemicals or phytochemical-rich extracts can consider as potential alternatives to synthetic drugs, scientific investigations are needed to evaluate the activity against specific infectious bacteria of potential plant extracts.

2.9.2. Major phytochemicals in selected medicinal plant

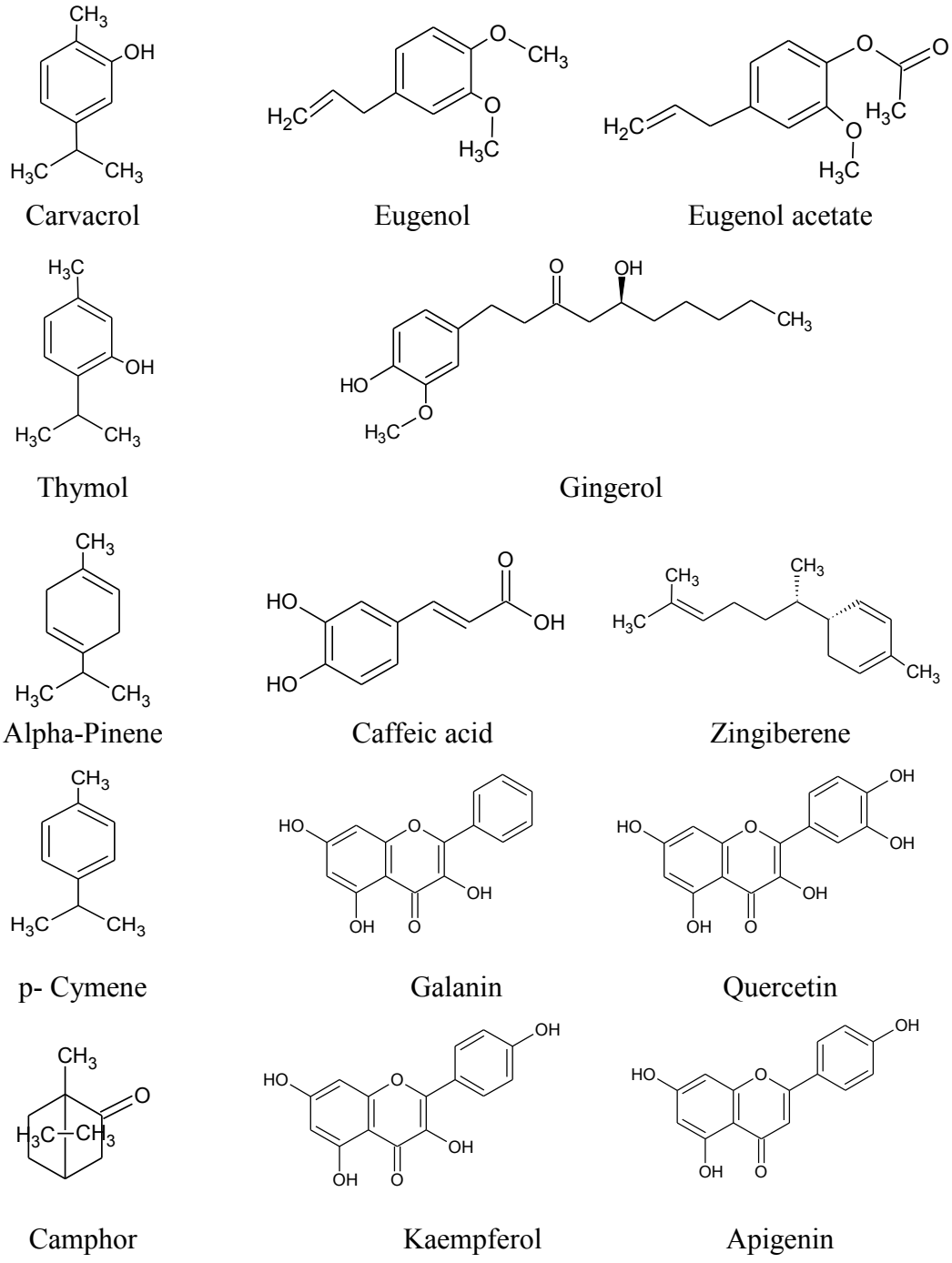

Figure 2.2: Chemical structures of some polyphenols in plants

Table 2.3: Major phytochemicals and therapeutic uses of selected herbal plant used in Canadian traditional medicine

Plant Name		Family	Parts used	Major phytochemicals	Therapeutic uses	Reference
Common	**Botanical**					
Barberry	*Berberis vulgaris*	Berberidaceae	Root	Berberine, Berbamine, 5-Methoxyhydnocarpin, Berlambine, Jatrorrhizine, Palmatine, Quercetin, Rutin, Oxyberberine,	Antimicrobial, antiemetic, antipyretic, antipruritic, antioxidant, anti-inflammatory, hypotensive, antiarrhythmic, sedative, antinociceptive, anticholinergic anti-acetylcholinesterase, anti-diabetic and anticancer effects, treating diarrhea and gasterointestinal disorders, stimulation of bile secretion	(Abd El-Wahab et al., 2013; Mokhber-Dezfuli et al., 2014)
Clove	*Syzygium aromaticum*	Myrtaceae	Flower bud	Eugenol, Eugenyl acetate, β-Caryophyllene, α-Humulene, β-Ocimene, Caryophyllene oxide, α-Copaene, p-Allyl phenol	Treats for mouth sores and ulcers, and sore gums, treating worms, viruses, antimicrobial, antifungal, antioxidant, anti-inflammatory, blood-thinner, mental stimulant, anaesthetic properties, used for toothaches, bad breath, cough, earache, stop vomiting, relieve diarrhoea, bloating, intestinal spasms and nausea, slows down macular degeneration and aids vision	(Chaieb et al., 2007; Rani et al., 2012; Sfeir et al., 2013)

Plant Name		Family	Parts used	Major phytochemicals	Therapeutic uses	Reference
Common	**Botanical**					
Eastern purple cone-flower	*Echinacea purpurea*	Asteraceae	Leave, Stem, Flower	Caftaric acid, Chlorogenic acid, Caffeic acid, Cynarin, Echinacoside, Cichoric acid, Quercetin and Kaempferol	Antiinflammatory, anti-oxidant, treat upper respiratory problems, urinary tract infections, chronic wounds, and snake and mosquito bites	(Barrett et al., 2010; Dennehy, 2001; Kumar and Ramaiah, 2011)
Ginger	*Zingiber officinale*	Zingiberaceae	Rhizo-me	Gingerol, Galanolactone, Ginerdiol, Geranial, α-Zingiberene, (*E*,*E*)-α-Farnesene, Oleoresin: Eugenol, Zingerone	Treatment for nausea and vomiting of pregnancy, good for diarrhea, stomachaches, cholera, toothaches, bleeding, anti-inflammatory effect,	(Park et al., 2008) (Kondo et al., 2010; Policegoudra et al., 2010)
Licorice	*Glycyrrhiza glabra*	Papilionaceae	Root	Glycyrrhizin, Glabridin, Licoflavan, Narigenin, Asparegene and Liquirtin	expectorant, antitussive, included in cough medications, useful for peptic ulcer, anti-inflammatory, laxative, memory enhancing activity	(Chu et al., 2012; Nitalikar et al., 2010)

Plant Name		Family	Parts used	Major phytochemicals	Therapeutic uses	Reference
Common	**Botanical**					
Oregano	*Origanum vulgare*	Lamiaceae	Flowering shoots and leaves	Carvacrol, p-Cymene, Borneol, Thymol, Linalool, Linlyl acetate, Terpinene-4-ol, Thymohydroquinone, Rosmarinic acid, Naringin	Antioxidative properties, help heal cold sores, dandruff, and other skin conditions, ease sore throat (Respiratory Conditions), antibacterial**.** antifungal, antiviral, Anti-inflammatory effect, antiparasitic, anti-allergenic	(Fournomiti et al., 2015; Sfeir et al., 2013; Teixeira et al., 2013)
Rose geranium	*Pelargonium graveolens*	Geraniaceae	Leaves	Citronellol, Citronellyl Formate and Geraniol,	Antibacterial, antifungal , antiinflammatory, antiseptic; aromatherapy, antioxidant, anti-influenza, anticance astringent, coagulant/hemostatic, analgesic, antidepressant	(Ghannadi et al., 2012)
Sage	*Salvia officinalis*	Lamiaceae	leaves, root	1,8-Cineole, p-Cymene, Camphor borneol, α-Thujone, Ledene, β-Pinene, α-Humulene, trans-Caryophyllene, β-Thujone and Myrcene	Antioxidant, antibacterial, antiseptic, antiscabies, antisyphilitic, anti-inflammatory, antifungal	(Abu-Darwish et al., 2013; Fournomiti et al., 2015; Horiuchi et al., 2007)

Plant Name		Family	Parts used	Major phytochemicals	Therapeutic uses	Reference
Common	**Botanical**					
Slippery elm	*Ulmus rubra*	Ulmaceae	Innner bark	Oleanolic acid, Ursolic acid, Uvaol, Betulinic acid, Botulin, β-Arotene, β-Sitosterol and Citrostadienol	Use for coughs, sore throat, colic, diarrhea, constipation, GI tract inflammation, good for skin wounds, burns, cold sores, abscesses, ulcers, toothaches, sore throat, and as a lubricant to ease labor.	(Lesley, 2006)
Thyme	*Thymus vulgaris*	Lamiaceae	Flowering shoots and leaves	Thymol, γ-Terpinene, p-Cymene, Mycrene, α-Pinene , α-Thujone, α-Terpinene , Carvacrol , 1,8 Cineole, Thymol, Methyl ether, α-Terpinyl acetate, Linalool, γ-Terpinene, α-Terpineol and Geraniol	antihelminthic, expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antioxidative, antivirotic, carminative, sedative, and diaphoretic effects	(Asbaghian et al., 2011; Sfeir et al., 2013) (Fachini-Queiroz et al., 2012; Fournomiti et al., 2015)

2.9.3. Methods of extraction of phytochemicals

Phytochemicals have been extracted from many parts of plants, such as the inner or outer bark, leaves, shoots, flowers, flower buds, roots, fruits, seeds and peels. Plant materials are prepared for extractions by pre-washing, oven/air/freeze drying, cutting, chopping, and grinding, to obtain homogenous samples. However, extraction is considered to be the crucial first step in the analysis of medicinal plants for their phytochemical profiles or efficacy of phytochemicals (Sasidharan et al., 2011).

The principles, pros and cons of commonly used extraction techniques are discussed and summarized in Table 2.3. These methods of plant extraction include maceration (Pandey and Tripathi, 2014), percolation (Sasidharan et al., 2011), infusion, decoction (Pandey and Tripathi, 2014), Soxhlet extraction (Wang and Weller, 2006), microwave-assisted extraction (Wang and Weller, 2006), sonication-assisted extraction (Albu et al., 2004; Wang and Weller, 2006), supercritical fluid extraction (SFE) (Belliardo et al., 2006), and distillation (Sasidharan et al., 2011).

The extraction of aromatic compounds (Essential oil) from plants, such as rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*) and ginger rhizome (*Zingiber officinale*), can employ steam distillation, headspace trapping or solid phase micro extraction (SPME) (Belliardo et al., 2006). Solvents and/or a combination of solvents should have compatible polarity and hydrogen bonding ability with the desired solutes (bioactive compounds) to be extracted (Pangarkar, 2008). Polar solvents, such as methanol, ethanol or ethyl-acetate, are used for hydrophilic solutes, whereas a combination of dichloromethane and methanol are employed for lipophilic solutes (Sasidharan et al., 2011). Furthermore, based on the targeted compound, particular operational temperatures,

pressures and duration must selected. It has been found that the efficiency of these techniques improves with elevated temperature and pressure and with short duration (Wang and Weller, 2006).

Table 2.4: Different techniques of phytochemicals extraction.

Method	Principle	Pros and Cons	References
Maceration	Soak the whole or coarsely powdered plant with gradient solvent of different polarity (Methanol, ethanol, or mixture of alcohol and water) with or without agitation in a stoppered container for a defined period.	No need of special technical knowledge Low cost, time consuming	(Pandey and Tripathi, 2014; Sasidharan et al., 2011)
Percolation	Passing continuous flow of solvent through the sample in a percolator.	Not require special knowledge, Large volume of solvent used, time consuming, less efficient	(Pandey and Tripathi, 2014; Sasidharan et al., 2011)
Decoction	Extraction of water soluble and heat stable constituents from crude plant by boiling it in water for 15 min, cooling, straining and passing sufficient cold water	No need of special technical knowledge Low cost	(Sasidharan et al., 2011) (Parekh and Chanda, 2007)
Infusion	Immerse the coarsely powdered plant in hot water, allowed to stand 10-15 minutes and then filtered through a filter.	No need of special technical knowledge Low cost	(Bimakr, 2010; Sasidharan et al., 2011)

Method	Principle	Pros and Cons	References
Distillation (water distillation, steam distillation, phytonic extraction)	Plant material immersed in water is heated or allow steam to go through plant materials until water and compounds reach their boiling point in a round bottom flask which connect to a condenser. As vapor is condensed, water is recycled to flask. Then separated natural product can collected.	Good for volatiles Time consuming, energy consuming, Require large amount of sample	(Sasidharan et al., 2011)
Soxhlet extraction (hot continuous extraction)	Finely ground crude plant is placed in a porous thimble made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. Solvent in flask is heated and vapours send through a condenser. Remaining dry sample in the flask is collected.	Time consuming and require relatively large quantities of solvents	(Wang and Weller, 2006; Zygmunt and Namiesnik, 2003)
Microwave assisted extraction	Water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the recovery of nutraceuticals.	Reduced solvent consumption, fast and efficient, less time and consequently financial inputs, Improved extraction yield Additional filtration or centrifugation is necessary to remove the solid residue.	(Wang and Weller, 2006)

Method	Principle	Pros and Cons	References
Ultra Sonication assist extraction	Powdered plant mixed with solvent is placed in ultrasonic bath. Ultrasound frequencies ranging from 20-2000 kHz is used to induce a mechanical stress on the cells through the production of cavitations. Methanol, ethanol, or mixture of alcohol and water used as solvent. cellular breakdown cause increases the permeability of cell wall.	Undesirable changes in molecules (formation of free radicals by ultrasound energy), inexpensive, easy to operate, good for extraction of thermo labile compounds, fast and efficient, less solvent consumption	(Albu et al., 2004; Pandey and Tripathi, 2014; Zygmunt and Namiesnik, 2003)
Super-critical fluid	Extraction vessel is pressurized with fluid by a pump. Fluid and the dissolved compounds transported to separators, where salvation power of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid. Product is then collected via a valve located in the lower part of the separators.	No solvent residue, No need to concentrate, Fast & effective, low temperature avoids damage from heat and some organic solvents, Environmental friendly, High cost	(Huie, 2002; Wang and Weller, 2006; Zygmunt and Namiesnik, 2003)
Accelerated solvent extraction	Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state. Pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix.	Economic and environmental friendly, less amount of solvent and the extraction time	(Wang and Weller, 2006)

2.9.4. Anti-bacterial screening assays

Antimicrobial assays are important tools to evaluate the inhibitory effects of phytochemicals against different microorganisms before establishing their inhibitory ranges. Similarly, these assays can be employed for antibiotic bacterial resistance evaluation of compounds. There are several methods used in assessing antimicrobial properties of phytochemical-rich extracts/novel compounds, in comparison with known antibiotics (Sarker et al., 2007). Diffusion and dilution methods are widely used in antimicrobial assessment assays (Valgas et al., 2007). These methods and their pros and cons are summarized in Table 2.5.

Disc diffusion and agar well diffusion methods, which are expressed as zonal diameter of inhibition (ZDI) in mm, are known as qualitative techniques, since these methods only give an idea of the presence or absence of substances with antimicrobial activity (Hood et al., 2003). Dilution methods (agar dilution, broth dilution or broth micro dilution) are quantitative assays which determine the minimal inhibitory concentration (MIC) (Hood et al., 2003; Jorgensen and Ferraro, 1998). MIC is defined as the lowest concentration of a compound that inhibits the visible growth of a microorganism after overnight incubation (Jorgensen and Ferraro, 2009; Sarker et al., 2007; Valgas et al., 2007; Wiegand et al., 2008). After that, minimum bactericidal concentration (MBC) also can measure to confirm anti-bacterial efficacy. The MBC represents the lowest antimicrobial concentration, where 99.9% or more of the initial inoculum is killed after incubation for 18-24 hr (Denys et al., 2011). Time-kill curves are another method of assessment of the interaction between bacteria and anti-microbial agents. These describe microbial growth pattern, killing rate and growth, with respect to both time and concentration.

Table 2.5: Different antibacterial screening and testing methods and their pros and cons.

Method	Description	Parameter	Pros and cons	References
Disc diffusion	Filter paper discs loaded with antibacterial agents are placed on the surface of Agar with inoculum spread and plates are incubated.	ZDI	**Pros:** low cost, can test against large numbers of isolates **Cons:** time consuming, labour intensive, difficult to diffuse lipophilic oil components or large molecules through agar	(Baker et al., 1991; Hood et al., 2003)
Agar well diffusion	Bacterial susceptibility to the antimicrobial present in the well made in agar instead of disk.	ZDI	**Pros:** low cost **Cons:** time consuming, labour intensive, difficult to diffuse large molecules through agar	(Hood et al., 2003)
Agar dilution	Serial dilutions of antibacterial agents in agar medium, followed by inoculation of bacteria.	MIC MBC	**Pros:** Quantitative, ability to test multiple bacteria at the same time **Cons:** time-consuming, Labour intensive, require substantial economic and technical resource	(Baker et al., 1991; Hood et al., 2003; Wiegand et al., 2008)
Macro broth dilution /tube broth dilution	Serial dilutions of antibacterial agents in broth tubes tested with added bacterial inoculum and incubation.	MIC MBC	**Pros:** Quantitative, Precise, ability to test multiple bacteria at the same time **Cons:** time consuming, labour intensive	(Jorgensen and Ferraro, 1998; Wiegand et al., 2008)

Method	Description	Parameter	Pros and cons	References
Micro broth dilution	Serial dilutions of antibacterial agents in 96-well plates	MIC MBC	**Pros:** precise assessment Quantitative ability to test multiple bacteria at the same time	(Baker et al., 1991; Hood et al., 2003; Sarker et al., 2007; Valgas et al., 2007)

2.9.4. Anti-biofilm formation assays

Biofilm formation is often considered the underlying reason that treatment with an antimicrobial agent fails. Since GAS live as biofilms, there is a high demand for effective anti-biofilm therapy and a need to assay different herbal products for their anti-biofilm formation ability. There are two models of *in vitro* screening methods; 1) static methods which measure early stages of biofilm formation and 2) flow methods which measure developmental processes associated with biofilm formation (Bueno, 2014). However, both assays determine the minimal biofilm inhibitory concentration (MBIC) and minimal biofilm eradication concentration (MBEC). MBIC is defined as the minimum concentration of test compound which makes no biofilm. MBEC is the lowest concentration of drug/compound/extracts required to clearly confirm eradication of biofilm, compared to a positive control (Kim et al., 2010). Table 2.6 shows different static methods of biofilm susceptibility testing, focusing on total biofilm biomass and viability of the biofilm. Crystal violet (CV) staining, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) reduction assay and 1,9-dimethyl methylene blue (DMMB) assay are colorimetric methods, whereas resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) assay and fluorescein-di-acetate (FDA) assay are fluorometric methods (Bueno, 2014; Pantanella et al., 2013).

Table 2.6: Different static biofilm susceptibility testing methods and their pros and cons.

Method	Principle	Pros and cons	References
Crystal violet staining	Biofilm biomass quantification by staining negatively charged molecules and polysaccharides in EPS by CV dye.	**Pros:** Cheap **Cons:** Undistinguishable for live or dead cells, slow, error-prone due to possibility of incomplete removal from surface	(Bueno, 2014)
DMMB assay	DMMB forms an insoluble complexion product with sulphated polysaccharides in the biofilm matrix which is measured spectrometrically.	**Pros:** easy to perform, economic and low runtime **Cons:** poor and inaccurate information on quantity of living bacteria, limited only to few bacteria	(Pantanella et al., 2013)
MTT reduction assay	Serial dilutions of treatment before or after biofilm formation followed by staining biomass with MTT reagent	**Pros:** Distinguishable for live or dead cells; easy and fast **Cons:** Susceptible to respiration rate of bacteria and biofilm thickness	(Bueno, 2014; Pantanella et al., 2013)
FDA assay	FDA is hydrolyzed by cellular esterases to fluorescein (fluorescent yellow) measured spectrophotometrically	**Pros:** Distinguishable for live cells; easy and fast **Cons:** limited field of examination and the thickness of biofilm, only semi-quantitative	(Pantanella et al., 2013)
Live/dead BacLight assay	Stained samples (two nucleic acid binding stains) are observed using a fluorescent optical microscopy to evaluate live and dead bacterial population.	**Pros:** representative of the total population able to cross all bacterial membranes **Cons:** only semi-quantitative	(Pantanella et al., 2013)

Method	Principle	Pros and cons	References
Resazurin assay	Non fluorescent resazurin reduced by cellular metabolic activity and converted in the pink-fluorescent resorufin measured spectrophotometrically. Output is proportional to viable cell number.	**Pros:** quantify actual number of viable cells in biofilm, rapid **Cons:** highly susceptible to bacterial respiratory efficiency. so some experimental conditions are difficult to standardize	(Pantanella et al., 2013; Sandberg et al., 2009)
Colony counting method	Pre- or post-treated biofilm with serial diluted extracts scraped from surface. Supernatant re-suspended and plated on agar plate.	**Pros:** Numerable for active biofilm cell (quantitative) **Cons:** Time-consuming, labor-intensive, error-prone	(Bueno, 2014)

Other than the most common methods used for analytical studies, which have been listed in Table 2.6, many different approaches are applied for visualization and quantification of biofilm. These includes biochemical (e.g. quorum sensing, anti-biofilm detachment activity, bioluminescence), genetic (e.g. real time quantitative-reverse transcription, fluorescence in situ hybridization), advanced microscopic techniques (e.g. confocal laser scanning microscopy, electron microscopy) and mass spectrometry (Bueno, 2014; Kim et al., 2010; Pantanella et al., 2013; Post et al., 2004). Furthermore, interaction between antimicrobial phytochemicals or drugs with biofilms, can be determined by the time to kill assay (time-kill curves) (Bueno, 2014). Efficacious compounds can be tested for safety and efficacy, using *in vitro* cell biological assay and *in vivo* phase II and III trails (Lebeaux et al., 2013).

2.10. BIOACTIVITY OF HERBAL PLANT EXTRACTS AGAINST STREPTOCOCCAL PHYRINGITIS

2.10.1. Anti-*S. pyogenes* activity of phytochemicals

The use of plants, as a source of remedies for numerous microbial infections, dates back to prehistory. Several studies have demonstrated that some bioactive compounds of plants, such as alkaloids, tannins, flavonoids and phenolic compounds, have anti-*S. pyogenes* activity. An *in vitro* study showed antibacterial activities of ethanol extracts for 15 plant species from Jordan. Using the agar diffusion method, these extracts were tested on 14 pathogenic bacterial species and strains, including *S. pyogenes* (ATCC 12351). The MIC of active extracts ranged from 4-32 mg/mL, while the MBC was in the range of 8-62 mg/mL (Nimri et al., 1999). A similar study was carried out to evaluate anti-*S. pyogenes* activity of selected medicinal plant extracts used in traditional Thai medicine, against 11 isolates of *S. pyogenes* from patients with upper respiratory tract infections. It was found that *Boesenbergia pandurata*, *Eleutherine americana*, and *Rhodomyrtus tomentosa* have antibacterial potentials against *S. pyogenes*. *Boesenbergia pandurata* and *Rhodomyrtus tomentosa* demonstrated antibacterial activity of 3.91 µg/mL of MIC and 31.25 µg/mL of MBC, whereas *Eleutherine americana* displayed MIC and MBC values of 250 and 250-500 µg/mL, respectively against all the isolates (Limsuwan and Voravuthikunchai, 2013). Another study showed that rhodomyrtone from *Rhodomyrtus tomentosa* (Aiton) Hassk leaf extract has a strong antibacterial activity against *S. pyogenes,* with a very low MIC of 0.39 to 0.78 µg/mL and MBC of 1.56 µg/mL. Furthermore, based on the time-kill curve analysis, rhodomyrtone had a significant bactericidal activity (99.9% killing) within 24, 6, and 5 hr at 2 × MBC, 4 × MBC, and 8 × MBC, respectively (Limsuwan et al., 2009). *Pleurostylia*

capensis, commonly found throughout tropical Africa, showed a strong antimicrobial activity against *B. cereus, K. pneumonia, S. pyogenes,* and *M. smegmatis,* with MIC values of 0.39 and 0.78 mg/mL (Razwinani et al., 2014).

Essential oils are aromatic oily liquids obtained from different herbal plant parts, such as leaves, shoots, flowers, buds, seeds, bark, wood, fruits and roots. The essential oils have different chemical compounds derived from terpenes and their oxygenated compounds, which contribute to their beneficial antibacterial, anti-inflammatory and antioxidant effects (Prabuseenivasan et al., 2006). Most studies reported phytochemical profiles of efficacious extracts and identified the compounds responsible for the activity. Antibacterial efficacy and antioxidant properties of essential oils from *Matricaria chamomilla* L., were evaluated by disk diffusion and tube dilution methods (Owlia et al., 2007). This study determined that the MIC/MBC ratio against *S. pyogenes* was 0.1/0.2. The same study reported 18 phytochemicals, including guaiazulene, E-farnesen, chamazulene, rx-bisabclol oxide B, rx-bisabolol and hexadecanole, as potential anti-microbial agents.

Antibacterial effects against *S. pyogenes* have been reported for many other plant species. Ethanol, methanol, glycerin and aqueous extracts have been identified in *Satureja bachtiarica* (a Iranian medicinal plant) as being effective against *S. pyogenes* (Sureshjani et al., 2013). Extracts from two traditionally used plants, *Albizia gummifera* and *Ferula communis*, were efficacious against clinical isolates of *S. pyogenes* and *S. pneumonia* (Unasho et al., 2009). Similarly, extracts from two Hawaiian medicinal plants, *Pipturus albidus* and *Eugenia malaccensis,* were tested against several bacteria and showed growth inhibition of *Staphylococcus aureus* and *S. pyogenes.* (Locher et al., 1995).

2.10.2. Anti-biofilm activity of bioactive phytochemicals

Biofilm formation is a potentially important mechanism in contributing to treatment failure of *S. pyogenes.* Several plant extracts have exhibited the ability of anti-biofilm formation and mature biofilm eradication. Therefore, herbal extracts as single source or as in combination with other antimicrobial therapies, such as antibiotics could employ for the treatment of pharyngitis. Some of anti-quorum-sensing, antiseptic, or anti-virulence factor properties which provides the anti-biofilm activity of herbal extracts are explained in this section. *Lagenaria siceraria,* a fruit growing in Iraq, was screened for its phytochemical profile and for its antibacterial and anti-biofilm effects (Mutalib et al., 2015). Another study has shown *in vitro* anti-biofilm activity of ethanol extracts of *Piper longum* and *P. nigrum,* against clinical isolates of *S. pyogenes,* isolated from pharyngitis patients (Darsini et al., 2015). The methanol and ethyl acetate extracts of *P. longum* and *P. nigrum* showed MBIC in the range of 85% to 96%, whereas the MBIC of chloroform and petroleum ether extracts were significantly greater (71% to 84%), at a concentration of 2 mg/mL. Anti-biofilm formation and anti-quorum-sensing activity in *S. pyogenes* were observed in extracts of *Boesenbergia pandurata* (Roxb.) Schltr, *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk (Mutalib et al., 2015).

2.10.3. Anti-inflammatory activity of bioactive phytochemicals

Phytochemicals have the potential to be used in alternative therapies for controlling inflammatory conditions (Fawole et al., 2010). Various studies have demonstrated the effect of herbal remedies on the production of human inflammatory and anti-inflammatory cytokines. For example, the anti-inflammatory effect of *Echinacea purpurea* extracts, used

traditionally in North America, has been reported to reduce inflammation caused by upper respiratory bacteria, including *S. pyogenes*. Inflammatory effects were measured with 20 different pro-inflammatory cytokines (TNF-α, IL-4, IL-6, IL-8, IL-12) secreted by bronchial epithelial cells. Moreover, the cellular response of echinacea targeted multiple sites and mechanisms that led to the relief of symptoms (Sharma et al., 2010). Leaf extracts of some medicinal plants from eastern Ethiopia, *Dodonaea viscosa, Rumex nervosus* and *Rumex abyssinicus,* were also reported to have anti-microbial effect against *S. pyogenes* (clinical isolate). Several phytochemicals were reviewed, including: flavonoids, terpenes, coumarins, steroids, oxalic acid, chrysophanic acid, chrysophanol, emodine, physcion, tannins, tartaric and citric acids. However, only methanol extracts of *R. abyssinicus* showed anti-inflammatory activity by inhibiting the synthesis of prostaglandin (PGE2), without being cytotoxic, (Getie et al., 2003). Another study indicated that *Pleurostylia capensis* extracts have significant anti-inflammatory activity against both COX-1 and COX-2, by 98% and 83%, respectively (Razwinani et al., 2014). A similar phenomenon was reported, with inhibitory effect of pro-inflammatory cytokines, in nine plants used in South African traditional medicine. They were evaluated for therapeutic potential as anti-inflammatory and anti-microbial agents, but only five methanolic extracts of *Cyphostemma natalitium* root, *Rhoicissus digitata* leaf*, R. rhomboidea* root, *R. tomentosa* leaf/stem and *R. tridentate* root showed significant inhibition of COX-1. Another set of extracts was employed for testing PG synthesis inhibition. *R. digitata* leaf and of *R. rhomboidea* root exhibited significant inhibition of PG synthesis with 53% and 56%, respectively (Lin et al., 1999).

CHAPTER 3. INHIBITION OF GROWTH AND BIOFILM FORMATION OF *STREPTOCOCCUS PYOGENES* BY PHYTOCHEMICAL EXTRACTS OF SELECTED CANADIAN HERBS

3.1. ABSTRACT

Streptococcus pyogenes, continues to be a significant public health problem worldwide. Natural health industries are increasingly interested in complementary and alternative medicines, including herbal medicine, as they notice that these treatment are both safe and effective. The present study was conducted to evaluate antibacterial and anti-biofilm formation activities of 32 phytochemical extracts of 12 selected Canadian medicinal plants against two strains of *S. pyogenes* (ATCC 19615, ATCC 49399) and a clinical isolate. Ethanol extracts (EE) showed minimum inhibitory concentration (MIC) of 62.5 to 1000 μg/mL, while minimum bactericidal concentration (MBC) was 125-1000 μg/mL. Among AE (AE), licorice root, oregano flowering shoots and thyme flowering shoots were the most effective, with MIC ranging from 1560 to 12,500 μg/mL and MBC was 6250 μg/mL. Essential oils (EO) prepared from oregano flowering shoot and sage leaves showed significantly lower MIC and MBC values, ranging from 250-500 μg/mL and 500 μg/mL, respectively. Penicillin G was used as the positive control and showed the most significant inhibition against planktonic *S. pyogenes* with MIC and MBC of 0.0078 and 0.0156 μg/mL, respectively. Oregano EO sage EE, sage EO, echinacea flower EE and licorice root EE showed the prompt killing ability of the initial bacterial burden, whereas it was 24 hr for penicillin for the same burden of *S. pyogenes*. The scan electronic microscopic (SEM) analyses of biofilms complied with minimum biofilm inhibitory concentrations (MBICs) ranging from 31.5-6250 μg/mL. These findings demonstrated that EE of licorice root,

echinacea stem and flower, sage leaves and EO from sage leaves and oregano flowering shoots could be an important alternate therapeutic agent in the management of streptococcal pharyngitis.

Key words: Pharyngitis, *Streptococcus pyogenes*, phytochemicals, biofilm inhibition, herbal, antibacterial

3.2. INTRODUCTION

S. pyogenes (group A streptococci-GAS) is a major upper respiratory tract bacterial pathogen that causes a wide variety of infections (Martin and Green, 2006). These infections range from mild infections, such as streptococcal pharyngitis, impetigo; severe infections, including toxic shock syndrome and necrotizing fasciitis (Stevens, 2000) and chronic debilitating non-suppurative sequelae, including acute glomerulonephritis (Eison et al., 2011) rheumatic fever and rheumatic heart disease (Seckeler and Hoke, 2011). In 2005, it was estimated that approximately 18.1 million people suffered from severe infections and 1.78 million new cases occur each year, accounting for more than 517,000 deaths due to severe invasive infections annually (Carapetis et al., 2005). Streptococcal pharyngitis is more common in children than adults, with 37% diagnosed in 5 to 15 year old children and adolescents, and 5-15% in adults (Shaikh et al., 2010; Shulman et al., 2012), often during the colder months (late winter and early spring) of the year (Cirilli, 2013; Pichichero et al., 1998). In 2005, over 616 million incident cases per year of GAS pharyngitis were reported worldwide (Carapetis et al., 2005). Economic burden per case of streptococcal pharyngitis is very high, including medicinal costs, such as antibiotics and Nonsteroidal anti-inflammatory drugs (NSAIDs), as well as non-medicinal expenses which account for almost one half of total expenditure, such as time, child care, non-prescription

drugs, transportation, throat lozenges, throat sprays, vapor rubs and herbal teas (Pfoh et al., 2008).

As pathogen, *S. pyogenes* has developed complex virulence factors and mechanisms to avoid host defenses and invasion, including several structural proteins, such as M protein and fibronectin-binding protein; cell surface components, such as peptidoglycan and lipoteichoic acids; capsule hyaluronic acid, streptokinase; streptolysins and erythrogenic toxin (Ogawa et al., 2011; Terao, 2012). Pathogenesis of *S. pyogenes* was reported to be initiated with adhesion through specialized receptors to host tissues and invasion followed by biofilm formation and spread to deep tissues (Terao, 2012; Valgas et al., 2007). Sudden onset of sore throat, tonsillar exudate, tender cervical adenopathy, and fever are major symptoms of streptococcal pharyngitis (Choby, 2009)

Although streptococcal pharyngitis is self-limited and localized inflammation of the tonsillopharynx resolves even without treatment, risk of complications increases in non-treated patients (Chiappini et al., 2011; DuBose, 2002). Antibiotics are often prescribed for the treatment of streptococcal pharyngitis and penicillin is the first choice of drug in many cases (Brook and Foote, 1997; Choby, 2009). Despite the fact that *S. pyogenes* retains robust susceptibility to penicillin, clinical failures of penicillin-treated patients were reported (Brook, 2001). *In vitro* studies have reported failure of penicillin, of rates up to 10% to 15% of clinical practices (Kuhn et al., 2001; Pichichero and Margolis, 1991). It was found that other antibiotics, such as cephalosporins, clindamycin, macrolides, and amoxicillin-clavulanate, were more effective than penicillin against streptococcal pharyngitis, especially in those which were unsuccessful in previous penicillin therapy (Brook, 2001). However, macrolide- and lincosamide-resistant *S. pyogenes* isolates have

been reported in many countries and gradually spread worldwide (Richter et al., 2005).

There are several treatment challenges due to the lack of protective micro flora, co-pathogenicity of β-lactamase-producing bacteria, early initiation of antibiotics resulting in insufficient immune response and lack of compliance (Ogawa et al., 2011). Also, it has been demonstrated that antibiotic treatment failure of *S. pyogenes* infection is associated with biofilm formation, across all types of antibiotic therapies (Conley et al., 2003). For example, therapeutic failures of antibiotics used to treat macrolide-susceptible streptococcal pharyngitis were reported to be due to biofilm formation (Baldassarri et al., 2006). A biofilm is a structure comprised of clusters of bacteria and the matrices of extracellular polymeric substances are interrupted by a complex network of channels (Conley et al., 2003; Fieber et al., 2015). Biofilm formation is inherently less susceptible to antimicrobial therapies than the same bacteria grown in planktonic form because it acts as a barrier to the action of toxic compounds and reagents, such as antibiotics and innate immunity products present in the environment (Hall-Stoodley et al., 2004; Lewis, 2001; Manetti et al., 2007).

The interest in using plant extracts for treatment of streptococcal pharyngitis has increased as several treatment challenges associated with conventional antibiotics, such as treatment failures, patient's allergy, poor patient compliance and unnecessary side effects (Cowan, 1999). Medicinal plants are a great source of alternative treatments for many infections. Plants are rich in a wide variety of secondary metabolites (phytochemicals), such as tannins, terpenoids, alkaloids, and flavonoids (Cowan, 1999), which have been shown to possess antimicrobial properties (Ghannadi et al., 2012; Li-Weber, 2009; Sfeir et al., 2013). For centuries, the therapeutic properties of various medicinal plants have been

used to treat human diseases. On the other hand, perception about medicinal plant-derived compounds is that they are safe and they have a long history of use in folk medicine for the treatment of infectious diseases. Throughout North America, both Native Americans and Americans of European origin have traditionally employed plants for medicinal uses. The employment of medicinal plants by traditional healers of native America has been reviewed and it has been reported that approximately 2,564 species of plants have been used as drugs (Moerman, 1996). Traditional herbal medicines for streptococcal pharyngitis are being re-evaluated in clinical laboratories and hence, few side effects and their suitability for long-term administration have been reported, compared with synthetic antibiotics. Plant extracts, such as thyme, oregano, sage, barberry, echinacea, and licorice, are used in traditional or folk healing practices in Canada to treat bacterial infections, including streptococcal pharyngitis (Table 3.1). However, inhibition of growth and biofilm formation of *S. pyogenes* by phytochemical extracts of these plants has not been reported. Therefore, the current investigation was carried out to identify the most efficacious plant source from 12 different herbal plants. The specific objectives were to: (1) identify the most effective extracts against planktonic *S. pyogenes* by determining the MICs and MBCs; (2) investigate the time required to kill bacteria by various concentrations of plant extracts; (3) explore the inhibitory effects of efficacious extracts on inhibition of biofilm formation by determining the MBICs; and (4) visualize bacterial biofilm at their minimum sub-inhibitory biofilm concentrations using SEM.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and instruments

A class II-type A2 biological safety cabinet (Model LR2-452, ESo Technologies Inc. Hartboro, PA, USA), Field Emission Gun Scanning Electron Microscope (Hitachi FEG-SEM 4700), water bath (BVS Hetomix, Heto-Holten, Allerod, Denmark); rotary evaporator (Heidolph, thermo electron corporation, Milford, MA, USA), micro-plate reader (Tecan Infinite® M200 PRO, Morriscille, NC, USA), nitrogen evaporator (N-EVAP™, Organomation Association Inc., Berlin, NJ, USA); micro-titer plate reader (Epoch™, Biotek, Winooski, VT, USA); centrifuge (Sorvail Legend Micro 21 R, Thermo Scientific Thermo Fisher Scientific Inc., Waltham, MA, USA); micro centrifuge (Sorvail ST 16, Thermo Scientific Thermo Fisher Scientific Inc., Waltham, MA, USA) and gas liquid chromatography (Bruker 430-GC with FID), Kinetics freeze dryer (Kinetics, FTS Systems Inc., Stone Ridge, NY, USA), ultrasonic bath of 20 kHz/1000 Watts (model 750D, VWR, West Chester, PA, USA) were used for extractions and experiments. Brain heart infusion (BHI), bacteriological agar and trypton were purchased from Oxoid Ltd. (Nepean, ON, Canada). Sodium chloride (≥ 99.0%, ACS reagent), dimethyl sulfoxide (DMSO) (≥ 99.8%), gas chromatography (GC), penicillin G sodium salt were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Life Technologies (Burlington, ON, Canada). Gas chromatography standards, such as carvacrol, p-cymene, α-terpenine and γ-terpenine, β-myrcene and caryophyllene, campene, α-thujone, fenchyl alchohol, citral, cinamaldehyde, thymol, eugenol, eugenyl acetate and trans-caryophyllene were purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.3.2. Collection of plant materials

Twelve different herbal plants: thyme (*Thymus vulgaris*), oregano (*Origanum vulgarem*), sage (*Salvia officinalis*), danshen (*Salvia miltiorrhiza*), clove (*Syzygium aromaticum*), barberry (*Berberis vulgaris*), licorice (*Glycyrrhiza glabra*), ginger (*Zingiber officinale*), slippery elm (*Ulmus rubra*), gernaium (*Pelargonium graveolens*), olive (*Olea europeus*), and purple coneflower/echinacea (*Echinacea purpurea*) were selected for the study. Sage, thyme, oregano, geranium and echinacea were collected from the university's herbal garden, Faculty of Agriculture, Dalhousie University, Canada during August-September, 2014. Fresh Chinese ginger, Canadian ginger and dried clove flower buds were purchased from Truro and Halifax super markets. Dry powder of licorice root, barberry root, were purchased from Mother Earth Natural Health, Ottawa, Ontario. Plants were authenticated by a taxonomist, Jeff Morton, Department of Environmental Sciences, Faculty of Agriculture, Dalhousie University, Canada. All the fresh plant parts were washed with distilled water and were rinsed off. Samples were dried at 50 °C and finally reduced to fine particles using a laboratory blender for 5 min at high speed and then stored in airtight closed containers until being used for further extraction steps. The 12 selected plants which are used in Canadian traditional medicine are shown in Table 3.1.

3.3.3. Preparation of extracts of medicinal plants

Aqueous extraction (AE):

The aqueous extracts were prepared using a previously described method (Gunathilake and Rupasinghe, 2014). Briefly, ten grams of dried powder samples in 100 mL distilled water (1:10 solid to water ratio) were placed in an Erlenmeyer flasks and extracted in a boiling water bath for about 10 min. The residues were removed by filtering through a Whatman No.5 filter paper under vacuum and the filtrates were collected into pre weighed plastic cups and were frozen overnight at -20 °C. Finally, they were dried in a freeze dryer under 3600 mT vacuum and -20 °C for 48 hr. After drying, dried sample were scraped out and were stored in labeled sterile screw capped bottles, in the freezer at -80 ^{o}C until use for further analysis.

Ultrasonic-assisted Ethanol extraction (EE):

Thirty five grams of powdered sample was dissolved in 350 mL of 95% ethanol (1:10 ratio) in a conical flask, plugged with cotton wool and then kept in a sonication bath of 20 kHz/1000 Watts at 35±2 °C for 45 min at 40 kHz frequency and 150W ultrasonic power. The residues were removed by filtering through a vacuum pump. The filtrates were collected and were evaporated to dryness (to evaporate the ethanol) using a rotary evaporator at 45±2 °C for 20-30 min to obtain the crude extract in the form of a paste. Solids in the flask were dissolved in 2-5 mL of 100% ethanol and placed in a nitrogen evaporator to remove residual ethanol. After the solids were completely dry, they were preserved in airtight amber glass bottles at -80 °C.

Hydro-distilled extraction:

Fifty grams of each dry, powdered plant sample was mixed with 750 mL distilled water (1:15 ratio). Mixtures were subjected to hydro-distillation for approximately 3 hr in a Clevenger apparatus, as previously described (Erkan et al., 2012). Collected EOs were dried with anhydrous sodium sulphate at a concentration of 2% w/v oil and stored in an airtight amber bottles at -20 ºC.

3.3.4. Phytochemical analysis and antioxidant capacity analysis

Determination of total phenolic content:

The Folin Ciocalteu assay was performed to estimate the total phenols present in the herbal extracts, as described by (Ainsworth and Gillespie, 2007) with some modifications. The assay was conducted using a 96-well micro-plate. Briefly, 20 µL of the plant extract was mixed with 100 µL of 0.2 N Folin-Ciocalteu reagent in the micro-plate wells of the clear 96-well micro-plates. After 5 min, 80 µL of a 7.5% sodium carbonate solution (pH=7) was added, mixed gently and was coved with a parafilm and a foil. Then, plates were left in the dark for 2 hr at ambient temperature. Absorbance values were measured at the wave length of 760 nm. The total phenol was calculated using a standard curve prepared with Gallic acid standards at concentrations of 10, 25, 50, 75, 100, 200, 300, 400, 500 and 1000 ppm. Total phenolic content were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry solid of extract. The solutions were made fresh under reduced light conditions and the reaction was carried out in darkness.

GC-FID analysis for EOs:

The major phytochemicals of the four EOs, prepared from oregano, sage, ginger and clove, were analyzed using gas chromatography-flame ionization detection (GC-FID), with a BR-

1 ms fused silica capillary column (15 m × 0.25 mm i.d.; film thickness 0.25 μm). Helium was the carrier gas with a column flow rate of 1 mL/min. Samples were dissolved to a concentration of 12.5 mg/mL in hexane; 1 μL of each sample was injected at a split ratio of 25:1. Standards and samples were prepared in triplicate. A method, with a total run time of 23.5 min, was programmed into the 430-GC as follows: initial temperature of 50 ºC for 2 min, temperature increase to 135 ºC at a rate of 10 ºC/min for 3 min, followed by an increase to 210 ºC at a rate of 15 ºC/min, with a final holding period of 5 min.

Determination of Ferric Reducing Antioxidant Power:

The ferric reducing antioxidant power (FRAP) assay was performed to determine the electron donating potential of the samples, based on the assay described by (Benzie and Strain, 1996) and modified by (Rupasinghe et al., 2008). The working reagent, consisting of 300 mM acetate buffer (pH 3.6), 1 mM 2,4,6-Tris (2-pyridyl)-s-triazine solution, and 20 mM ferric chloride, was combined in the ratio of 10:1:1 directly before analysis and preheated to 37 ºC. Twenty microliters of each sample or standard was placed in the wells of the 96-well clear polystyrene micro-plate and 180 μL of the working reagent was added to each well. The absorbance was read at 593 nm after a 6 min reaction time and antioxidant capacity was calculated based on Trolox standards at concentrations of 5, 10, 25, 50, 75, 150, 250, 500 and 750 mM. The ferric reducing antioxidant power of extracts was expressed as milligrams of Trolox equivalents (TE) per gram of dry solid of extract.

3.3.5. Bacteria strains

Three strains of *S. pyogenes* (ATCC 19615, ATCC 49399 and clinical isolate) were used in this study. Two *S. pyogenes* commercial strains (ATCC 19615™ and ATCC 49399™) were obtained from the American Type Culture Collection (ATCC) and inoculum was

prepared according to the manufacturer's instructions. The vials were enclosed using sterilized scissors and the hydrated suspension was cultured in BHI broth, as well as on agar medium by the streak plate method. Subsequently, the broth and plates were incubated (35±2 ^{o}C; 24 hr). Overnight culture in BHI broth was diluted to a 1:2 ratio with 40% glycerol in a 2 mL screw top tube or cryovial (final glycerol concentration will be 20%) and stored at -80 ^{o}C. A clinical isolate, obtained from a pharyngitis patient was kindly provided by Dr. R. J. Davidson (Director, Division of Microbiology and Director of the Molecular Epidemiology laboratory, Queen Elizabeth II Health Sciences, Halifax, NS, Canada) and was cultured and stored similarly.

3.3.6. Media preparation

Thirty seven grams of Brain Heart Infusion (BHI) was dissolved in 1 L of distilled water and brought to the boiling point. Similarly, 37 g of BHI and 15 g of Agar were mixed together and brought to a boil. Then, both media were autoclaved for 15 min at 121 °C and left to cool at room temperature. Once the BHI agar medium was cooled (about 45 °C), it was poured into petri dishes. Each petri dish was left on a flat surface for 30-40 min until completely set.

3.3.7. Culture and maintenance of microorganisms

All *S. pyogenes* strains, which were cultured on BHI agar plates, were maintained for around 7 days at 35 ± 2 ^{o}C. One or two colonies from these cultures were inoculated onto BHI broth and incubated at 35 ± 2 ^{o}C for 24 hr prior experiment and were diluted.

3.3.8. Standardization of bacteria

The bacteria density was standardized to 1×10^{9} CFU/mL, as previously described method with slight modifications (Ells and Truelstrup Hansen, 2006). Overnight bacterial cultures

were diluted two times in BHI broth. The absorbance of diluted bacterial cultures was measured at optical density of 600 nm (OD 600) with BHI broth as the blank, using a spectrophotometer. Each suspension (1.5 mL) was centrifuged at 28,342 g forces for 10 min. Then, supernatants were discarded and the pellets were re-suspended in v mL of fresh BHI broth calculated using the following formula:

v = (OD 600 * 2 * 1.5 mL)/1

3.3.9. Determination of MIC and MBC

Inhibition of bacterial growth were determined using the micro-broth dilution method (Wu et al., 2015). The cultures were incubated overnight in BHI broth and were standardized using the method described in Section 5.3.2 and then 10-fold diluted to 1×10^6 CFU/mL with Trypton water (1% Trypton, 0.85% NaCl, pH=7.0 ± 0.1). Plant extract of ethanol, aqueous and EOs were dissolved in 100% ethanol, BHI media and DMSO, respectively. In sterile 96-well micro-titer plates, 100 µL of the bacterial suspension in broth was added to wells containing 100 µL of a plant extract, which was diluted with broth. Serial two-fold dilutions of samples were performed to obtain concentrations ranging from 0.75 to 1000 µg/mL for the ethanol extracts; from 48 to 50000 µg/mL for the aqueous extracts; from 1.95 to 2000 µg/mL for the essential oils; and 0.0004 to 0.5 µg/mL for penicillin G. in a total volume of 200 µL BHI with 1×10^6 CFU/mL bacteria. Each plate included positive controls (bacteria without an antimicrobial), negative controls (medium only), diluent controls and serial two-fold dilutions of each of the plant extracts. After incubation for 24 hr at 35 ± 2 °C, growth of planktonic bacteria was determined by measuring absorbance at OD 600 nm, using a micro plate reader. The MIC was defined as the lowest concentration of test compounds inhibiting bacterial growth, where the absorbance showed significant change, as compared to the level of positive controls ($p \leq 0.05$). To determine MBC, 100

μL of suspension from each well showing no increase in absorbance compared to negative controls, was spread out on plate medium and then incubated (35 ± 2 °C; 24 hr). The MBC represents the lowest antimicrobial concentration where 99.9% or more of the initial inoculum is killed. The each of the three independent experiments was performed in triplicate.

3.3.10. Time-kill assay

The bactericidal activity of the extract was studied using a time-kill assay, as described by (Noviello et al., 2003) with minor modifications. An overnight culture of *S. pyogenes* ATCC 19615 was standardized and diluted to 1 x 10^6 CFU/mL, as described in Section 5.3.3. The bacterial suspension was added to BHI broth containing the extract (at 1/2 × MIC, MIC and 2 × MIC), or DMSO vehicle alone (as a negative control) in the 96 well plates. Then the plates were incubated in the incubator at 35 ± 2 °C and bacterial growth was monitored at different time intervals, based on the preliminary assay results. The bacterium was incubated over a 6 hr period for oregano EO, sage EO, licorice EE, echinacea flower EE and sage EE; at 2 hr or 3 hr intervals over a 24 hr period used for the rest of the treatments (echinacea stem EE, slippery elm EE, licorice root AE, oregano AE, thyme AE and penicillin). Viable colony count by a serial dilution method was performed. Briefly, 100 μL of samples from each well were collected at different time intervals and serially diluted 10-fold to countable numbers (30 CFU/mL to 300 CFU/mL), using 900 μL Trypton water. Each dilution was spotted onto a BHI agar plate and dried before the 24 hr incubation time at 35 ± 2 ºC. Penicillin G was tested as the positive control with the same corresponding concentrations within a 24-hr exposure period.

3.3.11. Determination of minimum biofilm inhibitory concentration (MBIC)

The anti-biofilm formation assay was adapted from (Jadav et al., 2013) and performed for five EEs, three AEs, two EOs, solvent controls and penicillin G. An extract or antibiotic control penicillin (100 µL) was added into each well, followed by addition of standardized bacteria (100 µL), as described in Section 5.3.3. Two-fold serial dilutions were made in wells so that each of the eleven different concentrations was tested. The last 4 rows of each micro-plate were used as controls (as color blank, medium control and as bacterial growth control). After three days incubation at 35 ± 2 ºC, the plates were emptied by flipping them over to remove the planktonic bacteria. Fresh BHI broth (100 µL) supplemented with 10 µL of 12 mM MTT was then added into each well, followed by incubation for 3 hr s at 35 ± 2 ºC. DMSO (50 µL) was added after the careful removal of 85 µL of BHI broth from each well. The insoluble purple formazan, obtained by reduction of MTT due to activity of dehydrogenase enzymes in living *S. pyogenes* biofilm cells, was detected by measurements of the absorbance of 540 nm, using a micro-plate reader.

3.3.12. SEM visualization

Sub-inhibitory biofilm inhibitory concentration of selected extracts (100 µL) was added into wells, followed by the addition of standardized bacteria (100 µL), similar to the anti-biofilm formation assay. After incubation for three days at 35 ± 2 ºC, the plates were emptied by flipping them to remove the planktonic bacteria. Biofilms were fixed by 0.1 M sodium cacodylate trihydrate solution with 2% gluteraldehyde for 2 hr. Then, fixed biofilms were rinsed three times at 10 min intervals, using 0.1M cacodylate buffer with 3% glucose. A second fixation of biofilms was performed by submerging them in 1% osmium tetroxide solution in 0.1 M cacodylate for 4 hr, followed by three washings with 0.1 M

cacodylate. Sample wells were dehydrated with ascending ethanol gradients series as follows: 35; 50; 75; 90; 100%, 15 min with each concentration except the last one (100:0) which was repeated 3 times. Then, sample wells were dried in four different hexamethyldisilazane (HDMS)/ethanol mixtures series as follows 25:75; 50:50; 75:25; 100:0 (15 min with each concentration except the last one (100:0) which is repeated twice). After discarding the last solution, the plates were air dried for 2 hr under the fume hood without the lid. Finally, fixed biofilms were cut with a heat blade and were mounted on aluminum stubs Carbon tabs, using a sterilized twister. Then, Sputters were coated with gold-palladium and were visualized in SEM using operational conditions of 10 kilovolts (kV) of acceleration voltage (Vacce), 14-16 microamps (μA) of emission current, 10-12 mm working distance and the analysis lens mode. Micrographs were captured at magnifications of × 1000, × 5000, × 10000, × 15000, × 25000 and × 50000.

3.2.13. Statistical analysis

Complete randomized design was used as the experimental design. All the experiments were performed in triplicate and independently, three times. One-way ANOVA analysis was carried out by using Minitab 17.0 statistical software and statistical differences ($P < 0.05$) between means of pairs were resolved by means of confidence intervals using Tukey's tests. Results were expressed as mean ± standard deviation.

3.4. RESULTS

3.4.1. Extraction yield and phytochemical study of herbal extracts.

Plant parts used in this study and extractions yield results are as shown in Table 3.2. GC-FID analysis was used to quantify the major phytochemicals of four EOs. A total of fifteen compounds were revealed and representing % area of all the compounds. Phytochemicals were confirmed based on the peak area, height percentage and retention time. Carvacrol, eugenol, alpha-thujone, p-cymene, and trans-caryophyllene were found to be the major components (Table 3.1. and Figure 3.4).

Table 3.1: Chemical composition of the four essential oils.

	Compounds	% Amount in EO			
		Sage	**Oregano**	**Ginger**	**Clove**
1	α-Pinene	0.28 ± 0.00^{i}	0.05 ± 0.00^{h}	0.27 ± 0.00^{de}	-
2	Camphene	0.68 ± 0.00^{g}	-	$1..25\pm0.03^{b}$	-
3	Myrcene	1.56 ± 0.00^{e}	0.24 ± 0.00^{f}	0.40 ± 0.01^{c}	-
4	α-Terpinene	0.12 ± 0.00^{k}	0.15 ± 0.00^{g}	-	-
5	p-Cymene	0.11 ± 0.00^{k}	1.23 ± 0.00^{d}	-	-
6	γ-Terpinene	0.36 ± 0.00^{h}	0.67 ± 0.00^{e}	-	-
7	α-Thujone	28.46 ± 0.06^{b}	0.17 ± 0.00^{g}	0.21 ± 0.00^{ef}	-
8	Fenchyl Alcohol	5.48 ± 0.01^{d}	-		-
9	Thymol	0.74 ± 0.00^{f}	0.27 ± 0.00^{f}	0.076 ± 0.00^{h}	-
10	Carvacrol	0.28 ± 0.00^{i}	91.60 ± 0.0^{a}	0.12 ± 0.00^{gh}	-
11	Eugenol	0.19 ± 0.00^{k}	0.14 ± 0.00^{g}	0.32 ± 0.00^{d}	75.67 ± 0.03^{a}
12	trans-Caryophyllene	5.76 ± 0.00^{c}	1.88 ± 0.00^{c}	0.17 ± 0.00^{fg}	12.00 ± 0.16^{b}
13	Eugenyl Acetate	-	-	0.21 ± 0.04^{ef}	10.40 ± 0.22^{c}
14	Citral 1	-	-	0.22 ± 0.00^{ef}	-
15	Citral 2	-	-	0.33 ± 0.00^{cd}	-
16	Others	46.24 ± 0.02^{a}	3.80 ± 0.00^{b}	96.76 ± 0.06^{a}	1.94 ± 0.02^{d}

Data were presented as % amount with mean±SD (n=3), Tukey's test. Means with different letters are significantly different from each other within the same column ($p < 0.05$). EO: Essential oil.

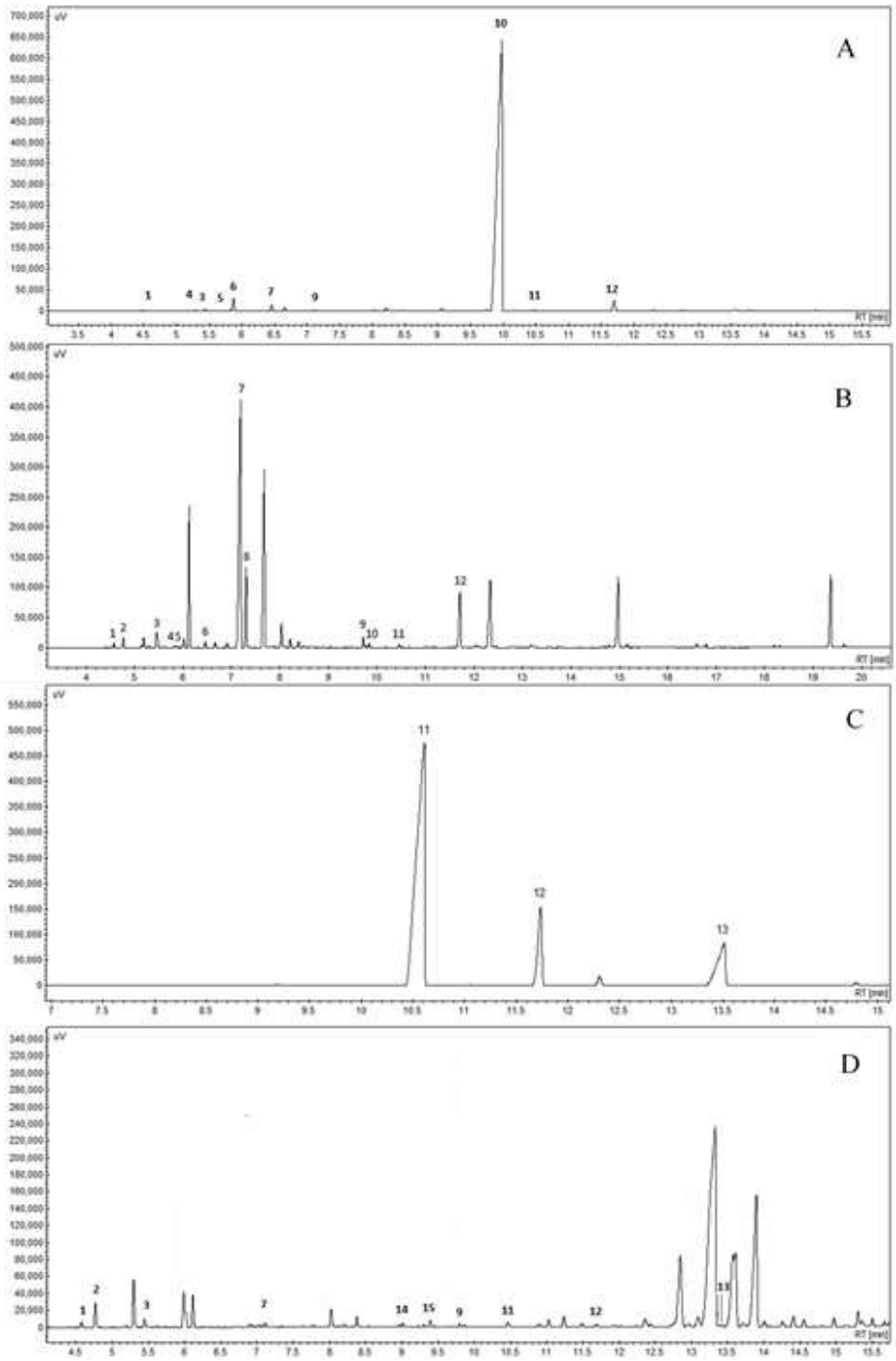

Figure 3.1: Gas chromatograms of (A) oregano EO; (B) sage EO; (C) clove EO and (D) ginger EO. Components: α-Pinene (1), Camphene (2), Myrcene (3), α-Terpinene (4), p-Cymene (5), γ-Terpinene (6) α-Thujone (7) Fenchyl Alcohol (8), Thymol (9), Carvacrol (10), Eugenol (11), trans-Caryophyllene (12) Eugenyl Acetate (13) Citral 1 (14) and Citral 2 (15). EO: EOs.

Table 3.2: Plant parts used in the study, extraction yield, total phenolic content and antioxidant capacity of extracts.

Common Name	Parts used	Extract yield (%) (DW basis)			TP (mg GAE/g DW)		FRAP (mg TE/g DW)	
		EE	**AE**	**EO**	**EE**	**AE**	**EE**	**AE**
Barberry	R	1.86±0.1^{g}	5.93±0.18^{e}	NA	2.58±0.1^{f}	2.31±0.17^{e}	4.861±0.1^{gh}	4.70±3.8f^{g}
Clove	FB	21.27±2.2^{b}	24.59±0.73^{bcd}	3.16±0.2^{a}	19.07±0.1^{a}	15.96±0.4o^{a}	34.12±1.4^{a}	56.55±1.2^{a}
Eastern purple cone flower	L	7.05±0.5^{efg}	31.50±2.98^{a}	NA	1.70±0.1^{fg}	2.14±0.17^{e}	0.29±0.0^{i}	1.48±0.2^{fgh}
	S	5.56±0.1^{fg}	18.92±0.76^{cd}	NA	1.08±0.5^{g}	1.16±0.21^{f}	0.46±0.0^{i}	0.22±0.1^{h}
	F	6.75±0.2^{efg}	20.34±2.15^{d}	NA	0.99±0.1^{g}	2.58±0.22^{e}	1.02±0.1^{i}	1.90±0.1^{fgh}
Geranium	L	9.31±0.2^{def}	28.96±3.79^{ab}	NA	7.12±0.7^{cd}	10.56±0.62^{b}	9.16±0.5^{ef}	38.67±1.4^{b}
Ginger-Chinese	Rh	19.62±1.6^{bc}	19.85±1.26^{d}	1.50±0.1^{d}	6.86±0.8^{cd}	0.53±0.68^{f}	23.24±1.8^{c}	0.95±0.2^{gh}
Ginger-Canada	Rh	NA	26.18±1.28^{abc}	NA	NA	0.64±0.16^{f}	NA	0.83±0.01^{gh}
Licorice	R	39.0±3.4^{a}	19.46±0.43^{d}	NA	4.63±0.1^{e}	2.76±0.22^{e}	2.91±0.5^{hi}	0.257±0.1^{h}
Olive	L	22.16±4.6^{bc}	24.18±4.09^{bcd}	NA	5.28±0.1^{e}	5.72±0.27^{c}	6.70±0.3^{fg}	15.20±0.8^{d}
Oregano	FS	14.5±0.9^{cd}	8.16±0.32^{e}	2.29±0.0^{b}	9.20±0.8^{b}	9.83±0.23^{b}	5.33±0.3^{gh}	26.10±1.5^{c}
Sage	L	8.915±1.6^{def}	19.82±1.92^{d}	2.06±0.0^{c}	5.18±0.4^{e}	4.06±0.18^{d}	4.36±0.3^{gh}	4.93±1.2^{f}
Danshen	R	NA	NA	NA	9.95±0.8^{b}	NA	28.54±0.6^{b}	NA
Slippery elm	IB	7.58±0.6^{ef}	7.103±0.28^{e}	NA	7.46±0.2^{c}	1.98±0.20^{e}	11.61±0.4^{de}	3.56±0.2^{fgh}
Thyme	FS	10.45±0.7^{de}	18.64±2.00^{d}	0.6±0.1^{e}	6.00±0.3^{de}	5.07±0.17^{c}	13.40±0.6^{d}	9.13±0.6^{e}

Means ± SD (n=3) of different extracts analyzed individually in triplicate. Different superscript letters within the same column indicate significant differences of means among extraction solvents used in study. EE: ethanol extracts, AE: Aqueous Extracts, EO: EOs; TP: total phenolic content; FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalents; TE: Trolox equivalents; NA: not analyzed and DW: dry weight of extracts; F: flowers; FB: flowering buds; IB: inner bark; FS: flowering shoots; Rh: rhizome; R: roots; L: leaves and S: stem.

3.4.2. Inhibitory effects of plant extracts against planktonic *S. pyogenes* growth.

The antibacterial activities of 32 medicinal plant extracts used in Canadian traditional medicine for streptococcal infections were evaluated against *S. pyogenes* ATCC 19615, ATCC 49399 and a clinical isolate from a pharyngitis patient. Penicillin G was used as the positive control. The MIC and MBC values are presented in Tables 3.3., 3.4. and 3.5. Out of these total 32 extracts, 22 of them possessed antibacterial effect against *S. pyogenes*. The MIC and MBC values of the rest of the extracts were beyond the highest concentrations. Licorice root EE, sage leaves EE, slippery elm EE, echinacea flower EE and stem EE showed highest inhibitory effects on all tested strains, presenting MIC values between 31.25 - 62.5 µg/mL, whereas all five EE showed the same lowest MBC value, which was 125 µg/mL (2 × MIC) for the bacteria strains (Table 3.4). Among the AEs tested, licorice root, thyme and oregano flowering shoots produced better activity against *S. pyogenes*, as indicated by lower MIC and MBC values, which ranged from 1560 to 50000 µg/mL and 6250 to 50000 µg/mL, respectively (Table 3.3). Due to the limited available dry material, only ethanol extraction was performed and tested for danshen root. Furthermore, the highest concentration for danshen root EE was 250 µg/mL, which was similar to MIC for both ATCC strains and the MBC value did not fall within the detection limit. Oregano EO and sage EO were the most effective EOs, yielding the lowest MIC value of 500 µg/mL. All strains and the isolate were showed significantly lower concentrations penicillin G (MIC = 0.0075 µg/mL, MBC = 0.016 µg/mL).

Table 3.3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of aqueous extracts of selected herbal plants against three strains of *Streptococcus pyogenes* using micro-broth dilution method.

	Plant source / reference	ATCC 19615			ATCC 49399			Clinical isolate		
		MIC (μg/mL)	MBC (μg/mL)	MBC /MIC	MIC (μg/mL)	MBC (μg/mL)	MBC/ MIC	MIC (μg/mL)	MBC (μg/mL)	MBC/ MIC
1	Clove FB	12,500	25,000	2	12,500	25,000	2	12,500	25,000	2
2	Sage L	12,500	25,000		12,500	25,000		12,500	25,000	
3	Ginger-Canada Rh	50,000	>50,000	-	50,000	>50,000	-	NA	NA	-
4	Ginger-Chinese Rh	50,000	>50,000	-	50,000	>50,000	-	NA	NA	-
5	Oregano FB	3125	6250	2	3125	6250	2	3125	6250	2
6	Thyme FB	3125	6250	2	3125	6250	2	3125	6250	2
7	Licorice R	1560	6250	4	1560	6250	4	1560	6250	4
8	Barberry R	3125	6250	2	3125	6250	2	3125	6250	2
9	Echinacea L	50,000	>50,000	-	50,000	>50,000	-	NA	NA	-
10	Echinacea S	6250	12,500	2	6250	12,500	2	6250	12,500	2
11	Echinacea F	50,000	>50,000	-	50,000	>50,000	-	50,000	>50,000	-
12	Geranium L	25,000	50,000	2	25,000	50,000	2	NA	NA	-
13	Slippery elm IB	>50,000	>50,000	-	>50,000	>50,000	-	NA	NA	-
14	Olive L	>50,000	>50,000	-	>50,000	>50,000	-	NA	NA	-
15	Penicillin	0.0078	0.0156	2	0.0078	0.0156	2	0.0078	0.0156	2

NA: Not analyzed; FB: Flowering buds; F: Flowers; FS: Flowering shoots; Rh: Rhizome; R: Roots; IB: Inner bark; L: Leaves.

Table 3.4: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanol extracts of selected herbal plants against three strains of *Streptococcus pyogenes* using micro-broth dilution method.

		ATCC 19615			ATCC 49399			Clinical isolate		
	Plant source/ reference	**MIC (μg/mL)**	**MBC (μg/mL)**	**MBC /MIC**	**MIC (μg/mL)**	**MBC (μg/mL)**	**MBC /MIC**	**MIC (μg/mL)**	**MBC (μg/mL)**	**MBC /MIC**
1	Clove FB	500	1000	2	500	1000	2	500	1000	2
2	Sage L	62.5	125	2	62.5	125	2	62.5	125	2
3	Sage/danshen R	250	>250	-	250	>250	-	NA	NA	-
4	Ginger-Chinese Rh	>1000	>1000	-	>1000	>1000	-	NA	NA	-
5	Oregano FS	500	1000	2	500	1000	2	500	1000	2
6	Thyme FS	500	1000	2	500	1000		500	1000	-
7	Licorice R	62.5	125	2	62.5	125	2	62.5	125	2
8	Barberry R	250	500	2	250	500	2	250	500	2
9	Echinacea L	>1000	>1000	-	>1000	>1000	-	NA	NA	-
10	Echinacea S	62.5	125	2	62.5	125	2	125	250	2
11	Echinacea F	62.5	125	2	31.25	125	4	62.5	125	2
12	Geranium L	>1000	>1000	-	>1000	>1000	-	NA	NA	-
13	Slippery elm IB	62.5	125	1	62.5	125	2	62.5	125	1
14	Olive L	>1000	>1000	-	>1000	>1000	-	NA	NA	-
15	Penicillin	0.0078	0.0156	2	0.0078	0.0156	2	0.0078	0.0156	2

NA: Not analyzed; FB: Flowering buds; F: Flowers; FS: Flowering shoots; Rh: Rhizome; R: Roots; IB: Inner bark; L: Leaves.

Table 3.5: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of selected EOs against *Streptococcus pyogenes* strains ATCC 19615, ATCC 49399 and clinical isolate using micro-broth dilution method.

Plant source		ATCC 19615			ATCC 49399			Clinical isolate		
		MIC (μg/mL)	**MBC (μg/mL)**	**MBC /MIC**	**MIC (μg/mL)**	**MBC (μg/mL)**	**MBC /MIC**	**MIC (μg/mL)**	**MBC (μg/mL)**	**MBC /MIC**
1	Clove FB	1000	2000	2	1000	2000	2	1000	2000	2
2	Sage L	500	500	1	500	500	1	62.5	250	4
3	Ginger Rh	1000	1000	1	1000	1000	1	1000	1000	1
4	Oregano FS	500	500	1	500	500	1	62.5	250	4
5	Penicillin	0.0078	0.0156	2	0.0078	0.0156	2	0.0078	0.0156	2

FB: Flowering buds; FS: Flowering shoots; Rh: Rhizome; L: Leaves.

3.4.3. Time to kill *S. pyogenes*

Time-kill assays were performed to investigate how long it took to achieve 99.99% planktonic *S. pyogenes* kill with the phytochemical-rich extracts. Ten herbal extracts (Licorice root EE, sage leaves EE, echinacea stem EE, echinacea flower EE , slippery elm inner bark EE, sage leaves EO, oregano flowering shoots EO, licorice root AE, thyme and oregano flowering shoots AE) were selected, based on their significantly low MIC and MBC values. Time-kill assay results of those plant extracts and penicillin G are shown in Figures 3.2, 3.3 and 3.4 in a concentration- and time-dependent manner. Time duration of incubation and the multiple of MIC being studied were determined, based on the preliminary assays. The most intense inhibitory effect was shown by oregano EO at 2 × MIC concentration, causing a total elimination of the initial bacterial inoculum after 5 min of exposure and at MIC (500 μg/mL) after 1 h. Licorice root EE and Sage EE presented a bacteriostatic effect for the first 2 hr of exposure and sage EO for 3 hr; from this time on, they established a progressive decrease in bacteria cell count and kill, up to 99.99%, showing bactericidal effects after 3, 3 and 4 hr in licorice root EE, sage EE and sage EO, respectively. Echinacea flower EE provided a bacteriostatic effect for the first 4 hr of exposure, after which, it established its bactericidal effect. More than 99.9% of the initial inoculum of the strain of *S. pyogenes*, was killed following 9 and 12 hr exposure to concentrations of their respective 2 × MIC concentrations of slippery elm EE, echinacea stem EE and licorice root AE. No regrowth was observed during the 24 hr duration of the experiments for all five EE and two EO. Furthermore, moderately slower killing rate was observed for all other AE and penicillin G. At MIC, echinacea flower EE and slippery elm EE rapidly became bactericidal against *S. pyogenes,* producing a more than 3 - *log* units

decrease in viable counts within 24 hr. There was no inhibition of growth in cells treated with 0.1% DMSO, which does not show significant difference in bacterial density with normal growth media.

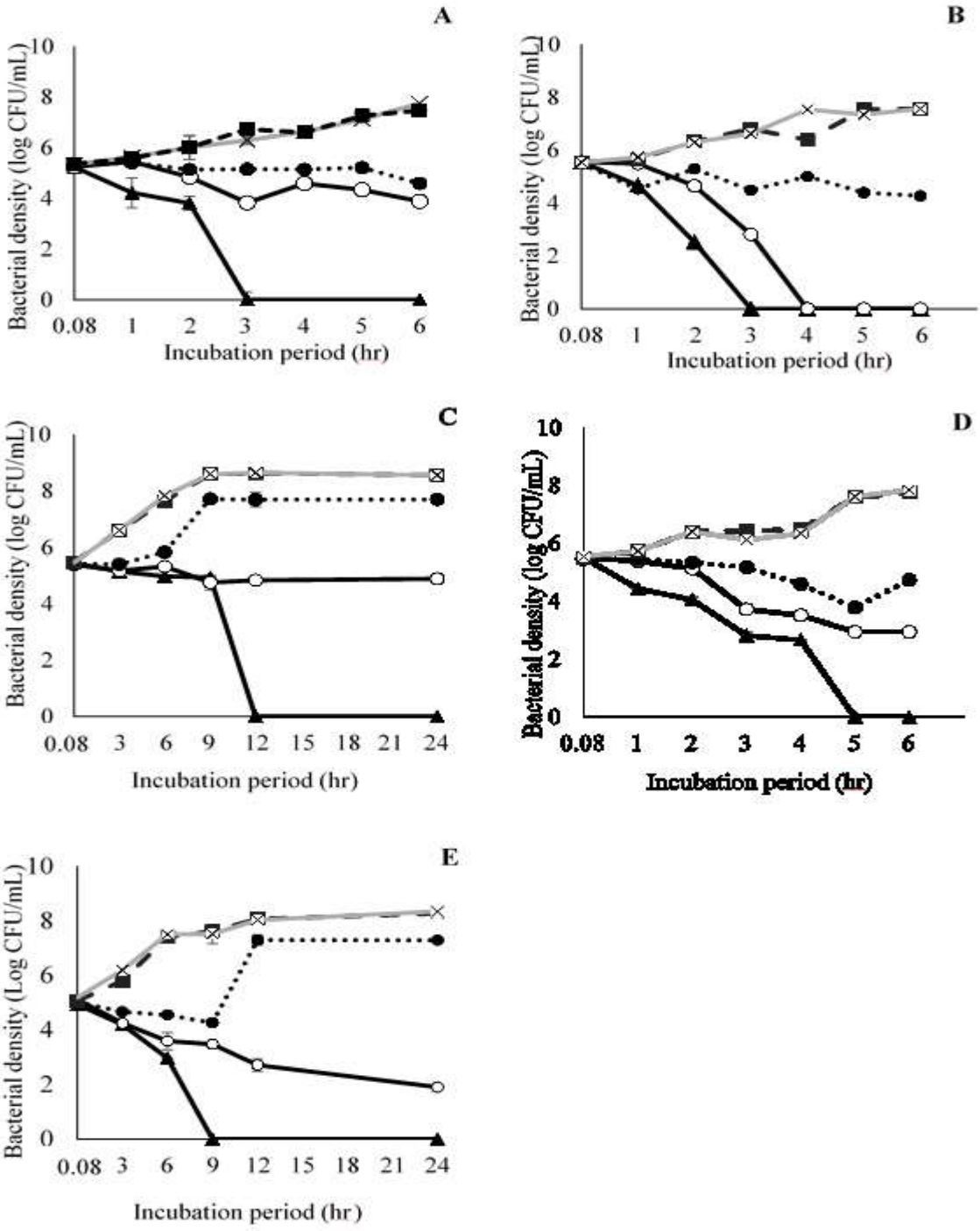

Figure 3.2: Time-killing curves for (A) licorice root EE; (B) sage leaves EE; (C) Echinacea stem EE; (D) Echinacea flower EE and (E) slippery elm inner bark EE on the growth of *Streptococcus pyogenes* ATCC 19615. A viable count was performed for different concentrations at 0.08, 1, 2, 3, 4, 5 and 6 hr in A, B and D and 3 hr intervals of 24 hr incubation in C and E. The killing curve was prepared at 35 ± 2 °C incubation in duplicate and results were identical to within one dilution. ▲= 2 × MIC; ○ = MIC; ●= ½ × MIC; ■= Diluent (1% ethanol); ×= bacteria control. EE: Ethanol extract and MIC: minimum inhibitory concentration.

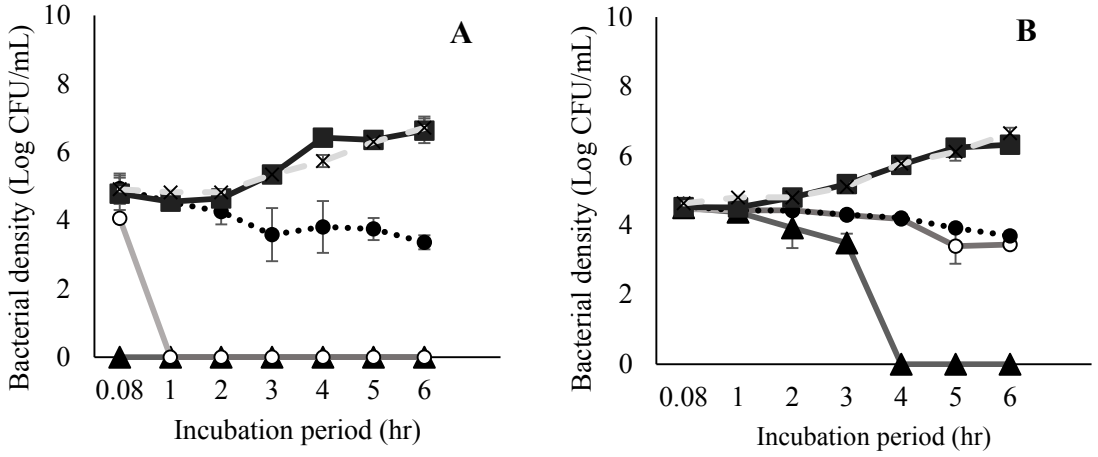

Figure 3.3: Time-killing curves for (A) Oregano flowering shoot EO on the growth of *Streptococcus pyogenes* ATCC 19615. A viable count was performed for different concentrations at 0.08, 1, 2, 3, 4, 5 and 6 hr incubation. The killing curve was prepared at 35 ± 2 °C incubation in duplicate and results were identical to within one dilution. ▲= 2MIC; ○ = MIC; ● = 1/2× MIC; ■= Diluent (1% ethanol); ×= bacteria control. EE: Ethanol extract and MIC: minimum inhibitory concentration.

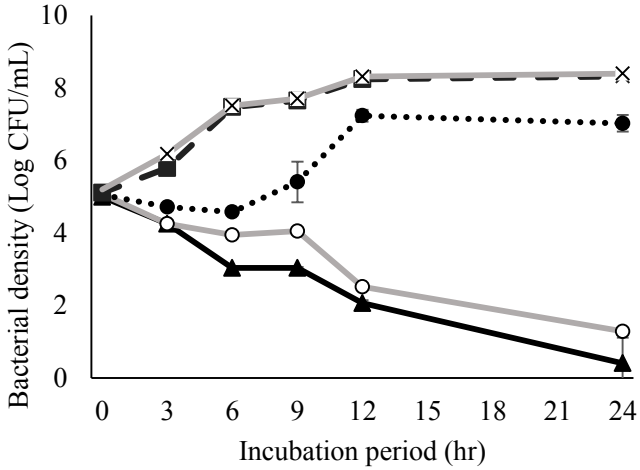

Figure 3.4: Time-killing curves for penicillin G on the growth of *Streptococcus pyogenes* ATCC 19615. A viable count was performed for different concentrations during 24 hr incubation. The killing curve was prepared at 35 ± 2 °C incubation in duplicate and results were identical to within one dilution. ▲= 2MIC; ○ = MIC; ● = 1/2× MIC; ■= Diluent (1% ethanol); ×= bacteria control. EE: Ethanol extract and MIC: minimum inhibitory concentration.

3.4.4 Anti-biofilm formation activity

The effects of sub-inhibitory concentrations of the five ethanol extracts (licorice root, sage leaves, echinacea flower, echinacea stem and slippery elm inner bark), three AE (oregano flowering shoots, thyme flowering shoots and licorice root) and two Eos (oregano flowering shoots and sage leaves) on biofilm formation by *S. pyogenes* ATCC 19615, ATCC 49399 and clinical isolate from a pharyngitis patient at 72 hr, were quantified by MTT staining and subsequently by measuring absorbance at 594 nm (Table 3.7). Penicillin G possessed the most pronounced inhibition of biofilm, with the lowest MBIC values for all three strains. Plant extracts exhibited inhibitory activity on biofilm formation, ranging from 31.5 - 6250 μg/mL and licorice root EE and slippery elm inner bark EE showed the next lowest values after penicillin G. MBIC values were reported in the order of AEs > EO>EE.

Table 3.6: Minimum biofilm inhibitory concentration (MBIC) of selected herbal extracts against *Streptococcus pyogenes* strains ATCC 19615, ATCC 49399 and clinical isolate.

Plant	Extract type	MBIC (μg/mL)		
		ATCC 19615	ATCC 49399	Clinical
Licorice R	EE	250 (4 × MIC)	250 (4 × MIC)	62.5 (MIC)
Echinacea S	EE	125 (2 × MIC)	250 (4 × MIC)	250 (2 × MIC)
Echinacea F	EE	125 (2 × MIC)	31.5 (MIC)	62.5 (MIC)
Sage L	EE	125 (2 × MIC)	125 (2 × MIC)	125 (2 × MIC)
Slippery elm IB	EE	62.5 (MIC)	62.5 (MIC)	125 (2 × MIC)
Sage L	EO	500 (MIC)	500 (MIC)	500 (MIC)
Oregano FS	EO	500 (MIC)	500 (MIC)	500 (MIC)
Oregano FS	AE	6250 (2 × MIC)	6250 (2 × MIC)	6250 (2 × MIC)
Licorice R	AE	3125 (2 × MIC)	12500 (8 × MIC)	3125 (2 × MIC)
Thyme FS	AE	6250 (2 × MIC)	6250 (2 × MIC)	6250 (2 ×MIC)
Penicillin G	AE	0.0156 (2 × MIC)	0.0625 (8 × MIC)	0.0625 (8 × MIC)

EE: Ethanol extracts, EO: Essential oils, AE: Aqueous extracts, MIC: Minimum inhibitory concentration; F: Flowers; FS: Flowering shoots; R: Roots; IB: Inner bark; L: Leaves

3.4.5. SEM visualization of fixed biofilms of *S. pyogenes*

Changes in morphology and density of biofilm formed by *S. pyogenes* ATCC 19615 were observed after 72 hr of treatment with all 10 plant extracts at their particular sub-inhibitory concentrations. The remaining biofilms/cells were visualized under a SEM and the results are shown in Figures 3.5, 3.6 and 3.7.

No morphological changes were observed in the diluent controls with the incorporation of 1% DMSO and 1% ethanol; however, bacterial cell density was lower than in the BHI media. SEM microscopy of penicillin G confirmed its high efficiency in inhibiting bacterial growth and biofilm formation, as demonstrated by the destroyed cell debris (Figure 3.6). Other than reduction of cell density, several morphological changes, such as reduction of cocci sizes and shape of the cells, incomplete separation of cocci from the strep, an abnormal cell division, ruptured cell structure and swelling were observed in the all type of extracts (Figure 3.6 and 3.7). Thyme AEs treated cells were clumped together, which may suggest an attempt to increase cell survival of *S. pyogenes* against the activity of phytochemicals present in the extracts. Interestingly, oregano flowering shoots essential oils showed significant biofilm inhibition by leaving only a cluster of dead cell debries. Furthermore, licorice EE and oregano AE also showed effective in cell destruction. Cell density is significantly reduced due to treatment of sage EO. Moreover, sage leave EE and Echinacea stem EE treatment led to morphological damages to cocci and strep of the bacteria while able to reduce the bacterial density as well.

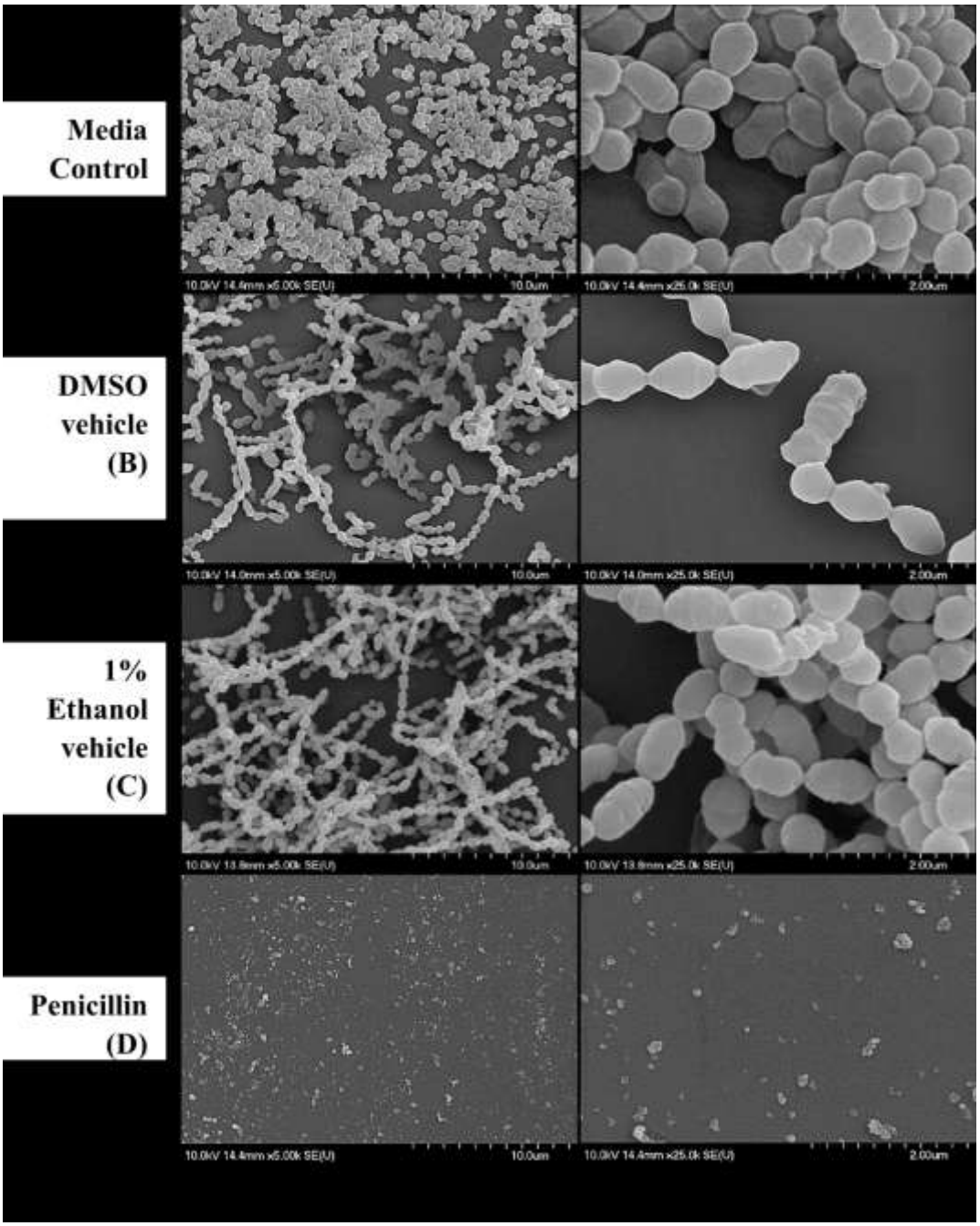

Figure 3.5: Scanning electron micrographs of *Streptococcus pyogenes* biofilms formed on 96 well plate well surface with media, and solvent controls.

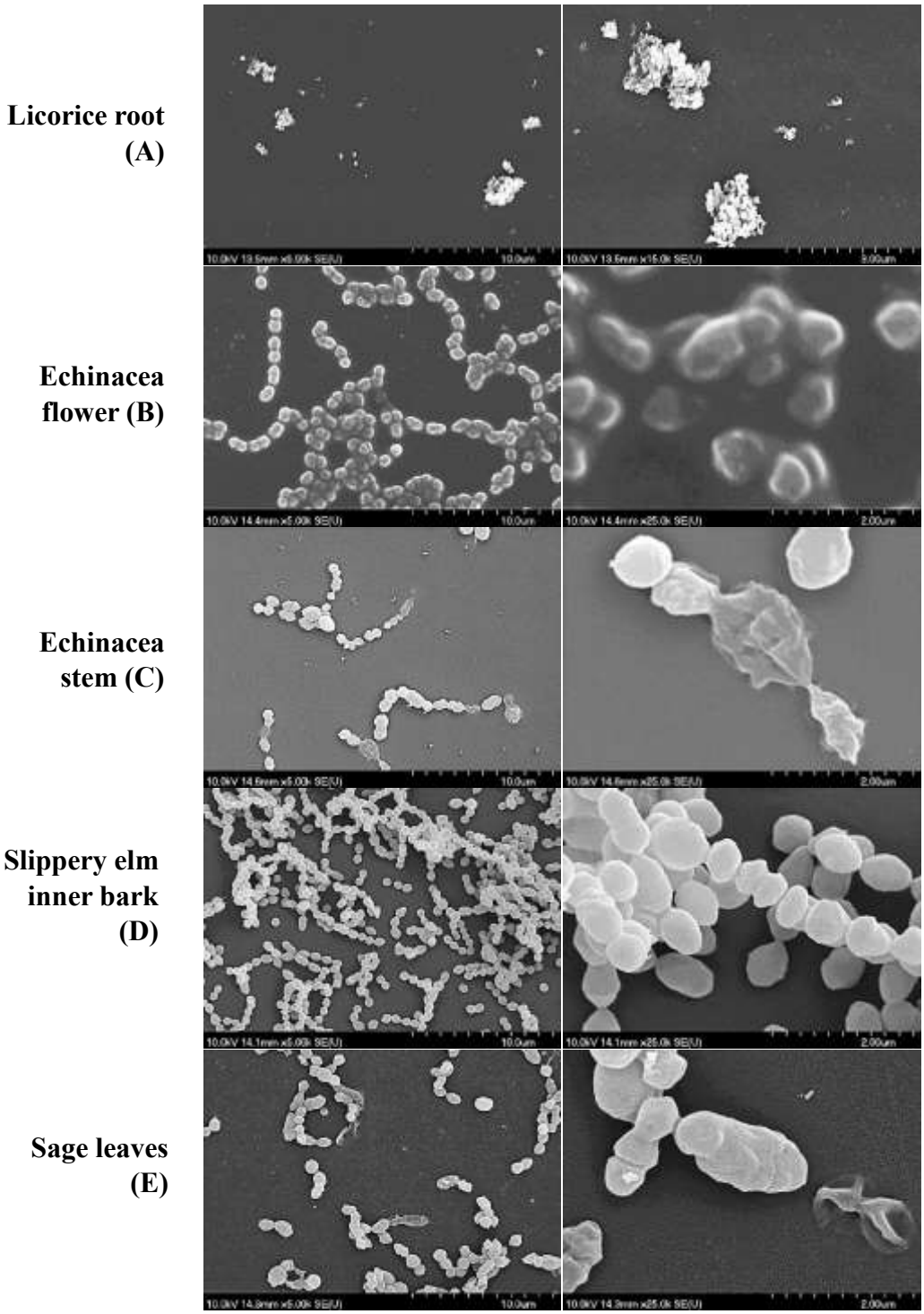

Figure 3.6: Scanning electron micrographs of *Streptococcus pyogenes* biofilms formed on 96 well plate well surface treated with ethanol extracts.

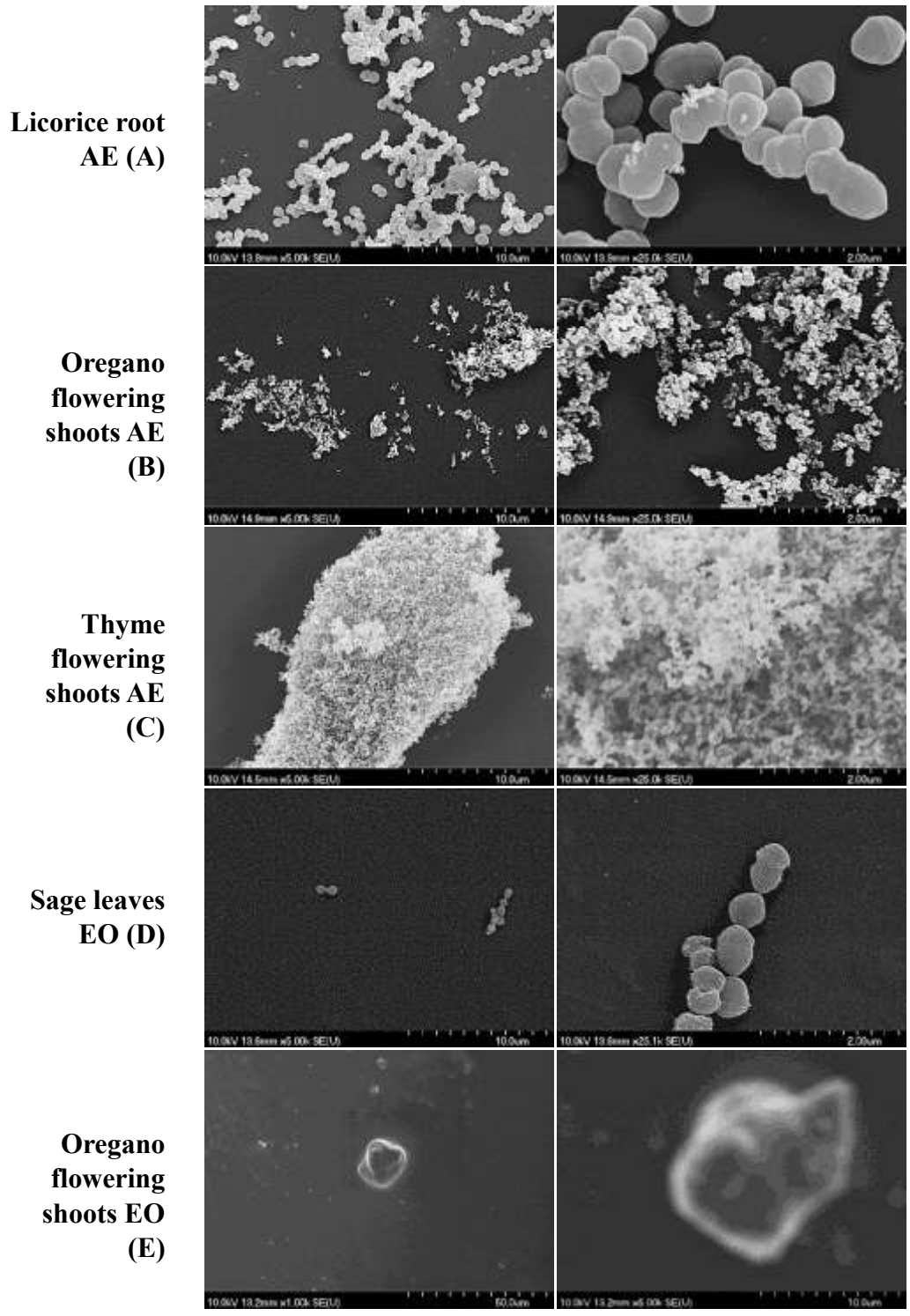

Figure 3.7. Scanning electron micrographs of *Streptococcus pyogenes* biofilms formed on 96 well plate well surface treated with aqueous extracts (AE) and essential oils (EO).

3.5. DISCUSSION

Plants consist of a wide range of phytochemicals, such as flavonoids, terpenoids, alkaloids, and tannins, which show various biological activities and are adapted to be defensive mechanisms (Cowan, 1999). A large number of traditional medicinal plants, all over the world, have been assessed for their potential applications in the treatment of streptococcal diseases. For example, a previous research investigated the anti-bacterial activities of 39 plants from traditional Australian aboriginal medicine against several Gram-positive bacteria, including *S. pyogenes* (Palombo and Semple, 2001). Another study showed antibacterial activities of native Californian medicinal plant extracts against *S. pyogenes* (Carranza et al., 2015). Some studies have investigated the activity of plant extracts and phytochemicals to inhibit the planktonic growth of *S. pyogenes*, while others have focused on the ability of the extracts to inhibit the formation of biofilms. The present study evaluated the inhibition of planktonic growth and biofilm formation of *S. pyogenes* by EE, AE and EOs of medicinal plants known to traditional healers in Canada, for use against streptococcus pharyngitis infection.

Anti-bacterial effect of aqueous extracts

In the present study, *in vitro* anti-bacterial efficacy of the fourteen extracts was assessed on the basis of MIC and MBC values. Among the AEs tested, licorice root was the most efficacious extracts which showed the lowest MIC value of 1560 µg/mL and MBC value of 6250 µg/mL. Thyme and oregano flowering shoots AEs as well as barberry roots AE also showed significantly higher anti-bacterial effect against *S. pyogenes*. Other than licorice root AE, three of these extracts shared the similar MIC and MBC values where 3125 µg/mL and 6250 µg/mL, respectively. However, time to kill analysis was conducted

only for licorice, thyme and oregano extracts. Although thyme and oregano extracts showed only a time dependent bacteriostatic effect, interesting bactericidal effect was observed within 12 hr of exposure of 4 × MIC (MBC) licorice root (data not shown). The existence of anti-microbial activity in a particular parts of a plant species may be due to the presence of specific phytochemical(s) such as flavonoids, alkaloids, steroids and/or saponins (Cowan, 1999; Nimri et al., 1999). Different species of thyme and oregano are known to possess some antimicrobial activities (Fournomiti et al., 2015). Both of plant leaves or young shoots are used in various food preparations as flavour enhancers as well as in herbal remedies. Although the modes of actions of the extracts are not clearly identified, it may be due to the major bioactive compounds, including thymol, terpenes, eugenol, flavones, glycosides of phenolic monoterpenoids and aliphatic alcohols among other elements (Fournomiti et al., 2015; Nzeako et al., 2006).

Licorice plant exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria. Most of antimicrobial effects of licorice roots as well as leaves has reported due to isoflavonoid components particularly hispaglabridin and B 4'-O-methylglabridin, glabridin, glycyrrhizin, glabriol and 3-hydroxyglabrol (Fu et al., 2013). Furthermore, effect of licorice roots against several oral pathogens can be attributed to several mechanisms, for example, glycyrrhizin and glycyrrhizic acid have been displayed inhibition of bacterial growth (Sedighinia et al., 2012). However, some studies reported side effects with licorice supplementation, mainly elevated blood pressure (Zadeh et al., 2014).

The relative antibacterial activities of AEs based on the MICs and MBCs were lower than EEs. However, water-based extraction process is acceptable for food

applications and allows incorporation of phytochemicals in natural health products without a concern of potential toxic solvent residuals (Wang and Weller, 2006). Therefore, it is possible that licorice, thyme or oregano extracts could be used to incorporate into products such as dehydrated honey lozenges in the management of streptococcal pharyngitis.

Anti-bacterial effect of ethanol extracts

Out of the fourteen EEs tested, licorice root, sage leaves, echinacea flower, echinacea stem and slippery elm inner bark extracts exhibited a remarkable inhibition activities against planktonic growth of *S. pyogenes*. Plant bioactive constituents in the efficacious EEs of the present study may consists of polar compounds such as the phenolic compounds which may be the reason for exhibited activity against *S. pyogenes* strains. A previous study was observed a relationship between anti-microbial activity and the phytochemicals in the extracts. Ethanol extract of *Rhodomyrtus tomentosa* (Aiton) Hassk (Rose Myrtle) leaves showed a strong antibacterial activity against 47 clinical isolates of *S. pyogenes* with MIC values of 3.9 - 62.5 μg/mL and MBC values of 3.9 - 62.5 μg/mL (Limsuwan et al., 2012). Similarly, "rhodomyrtone", an isolated active compound from *R. tomentosa,* also revealed promising antibacterial activity against 14 isolates of *S. pyogenes,* with 0.39 - 1.56 μg /mL of both MIC and MBC values (Limsuwan et al., 2009).

These five EEs (licorice roots, sage leaves, slippery elm bark, echinacea flower and stem) showed significantly low MIC and MBC values, however, the values were similar in all five as 62.5 μg/mL and 125 μg/mL, respectively. Therefore, time to kill analysis results are useful when determining the most efficacious extracts. Licorice root EE and sage leaves were the most efficacious bactericidal agents among EEs, which kill 99.99% of the initial bacterial load within 3 hr exposure to the 2 × MIC. Areal parts of the Echinacea species,

such as *E. angustifolia, E. pallida* and *E. purpurea* are mostly used to treat respiratory infections (Kumar and Ramaiah, 2011; Sharma et al., 2010). However, in the present study antimicrobial activity of EEs was assessed using different plant parts (stem, flowers and leaves) of *E. purpurea* against *S. pyogenes.* Only stem and flower EEs showed significant effect with MIC value of 62.5 µg/mL whereas leaf EE was not effective. Furthermore, EE of Echinacea flowers exhibited bactericidal effect against S. *pyogenes* (ATCC 19615) within 5 hr of exposure of 2 × MIC where as it acquired 9 hr to possess the similar bactericidal effect in stem EE.

Studies have reported on the chemical nature of the phytochemicals of some of the species used in the present study or species belonging to the same genera of them (Fournomiti et al., 2015; Kumar and Ramaiah, 2011; Nitalikar et al., 2010; Sfeir et al., 2013). However, future investigations should be continued to identify the phytochemical profiles and concentrations of the five most efficacious species used in present study. However, bioactive compositions and concentrations in different plant species could vary due to several reasons, such as extraction method, maturity of the plant, the part of the plant chosen for examination, time of the harvesting, cultivation conditions and environmental conditions (Burt, 2004; Figueiredo et al., 2008). In this study, many plant parts were collected during August to September, 2014, which were grown in Nova Scotia under similar environmental conditions.

The chemical structure and concentration of the individual antimicrobial active components of the efficacious EEs is important to their mode of action. However, it is expected that the antibacterial activities of licorice, sage and echinacea flower EEs may attributable to several mechanisms due to number of phytochemicals reported in previous

work. The active compounds of the sage EEs such as, 1, 8-cineole, p-cymene, camphor, α-thujone, β-pinene, trans-caryophyllene, β-thujone, and naphthalene may have contributed to the anti-bacterial activity (Fournomiti et al., 2015). These compounds are reported to possess many modes of action, such as disturbing the cytoplasmic membrane integrity of bacteria, affecting to the electron transport chain, changing the pH homeostasis, disrupting to the proton motive force and coagulation of cell contents(Abu-Darwish et al., 2013; Burt, 2004; Fournomiti et al., 2015). Therefore, one or combined actions of phytochemicals bacterial growth inhibition and death.

Anti-bacterial effect of essential oils

EOs are natural volatile and odorous organic compounds and products of plant secondary metabolism, which are widely used in traditional medicine (Kalemba and Kunicka, 2003). Numerous studies have investigated various EOs and their constituents for antimicrobial properties against different bacteria. Gram-positive bacteria are known to be more susceptible to EOs or their derivatives than Gram-negative bacteria (Burt, 2004; Cristani et al., 2007). Among four EOs tested in the present study, oregano flowering shoots and sage leaf EO exhibited significant activity against *S. pyogenes*. However, EOs of clove and ginger also showed considerable low MIC values, compared with all AEs and some EEs. Oregano EO showed immediate bactericidal effect within 5 min of exposure at MBC (2 × MIC). A potential explanation for these bactericidal effects of oregano EO, may be the high content of carvacrol (91.63±0.0eb1%) and the presence of other phenolic compounds, such as p-cymene, myrcene and γ-terpinene. Several other studies supported the present results of phytochemical % and their anti-bacterial efficacy. Carvacrol, thymol, p-cymene, myrcene and γ-terpinene, derived from another species of oregano (*Origanum*

compactum), showed the time-dependent bactericidal kinetics within 15 min and 5 min at concentrations < 2% (v⁄v) against *S. aureus* and *S. pyogenes,* respectively (Mayaud et al., 2008). It was reported that EOs from Italian species of thyme and oregano shown great antimicrobial activity with MICs ranging from 256 - 512 μg/mL, which is compatible with the present study. Remarkably, carvacrol, a major component identified in both EOs, has been demonstrated to have antibacterial effect, either alone, or in combination with erythromycin against erythromycin-resistant GAS (Magi et al., 2015). In the present study, EO of sage leaf was also bactericidal within 4 hr at 2 × MIC (500 μg/ mL) against ATCC 19615, whereas at MIC exhibited only time dependent bacteriostatic effect. Sage leaf EO contains mainly α-thujone (26.46±0.02%) and also other active polyphenols, such as trans-caryophyllene, myrcene and carvacrol. There are additional five compounds which have been reported to present in the EO of sage leaf according to the GC analysis (Figure 3.1). Further analysis is required to identify those unknown compounds. Hence, they may also contribute to the antibacterial effect of sage leaf EO. Efficacy of eugenol, β-pinene and α-pinene in inhibiting the growth of potential infections caused by gram-positive bacteria, including *S. pyogenes,* were studied previously (Leite et al., 2007). The strongest inhibitory effect was exhibited by β-pinene, eugenol and α-pinene which showed bacteriostatic effect after 2, 2 and 4 hr of introduction, respectively. Furthermore, the initial bacterial load was eliminated after 8 hr of exposure to β-pinene and eugenol. Similarly, it was reported in a previous study that EO of *Monarda punctate* exhibited bactericidal effect within 6 hr and killed 99.99% bacteria in the inoculum of *S. pyogenes* within 12 hr (Li et al., 2014).

Although the previous studies have attempted to explain the actions of phytochemical-rich EOs, the antibacterial mechanisms of action are still unclear. However,

antibacterial mechanisms of some specific constituents of EOs are reported. For example, it was stated that microorganisms exposed to carvacrol derived from some EOs, such as oregano and thyme, exhibited morphological modifications, indicating a change in cell surface structure of bacteria. Moreover, microscopic observations reported that the both length and diameter of bacteria was decreased after contact with carvacrol (La Storia et al., 2011). This is in agreement with the present study, where administration of either sage or oregano EOs, which are rich in carvacrol, resulted in morphological changes to cocci length and diameter of *S. pyogenes,* even at sub inhibitory concentrations. It may be due to the lipophilic ability/ hydrophobicity of the EO's and its components which target the bacterial membranes (Burt, 2004). An important characteristic of EOs permits them to partition in the lipids of the membranes and enhance the permeability, causing damage, which leads to cell content leakage (Burt, 2004; Sfeir et al., 2013). Bacteria cells may tolerate a certain amount of cell content leakage; however extensive loss of critical ions and molecules leads to cell death (Labbe and Saleh, 2008). In the presence of EO of tea tree, there was stimulated autolysis in the exponential phase of *Escherichia coli* cells due to the loss of electron dense material and coagulation of cell cytoplasm (Gustafson et al., 1998). In addition, it was reported that oregano EO caused leakage of phosphate ions from *Staphylococcus aureus,* leading to cell death (Lambert et al., 2001). Destruction of cell membrane integrity after 1 hr of carvacrol treatment against microbial cells was demonstrated in a previous study, based on the morphological and cell surface roughness analysis. Therefore, it was confirmed that cell wall membranes are a major target of compounds, such as carvacrol (La Storia et al., 2011). On the other hand, phenolic alcohols or aldehydes interfere with membrane-integrated or associated enzyme proteins, stopping

their production and activity (Burt, 2004; Kalemba and Kunicka, 2003; Sfeir et al., 2013). It has also been argued that the components of EOs can also interfere with the electron transport chain from bacterial mitochondria, as well as mammalian mitochondria, and disturb energy production (Bakkali et al., 2008; Kalemba and Kunicka, 2003). Therefore, more studies are required to identify the appropriate dose of oils, or potentially active compounds, for safe human consumption. However, based on these previous studies, it may be hypothesized that there is a similar mechanism of action on the *S. pyogenes'* cell wall responsible for remarkable activity against *S. pyogenes* and SEM visualization results, even though the above authors only tested a Gram-negative microorganism.

Penicillin and penicillin derivatives are considered as the drugs of first choice for streptococcal infections. However, all of these extracts exhibited significantly weaker inhibitory activity than penicillin G, which showed 0.0078 μg/mL of MIC and 0.0156 μg/mL of MBC. Results of Penicillin G were consistent with a previous study on MIC and MBC values, where it was reported that MIC range was 0.008 - 0.015 μg/mL and MBC range was 0.008 - 0.015 μg/mL (Sakata, 2013). The MIC and MBC for penicillin were determined in another study and both were 0.015 μg/mL with broth micro-dilution and the "Epsilometer test" method resulted in an MIC of 0.012 μg/mL (Stevens et al., 1998). Antibiotic susceptibility of *S. pyogenes* strains isolated from throat cultures of children with tonsillo-pharyngitis were studied and it was reported that MIC range was 0.0004 - 0.03 μg/mL (Ciftici et al., 2003). Similarly, penicillin G MBC / MIC ratios of *S. pyogenes* strains were explored in the same study and it was reported that 246 strains among 263 total strains studied showed MBC / MIC of 2, whereas only 15 strains showed MBC / MIC

of 1. This present study is in agreement with those findings which demonstrated a ratio of 2.

Anti-biofilm formation ability of plant extracts

More importantly, out of thirty-two extracts, ten selected extracts (licorice root EE, sage EE, echinacea stem EE, echinacea flower EE, slippery elm inner bark EE, sage leaves EO, oregano EO, licorice root AE, thyme AE and oregano AE) demonstrated anti-biofilm formation activity in *S. pyogenes*. Although penicillin possessed the most suppressive activity, with the MBIC values of 0.0039 - 0.0078 µg/mL ($p > 0.05$), it was herbal plant extracts which also showed remarkable results, ranging from 31.5 - 6250 µg/mL. Several previous studies have been reported in a number of medicinal plants which prevent the formation of biofilm in different bacteria, including *S. pyogenes* (Darsini et al., 2015; Limsuwan and Voravuthikunchai, 2008; Mutalib et al., 2015). A recent study evaluated *in vitro* antibacterial activity of *Lagenaria siceraria* (bottle gourd/long melon) dried fruit using micro dilution assay and biofilm formation activity using crystal violet assay (Mutalib et al., 2015). Development of biofilm is a key mechanism involved in *S. pyogenes* virulence during pharyngitis infections which assures superior survival and protection from host defensive mechanisms, antibiotics and other environmental fluctuations (Ogawa et al., 2011; Post et al., 2004). It has been suggested that the treatment failure of antibiotic in the eradication of *S. pyogenes* may be due to the presence of bacterial biofilm, also known as communities, attached to host cell surfaces and covered by EPS (Conley et al., 2003; Hall-Stoodley et al., 2004; Manetti et al., 2007). Numerous studies have shown that MBIC of some herbal extracts/antibiotics can affect growth, morphology, surface properties, pathogenicity and biofilm formation of bacteria (Andersson and Hughes, 2014; Fonseca

and Sousa, 2007; Kaplan, 2011). MBIC values represented the metabolic activity of biofilm biomass and in the present study, were mostly 2 × MIC and MIC. Results indicated that the plant extracts possibly interfered with *S. pyogenes* biofilm formation at some unknown stage. Observation of the cell density and morphology of *S. pyogenes* biofilms by SEM further confirmed that *S. pyogenes* did not form thick biofilm layers when treated with MBIC of the extracts. (Fuqua et al., 1994) suggested that phytochemicals of herbal extracts suppress the expression of genes responsible for bacterial pathogenesis by interfering with the formation of biofilm. However, plant extracts comprise a large number of components and it is expected that their mode of action involves several targets in the *S. pyogenes* cells, rather than a single mechanism (Burt, 2004).

In the present study, a single effect of plant extracts on anti-biofilm activity was considered. However, the synergistic effect of several plant extracts against biofilm formation have been reported in a recent study (Dineshbabu et al., 2015). Accordingly, it could be hypothesized that the combined extract exhibited significantly higher anti-biofilm activity than the respective individual plant extracts. The biological significance of compounds, such as carvacrol, p-cymene and trans-caryophyllene in oregano EO; alpha-thujone, fenchyl alcohol in sage EO and eugenol in clove EO, were reported from GC-FID analysis for EOs (Burt, 2004). These compounds were reported to have anti-biofilm formation ability individually which may be attributed to their enhanced and combined efficacy, leading to a significant synergistic activity against *S. pyogenes.* The increase in biofilm formation was dose-dependent in penicillin G for ATCC 49399 and MIC (0.0078 μg/mL) for ATCC 19615 and clinical isolate had the strongest effects on biofilm formation, and were considered as their MBIC. No significant differences were shown in biofilm

formation of lower concentrations among these tested concentrations, other than sub MIBC, when compared to the untreated controls. Research has shown that solvent extracts (chloroform, ethyl acetate, methanol and petroleum ether) from the grains of *Piper longum* and *P. nigrum* exhibited biofilm inhibition against clinical isolates of *S. pyogenes* and a reference strain of *S. pyogenes* ATCC 1924. The anti-biofilm potential of methanol and ethyl acetate extracts were reported to be significant at 2 mg/mL concentration, for both plants. As well, these biofilm inhibitory effects were confirmed by microscopic visualization (Darsini et al., 2015).

Although no detailed previous studies have been reported, some have examined cell morphology after treating plant based extracts or EOs, by other microscopic techniques. This present study performed SEM to describe morphological changes, such as structural alterations, after EE, AE and EOs oil exposure, of *S. pyogenes* strains. Interestingly, a study has reported that the proteome of *Salmonella* cells treated with thymol showed the influence of this EO on some proteins involved in the cell division mechanism (La Storia et al., 2011). Furthermore, it could be hypothesized that compounds, such as carvacrol and thymol, move through the peptidoglycan layer and then act on the cytoplasmic membrane of *S. pyogenes*. The structural changes in the membrane, such as fluidity alteration, could lead to a slight modification in the external surface of the Gram-positive cell wall. The reduction in cell size, length and diameter was observed for all tested extracts, in response to their sub-inhibitory concentration treatment. This could be attributed to leakage of cytosolic fluids outside the cells.

3.6. CONCLUSION

In the last few decades, indigenous medicines have been used as new therapeutic agents for the prevention and treatment of streptococcus infections. This study reports the inhibitory effect on the growth and formation of biofilm of *S. pyogenes* by phytochemical extracts of selected Canadian traditional medicine, emphasizing its importance as an alternative treatment. It is also noticeable to observe the antibacterial properties of the oregano flowering shoot EO, sage leaf EO, sage leaf EE, echinacea flower EE and licorice root EE and their possible use in antimicrobial therapy against *S. pyogenes*. However, further studies are required to elucidate the major phytochemicals of the efficacious extracts and their mechanisms of action against *S. pyogenes*.

CHAPTER 4. ANTI-INFLAMMATORY EFFICAY OF PHYTOCHEMICAL-RICH EXTRACTS PREPARED FROM SELECTED MEDICINAL PLANTS AGAINST LIPOTEICHOIC ACID- AND PEPTIDOGLEYCAN-INDUCED INFLAMMATION IN HUMAN TONSIL EPITHELIAL CELLS IN VITRO

4.1. ABSTRACT

Streptococcal pharyngitis is one of the most common upper respiratory tract diseases, which is mainly caused by *Streptococcus pyogenes*. Interest has arisen for the use of anti-inflammatory herbal remedies as potential alternatives for synthetic medicine. This study was conducted to evaluate the anti-inflammatory effects of phytochemical-rich extracts, which are prepared from twelve selected herbal plants used in Canadian traditional medicine. The extracts were tested on lipoteichoic acid (LTA) - and peptidoglycan (PGN)-stimulated inflammation in human tonsil epithelial cells (HTonEpiC). These extracts were not cytotoxic at, or below, the concentrations of 5 μg/mL as measured by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Inflammation of HTonEpiC cells was triggered by a mixture of LTA (10 μg/mL) and PGN (10 μg/mL) for 4 hr, followed by the incubation with selected ethanol extracts (EE) or aqueous extracts (AE) in concentrations of 0.5, 1 and 5 μg/mL for 20 hr. All the extracts significantly suppressed the expression of proteins of pro-inflammatory cytokines, such as interleukin-8 (IL-8), human beta defensin-2 (hBD-2), epithelial-derived neutrophil activating protein-78 (ENA-78) and granulocyte chemotactic protein-2 (GCP-2). The EEs of danshen root, ginger, clove, echinacea flower and AEs of clove, ginger and echinacea flower were discovered to be the most effective. These efficacious extracts and/or their active phytochemicals are suggested to be used as novel

therapeutic agents against streptococcal pharyngitis. Further studies are required to understand the anti-inflammatory mechanisms of these phytochemical-rich extracts.

Keywords: Streptococcal pharyngitis, inflammation, plant extracts, phytochemicals, tonsil epithelial cells

4.2. INTRODUCTION

Streptococcal pharyngitis, also referred to as strep throat, is an extremely common infection worldwide, accounting for several million cases each year. It accounted for 1.3% of outpatient visits to health care providers in 2006, resulting in an estimated national economic burden of USD 224 million to USD 539 million (Pfoh et al., 2008) in the United States alone. *S. pyogenes*, a group A beta hemolytic streptococcus (GAS), is the main bacterial etiology responsible for 15% to 36% of acute pharyngitis infections in children aged from 5 to 15 (Carapetis et al., 2005; Martin and Green, 2006), causing inflammation of the pharynx, surrounding structures and lymphatic tissue (Dong et al., 2014).

The inflammatory response of epithelial cells of the upper respiratory tract plays an important role in host defense, acting as the first lines of defense recruited to combat GAS (Rock et al., 2010). Inflammation is a cascade of biochemical responses of a host against external stimuli. Once triggered by GAS, the inflammatory response can develop very rapidly, involving the local vascular system, the immune system, and damaged tonsil epithelial cells (Rock et al., 2010). Toll-like receptors (TLRs) in host cells are involved in the recognition of cell wall compounds of *S. pyogenes,* especially PGN and LTA, and certain membrane proteins and are responsible for host-pathogen interactions (Bisno et al., 2003). Cellular recognition, trigged by bacterial virulence factors, has been shown to induce damaged tonsil epithelial cells, blood plasma and white blood cells to *produce*

chemical mediators of inflammation, such as cytokine, chemokine, prostaglandin (PG) (Ricciotti and FitzGerald, 2011; White, 1999), resulting in the coordinated activation of transcription factor called nuclear factor-kappa B (NFκB) (Kumar et al., 2009).

Chemical mediators of inflammation contribute to the development of the cardinal signs and symptoms of acute inflammation, such as redness, heat, swelling and pain and in severe cases, loss of function (Ricciotti and FitzGerald, 2011). The incubation period of streptococcal pharyngitis is 1 to 4 days, leading to a self-limited, localized inflammation of the tonsillopharynx, lasting 3 to 5 days (DuBose, 2002). Sudden onset of sore throat, fever, headache, pain on swallowing, tonsillar exudates, and anterior cervical adenopathy, abdominal pain, nausea and vomiting are signs and symptoms of GAS pharyngitis (Choby, 2009; DuBose, 2002).

Penicillin remains the first choice of drug for treating pharyngeal patients (Snelling and Carapetis, 2010). Prompt antibiotic treatment within 24 hr of onset of signs and symptoms is recommended, with the aim to eradicate GAS, gradually reduce clinical signs and symptoms, prevent the risk of bacterial transmission and the development of complications (Chiappini et al., 2011). However, patients are more concerned about rapid symptomatic relief from swelling and throat pain. Swelling is due to fluid accumulation outside the blood vessels and pain is associated with alteration of tissues by swelling, local tissue destruction and irritation of sensory nerve receptors (Pfoh et al., 2008; Rock et al., 2010). Therefore, analgesic drugs, throat lozenges, throat sprays, vapor rubs, and home remedies, such as gargling with warm water mixed with honey or salt, sucking garlic, licorice root or marshmallow root, sniffling over a bowl of steaming water with essential oil and drinking warm liquids or herbal teas are used as common supportive treatments to

manage pain and swelling (Pfoh et al., 2008; Schams and Goldman, 2012).

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, ibuprofen, nimesulide, piroxicam and ketoprofen are widely recommended by physicians for the management of inflammatory conditions in soft tissues of the upper respiratory tract (Cremonesi and Cavalieri, 2015). NSAIDs inhibit the cyclooxygenase-2 (COX-2) production, which is an enzyme responsible for the synthesis of pro-inflammatory PGs (Ricciotti and FitzGerald, 2011). A recent study has shown significant reduction in pain in GAS-positive patients who take antibiotics, as well as steroids as adjuvant treatment. Time taken to relieve from the throat pain was quicker in steroid-treated patients, who have shown pain relieve beginning at 6.3 hr compared to that of 12.4 hr in the placebo treated patients group (Schams and Goldman, 2012). Another study has also shown the similar finding in children with moderate to severe GAS pharyngitis where a single dose of oral dexamethasone has significantly reduced the pain (Olympia et al., 2005). However, physicians and parents are sometimes reluctant to give steroids to children, due to possible long term side effects (Schams and Goldman, 2012). Although these side effects are not significantly common, some patients are allergic to NSAIDs and may develop shortness of breath after intake (Kim et al., 2013).

Natural products are potential alternatives for current synthetic medicines, for inflammatory disorders (Fawole et al., 2010). Medicinal plants are believed to be an important source for the discovery of potential anti-inflammatory phytochemicals. Numerous previous studies have reported the chemical composition of various plant extracts and their potential anti-inflammatory activity (Albu et al., 2004; Chaieb et al., 2007; Fachini-Queiroz et al., 2012; Hamalainen et al., 2007; Khouya et al., 2015; Rani et

al., 2012; Teixeira et al., 2013). Based on the reported literature and geographical availability the following herbs were selected for this study: *Thymus vulgaris* (thyme) flowering shoots, *Origanum vulgarem* (oregano) flowering shoots, *Salvia officinalis* (Canadian sage) leaves, *Salvia miltiorrhiza* (Chineese sage/danshen) roots, *Syzygium aromaticum* (clove) flower bud, *Berberis vulgaris* (barberry) roo*t, Glycyrrhiza glabra* (licorice) root, *Zingiber officinale* (ginger) rhizome, *Ulmus rubra* (slippery elm) inner bark*, Pelargonium graveolens* (geranimum) leaves*, Olea europeus* (olive) leaves, and *Echinacea purpurea* (ehinacea) flower, stem as well as leaves were selected for the present study. Most of them have been used in traditional medicines for centuries and are known to have different therapeutic effects, such as anti-microbial (Fournomiti et al., 2015; Ghannadi et al., 2012; Nitalikar et al., 2010), anti-inflammatory (Fawole et al., 2010), anti-oxidant (Asbaghian et al., 2011; Carlsen et al., 2010), anti-coagulant (Fawole et al., 2010; Policegoudra et al., 2010) and anti-cancer effects (Li-Weber, 2009; Policegoudra et al., 2010).

The objective of this study was to evaluate the anti-inflammatory effects of phytochemical-rich AEs and EEs prepared from the above mentioned medicinal plants, using an HTonEpiC cells induced by a mixture of LTA and PGN. Cell viability, production of pro-inflammatory biomarkers and cell morphological changes were determined to identify efficacious extracts, with the future aim of developing an herbal extract incorporated throat lozenge or a herbal natural health product for streptococcal pharyngitis.

4.3. MATERIALS AND METHODS

4.3.1. Materials

4.3.1.1. Chemicals and reagents

Diclofenac sodium salt, Dulbecco's phosphate buffered saline (DPBS), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), phenazine methosulfate (PMS), poly-L-lysine (PLL) hydrobromide and LTA were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). PGN was obtained from Cedarlane Labs (Burlington, ON, Canada). Human tonsil epithelial cells (HTonEpiC), tonsil epithelial cell medium (TEpiCM), tonsil epithelium cell growth supplement (TEpiCGS), penicillin/streptomycin solution (P/S solution), trypsin/ ethylenediaminetetraacetic acid (EDTA), trypsin neutralization solution (TNS) and were purchased from ScienCell Research Laboratory (San Diego, CA, USA). Fetal bovine serum (FBS) was bought from American Type Culture Collection (ATCC) (Manassas, VA, USA). IL-8 ELISA kit was purchased from BD Biosciences (Mississauga, ON, Canada); human ENA-78 and GCP-2 enzyme-linked immune sorbent assay (ELISA) kits from Ray Biotech, Inc. (Norcross, GA, USA); hBD-2 ELISA kit from PromoCell GmbH (Sickingenstrabe, Heidelberg, Germany).

4.3.1.2. Instruments

A class II-type A2 biological safety cabinet (Model LR2-452, Eso Technologies Inc. Hartboro, PA, USA), CO_2 incubator (Model 3074, VWR International, West Chester,PA, USA), inverted microscope (ECLIPSE TS 100/TS 100-F, Nikon Instruments Inc., Melville, NY, USA) coupled with a Lumenara Infinity camera (1-2 USB, 2.9 Megapixel) containing capture and analyzing software (Infinity Analyze, Lumenara Corporation,

Ottawa, ON, Canada), haemocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA), micro-plate reader (Tecan Infinite® 200 PRO, Morriscille, USA), water bath (ISOTEMP^{TM} Digital-Control Water Baths: Model 205, Fisher Scientific Company, Ottawa, ON, Canada); rotavapor (R-200, Buchi, Flawil, Switzerland), centrifuge (Sorvail Legend Micro 21 R, Thermo Scientific Thermo Fisher Scientific Inc., Waltham, MA, USA).

4.3.2. Human tonsil epithelial cell culture

HTonEpiCs cells were cultured and maintained according to the ScienCell Research Laboratory's guidelines. Briefly, PLL coated flask (2 μg/cm^2 T-75 flask) was prepared before culturing the cells. Ten milliliters of sterile water was added into the T-75 flask, followed by adding 15 μL of PLL stock solution (10 mg/mL). After overnight incubation at 35 ± 2 ºC in a humidified incubator with an atmosphere of 95% air and 5% CO_2, the T-75 flask was rinsed with sterile water twice and replaced with 20 mL of complete growth medium (CGM) (500 mL of TEpiCM mixed with 5 mL of TEpiCGS and 5 mL of P/S solution). The cryopreserved HTonEpiCs were completely thawed in a 35 ± 2 ºC water bath by rotating the vial and promptly removing it from the water bath. Then, re-suspension of cells was carried out by dispensing the contents of the vial into the equilibrated, PLL coated culture flask. Then, cells were allowed to evenly distribute by gently rocking the flask and was incubated for 16-24 hr without disturbing for attachments. Next day, after cell establishment, the medium was refreshed by new 15-20 mL CGM to remove residual dimethyl sulfoxide (DMSO) and unattached cells. Every three days, the medium was changed with fresh CGM until the culture was approximately 70% confluent (Figure 4.2) and then every other day thereafter.

Sub-culturing

When the culture reached approximately of 90% confluence (Figure 4.2), they were used for sub-culturing for the experiments. First, medium was aspirated out and discarded, followed by rinsing the cell layer with DPBS (Ca^{2+} and Mg^{2+} free). Then, 5 mL of DPBS and 5 mL of trypsin/EDTA solution were added into the flask and was incubated at 35 ± 2 ºC for 3-5 min. The detachment of cells was monitored under an inverted microscope for any morphological changes. Then 5 mL of TNS was added to stop the reaction of trypsinization and the solution was subsequently transferred to the 50 mL centrifuge tube, supplemented with 5 mL of FBS. The empty flask was then continuously incubated at 35 ± 2 ºC for 1 to 3 min and the side of the flask was gently tapped to dislodge cells. Afterwards, residual cells were harvested by rinsing additional 5 mL of TNS and detached cells were transferred from the flask to 50 mL centrifuge tube. Flask was examined again under the inverted microscope for a successful harvest by looking at the number of cells remaining (less than 5%). The harvested cells were then centrifuged at 1,000 rpm for 5 min. Following this procedure, the cells were re-suspended in 4-5 mL CGM. Trypan blue staining was performed to count the cell number. Briefly, 40 µL of cells were diluted 10 times with Trypton blue and 10 µL of the diluted cells were mounted on a haemocytometer, and cells were counted under the inverted microscope. Then, cells were seeded into a 96-well plate at the required density.

4.3.3. Cell viability assay

Cells were cultured in 96-well plates pre-coated with PLL at a density of 6,000 cells/well and treated with EEs and water extracts of selected herbal plants of different concentrations in 0.5, 1 and 5 µg/mL treatments, solvent control (DMSO), or PBS buffer. The final

concentration of solvent in culture medium did not exceed 0.5% (v/v). A combination of LTA and PGN, at a concentration of 10 μg/mL, was used as a bacterial antigen control. Nemesulide, a drug recommended for inflammation (0.5, 1 and 5 μg/mL), was tested as a positive control. Plates were placed in the incubator with 5% CO_2 at 35 ± 2 ºC for 24 hr. An assay of MTS was employed to determine cell viability. After 24 hr incubation, the cells were refreshed by adding 100 μL of fresh CGM, followed by 2 1/2 hr incubation in the presence of 20 μL of MTS mix (MTS: PMS=20:1). The absorbance was measured at 490 nm by using a micro-plate reader. Cell viability was calculated using the following equation.

$$\boldsymbol{Cell\ Viability}\ (\%) = \frac{\boldsymbol{Absorbance\ of\ the\ treated\ wells - blank}}{\boldsymbol{Absorbance\ of\ the\ control\ wells - Absorbance\ of\ the\ blank}} \times \mathbf{100}\%$$

Where, the treated wells contained cells incubated with test compounds, the control wells contained cells without compound treatments, and the blank wells contained CGM only.

4.3.4 Treatment of plant extracts

The cells were seeded at a density of 35,000 cells/well in 6-well plate which were percolated with PLL and incubated overnight at 35 ± 2 ºC for 20-22 hr. HTonEpiC cells were treated with 10 μg/mL of LTA and PGN mixture in each well, followed by 4 hr incubation at 35 ± 2 ºC to trigger inflammation. Then, ethanol and water extracts at concentrations of 1 and 5 μg/mL (selected based on the cell viability results) were treated and incubated at 35 ± 2 ºC for 24 hr. Wells containing 4 hr and 24 hr LTA and PGN stimulated as controls for inflammation, nemesulide as the positive control and 0.05% of DMSO in the absence of test extracts as a negative control, were used. Cell culture supernatants were collected and stored at -80 ºC for determination of pro-inflammatory biomarkers.

4.3.5. Cell morphological assessment

After the treatment with 1 and 5 μg/mL selected extracts, the cells were examined under an inverted microscope with 40 × magnification. The images were captured and saved using a Lumenara infinity camera, coupled with capture and analyzing software.

4.3.6. IL-8 assay

The concentration of pro-inflammatory chemokine IL-8 was measured by using a commercial ELISA kit. Detection was performed on anti-human IL-8 monoclonal antibody coated 96-well plates, provided by the supplier. First, 50 μL ELISA diluent was added into each well, and then standards and samples (100 μL) were pipetted into appropriate wells. Then plates were gently shocked for 5 s and were incubated for 2 hr at room temperature. The wells were decanted and washed with 300 μL wash buffer (diluting 20 × wash concentrate with deionized water) five times. Subsequently, 100 μL of detecting antibody was added into each well. The covered plates were incubated for 1 hr at room temperature, followed by seven rinsing steps with wash buffer. Then, 100 μL/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate reagent was pipetted and incubated for 30 min at room temperature in the dark. Then, 50 μL of Stop solution was added into each well and an absorbance reading at 450 nm was measured using a micro-plate reader (Tecan Infinite® 200 PRO, Morriscille, NC, USA). The concentration of IL-8 in each sample was expressed as pg/mL using the standard curve.

4.3.7. Human BD-2 assay

The content of hBD-2 in cell supernatants was determined by a hBD-2 ELISA kit. Anti-human BD-2 antibody coated plates were prepared using detection antibody and avidin-horseradish peroxidase (HRP) conjugate, according to the manufacturer's protocol. First,

the 96-well plates were coated with capture antibody and incubated overnight at room temperature with plate sealing. Then plates were washed four times with wash buffer. Then, samples and diluted standards, provided with the kit (100 µL), were immediately added into appropriate wells in triplicates and incubated for at least 2 hr at room temperature. The plates were washed four times with washing buffer, followed by adding detection antibody and incubated for 2 hr at room temperature. Then, avidin-HRP conjugate was pipetted into each well and incubated for 30 min. 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) liquid substrate was added into each well for color development. Then, plates were read at the absorbance of 405 nm.

4.3.8. ENA-78 assay

The human ENA-78 ELISA kit was used to detect the % secretion of ENA-78 protein in the cell culture supernatant. Pre-coated 96-well plates with specific antibody for human ENA-78 were used for this assay. After adding 100 µL standards and samples into appropriate wells, the plates were incubated overnight at 4 ºC after gentle shaking. The solution was then discarded and the plates were washed four times with wash solution. Then, 100 µL of biotinylated antibody was added into each well and incubated for 1 hr at room temperature with gentle shaking. After four washing steps, 100 µL of HRP-streptavidin solution (to provide enzyme activity) was added into each well. After an incubation period of 45 min at room temperature with gentle shaking, the plates were rinsed with wash buffer, followed by 100 µL of TMB substrate reagent was added into each well and incubated for 30 min at room temperature with gentle shaking, protected from light. The reaction was ended by adding 50 µL of the stop solution. Then, plates were read at the absorbance of 450 nm. The ENA-78 concentration was calculated using a standard curve

and the data is expressed as pg/mL.

4.3.9. GCP-2 assay

The human GCP-2 ELISA kit was used to measure the protein production of GCP-2. GCP-2 micro plate coated with anti-human GCP-2 was used. The ELISA procedure was similar to the description in 4.3.7.

4.3.10. Statistical analysis

Experimental designs for all the experiments were completely randomized design. Cell viability experiments were conducted in triplicate and independently three times whereas all the ELISA experiments were run in triplicate and independently twice. Results were expressed as mean ± standard deviation. Exponential regression analysis of data were performed using the Microsoft excel 2013 software (Microsoft Corporation, Redmond, WA, USA). One-way ANOVA analysis was performed by using Minitab 17.0 statistical software and statistical differences ($P < 0.05$) between means of pairs were resolved by using Tukey's tests.

4.4. RESULTS

4.4.1. Cytotoxic effect of selected phytochemical-rich extracts on human tonsil epithelial cells.

The cytotoxicity effects on human tonsil epithelial cells of 14 EEs and 13 of AEs from selected herbal plant parts were evaluated before the bioactivity study. MTS assay was performed after 24 hr of incubation of the cells with the extracts. The extracts did not significantly reduce cell viability when the concentrations were between 0.05 to 100 μg/mL in AEs (Table 4.1 and 4.2). However, the EEs of licorice root, sage and geranium showed toxic effects at higher concentrations (> 0.5 μg/mL). Therefore, for comparison purposes, concentrations of 0.5, 1, and 5 μL/mL were considered for final cytotoxicity analysis (Tables 4.1 and 4.2). Interestingly, except for geranium, oregano and echinacea stem, other AEs were shown to have considerable high cell viability up to 100 μg/mL, without demonstrating cytotoxicity to the HTonEpiCs (Table 4.2). Bacterial antigen mixture (LTA and PGN) had no cytotoxicity at the tested concentration of 0.1 μg/mL, whereas nemesulide showed low cytotoxicity to the cells at all the concentrations tested (0.1-100 μg/mL). The concentrations of 1 and 5 μg/mL of extracts were chosen to evoke inflammation without affecting the cell viability.

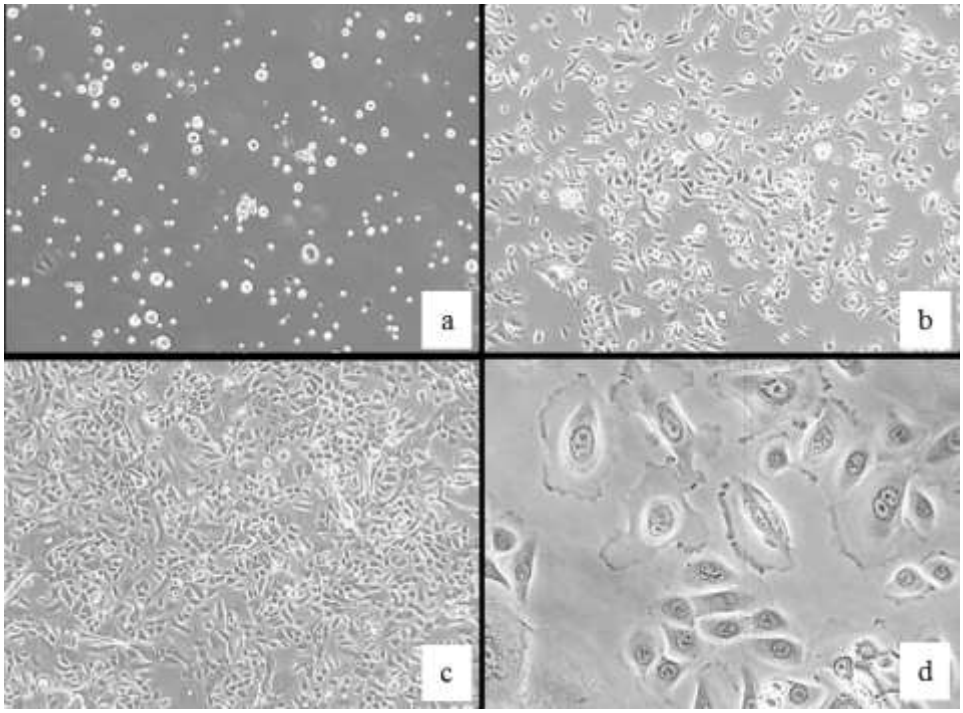

Figure 4.1: Morphologies of human tonsil epithelial cells in primary culture observed using phase-contrast microscopy. (a): before attachment (magnification, ×10); (b): undergoing subculture in poly-L-Lysine coated flask with tonsil epithelial growth medium with 70% confluence (magnification, ×10); (c): with 90% confluence (magnification, ×10) and (d): 90% confluence (magnification, ×40).

Table 4.1: Effect of ethanol extracts on percentage cell viability of human tonsil epithelial cells.

Test compounds	Cell viability (%)							
	0.1 µg/mL	0.5 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
PGN+LTA	96.8±1.1^{ab}	ND	ND	ND	ND	ND	ND	ND
Licorice R	99.0±1.6^{a}	99.1±1.6^{a}	98.0±1.7^{a}	98.4±1.7^{a}	97.1±1.7^{a}	32.6±1.3^{ef}	24.4±0.7^{f}	22.5±1.2^{fg}
Sage L	99.2±1.2^{a}	99.8±0.4^{a}	96.2±6.0^{a}	86.2±1.1^{c}	33.1±1.7^{a}	29.5±0.8^{ef}	24.4±0.9^{f}	25.3±1.8^{f}
Echinacea S	82.8±1.3^{c}	82.8±3.7^{c}	95.8±3.7^{a}	83.0±2.1^{c}	52.5±2.6^{d}	26.2±1.3^{ef}	20.9±0.6^{g}	20.4±1.0^{g}
Echinacea F	97.0±0.0^{a}	97.1±2.0^{a}	96.1±3.1^{a}	97.1±1.1^{a}	97.5±5.5^{a}	27.5±0.1^{ef}	18.2±1.3^{g}	18.2±0.6^{g}
Oregano FS	96.9±1.5^{a}	96.9±1.9^{a}	96.9±1.0^{a}	86.9±1.0^{c}	85.3±0.8^{c}	68.4±0.1^{d}	67.7±3.2^{a}	25.2±2.0^{f}
Theme FS	95.6±3.2^{a}	95.6±3.2^{ab}	96.6±3.2^{ab}	95.6±2.1^{a}	92.5±0.8^{b}	90.4±1.9^{b}	82.8±2.8^{c}	78.9±0.6^{c}
Barberry R	97.5±0.5^{a}	97.5±0.7^{a}	95.5±0.4^{ab}	97.5±1.0^{a}	97.7±0.4^{a}	92.8±0.4^{b}	98.2±0.2^{a}	87.8±1.5^{bc}
Slippery elm IB	95.2±1.5^{ab}	93.4±1.1^{b}	89.7±1.0^{b}	82.7±1.0^{c}	76.0±3.0^{c}	23.9±0.4^{fg}	20.7±1.2^{g}	21.0±1.7^{g}
Clove FB	96.1±1.1^{a}	97.5±1.9^{a}	96.5±1.9^{ab}	97.5±1.3^{a}	98.7±3.2^{a}	73.0±8.4^{c}	36.0±1.6^{e}	34.7±2.5^{e}
Ginger Rh	98.5±1.1^{a}	98.9±0.6^{a}	98.9±1.6^{a}	98.9±1.2^{a}	98.8±5.3^{a}	91.4±2.4^{b}	96.4±1.6^{a}	40.1±1.0^{e}
Olive L	99.8±1.3^{a}	96.8±1.6^{ab}	98.8±1.7^{a}	98.8±2.0^{a}	95.4±7.2^{a}	94.4±5.8^{ab}	20.7±1.2^{g}	21.0±1.7^{g}
Geranium L	95.2±1.3^{ab}	85.1±2.0^{c}	46.1±2.0^{de}	41.1±0.0^{e}	36.8±0.8 ^{a}	28.0±1.9^{ef}	31.8±2.7^{a}	21.4±2.0^{g}
Echinacea L	97.9±1.1^{a}	97.3±1.4^{a}	97.9±1.5^{a}	97.9±0.6^{a}	88.0±2.0^{b}	ND	42.0±1.6^{a}	28.1±1.5^{ef}
Danshen R	98.2±1.0^{a}	95.9±1.1^{ab}	98.9±1.0^{a}	98.9±1.1^{a}	65.3±1.5^{d}	ND	26.2±2.7^{f}	18.8±1.4^{g}
Nemesulide	97.1±1.2^{a}	97.4±1.0^{a}	95.8±1.8^{ab}	95.8±1.8^{ab}	95.7±4.5^{a}	96.6±5.0^{a}	89.9±2.1^{b}	86.0±2.3^{bc}

Cells were treated with various concentration of test compounds for 24 hr. Cell viability (%) was calculated relative to the control of 0.05% DMSO. Values of the same column are expressed as mean±SD (n=3), Tukey's test ($p \leq 0.05$). The value with different letters indicating the significant difference. ND: not determined; F: flowers; FB: flower bud; FS: flowering shoots; IB: inner bark; L: leaves; Rh: rhizome; R: roots; S: stem.

Table 4.2: Effect of aqueous extracts on percentage cell viability of human tonsil epithelial cells.

Test compounds	Cell viability (%)							
	0.1 µg/mL	0.5 µg/mL	1 µg/mL	5 µg/mL	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
PGN+LTA	96.4±1.6^{ab}	ND	ND	ND	ND	ND	ND	ND
Licorice R	98.7±1.0^{a}	96.4±1.6^{a}	97.1±1.0^{a}	80.7±1.8^{c}	95.0±3.5^{ab}	97.5±1.0^{a}	97.7±1.9^{a}	98.2±1.3^{a}
Oregano FS	97.8±1.1^{a}	98.7±1.1^{a}	98.7±1.0^{a}	89.4±5.1^{b}	90.2±1.9^{b}	88.1±3.8^{b}	97.8±2.0^{a}	25.2±4.0^{e}
Thyme FS	98.2±0.5^{a}	97.8±1.1^{a}	98.0±1.0^{a}	84.2±3.6^{c}	93.2±4.2^{ab}	96.4±2.9 ^{ab}	96.6±1.0^{a}	94.1±0.4^{b}
Barberry R	99.3±2.0^{a}	98.2±0.5^{a}	98.1±0.0^{a}	99.1±3.2^{a}	99.6±0.3^{a}	95.3±0.7^{a}	99.2±3.5^{a}	96.4±3.8^{a}
Echinacea S	99.1±1.1^{a}	99.3±2.0^{a}	97.1±0.1^{a}	97.4±1.0^{a}	96.3±0.5^{a}	91.5±1.3^{b}	95.5±2.7^{a}	58.3±5.3^{d}
Echinacea F	98.1±1.1^{a}	99.1±1.1^{a}	96.1±1.1^{a}	98.9±0.9^{a}	95.6±4.5^{a}	91.2±1.3^{b}	97.2±2.9^{a}	97.3±3.3^{a}
Echinacea L	99.1±1.5^{a}	98.1±1.1^{a}	97.0±1.2^{a}	96.0±0.6^{a}	ND	ND	99.7±1.9^{a}	99.6±3.9^{a}
Sage L	97.1±0.8^{a}	99.0±1.5^{a}	97.0±1.1^{a}	97.6±1.9^{a}	ND	ND	103.5±0.9^{a}	99.7±1.9^{a}
Clove FB	98.5±1.7^{a}	98.4±0.0^{a}	98.9±0.3^{a}	98.3±1.1^{a}	ND	ND	92.7±3.4^{b}	87.3±4.3^{b}
Ginger Rh	99.0±1.1^{a}	98.1±0.1^{a}	98.4±1.0^{a}	98.7±0.5^{a}	ND	ND	98.6±4.8^{a}	98.3±2.8^{a}
Olive L	97.2±1.0^{a}	97.0±1.7^{a}	96.0±1.6^{a}	96.2±1.0^{a}	ND	ND	99.7±1.9^{a}	94.6±2.4^{b}
Geranium L	97.1±0.2^{a}	98.2±1.2^{a}	99.0±1.1^{a}	93.1±1.0^{ab}	ND	ND	45.7±6.1^{d}	25.2±1.5^{e}
Slippery elm IB	99.1±1.3^{a}	98.0±1.5^{a}	96.3±1.7^{a}	97.1±1.3^{a}	ND	ND	99.7±4.8^{a}	99.6±2.4^{a}
Nemesulide	99.4±1.0^{a}	98.0±1.6^{a}	98.1±1.4^{a}	96.0±1.1^{a}	95.7±4.5^{a}	96.6±3.0^{ab}	89.9±2.1^{b}	86.0±3.0^{bc}

Cells were treated with various concentration of test compounds for 24 hr. Cell viability (%) was calculated with relative to the control of 0.05% DMSO. Values are expressed as mean±SD (n=3), Tukey's test ($p \leq 0.05$). The value with different letters indicating the significant difference. ND: not determined; F: flowers; FB: flower bud; FS: flowering shoots; IB: inner bark; L: leaves; Rh: rhizome; R: roots; S: stem.

4.4.2. Inhibitory effects of selected phytochemical-rich extracts on the secretion of pro-inflammatory biomarkers

In this study, LTA- and PGN-stimulated human tonsil epithelial cells were used as a model to test plant extracts for anti-inflammatory activity. HTonEpiCs were first treated with mixture of LTA and PGN (10 µg/mL) to induce the inflammation condition over 4 hr and then treated with various EEs and AEs. The concentrations of secreted IL-8, hBD-2, CGP-2 and ENA-78 in the supernatant were determined and compared with that of NSAID, nemesulide. Morphological changes are shown after treatment of plant extracts at the concentrations of 5 µg/mL (Figures 4.4 and 4.5) and 1 µg/mL (Figures 4.6 and 4.7). All the tested extracts showed a considerable range of reduction of pro-inflammatory cytokine secretion. For all four pro-inflammatory cytokines analyzed, two concentrations of 5 µg/mL and 1 µg/mL were tested for all the extracts. However, only the results of % total production of IL-8, hBD-2, CGP-2 and ENA-78 PGE-2 of LTA and PGN-induced HTonEpiCs at 5 µg/mL are reported in Figures 4.2 and 4.3. Both ethanol and aqueous extracts of geranium showed significant reduction of cytokine, but in spite of anti-inflammatory potential, it was excluded because of its cytotoxicity, which might have been responsible for decrease in IL-8, hBD-2, ENA-78 and GCP-2 levels. *In vivo* anti-inflammatory activities of both ethanol and aqueous extracts of all the plant materials tested diminished the production of cytokines in LTA and PGN-activated HTonEpiCs, in a dose-dependent manner.

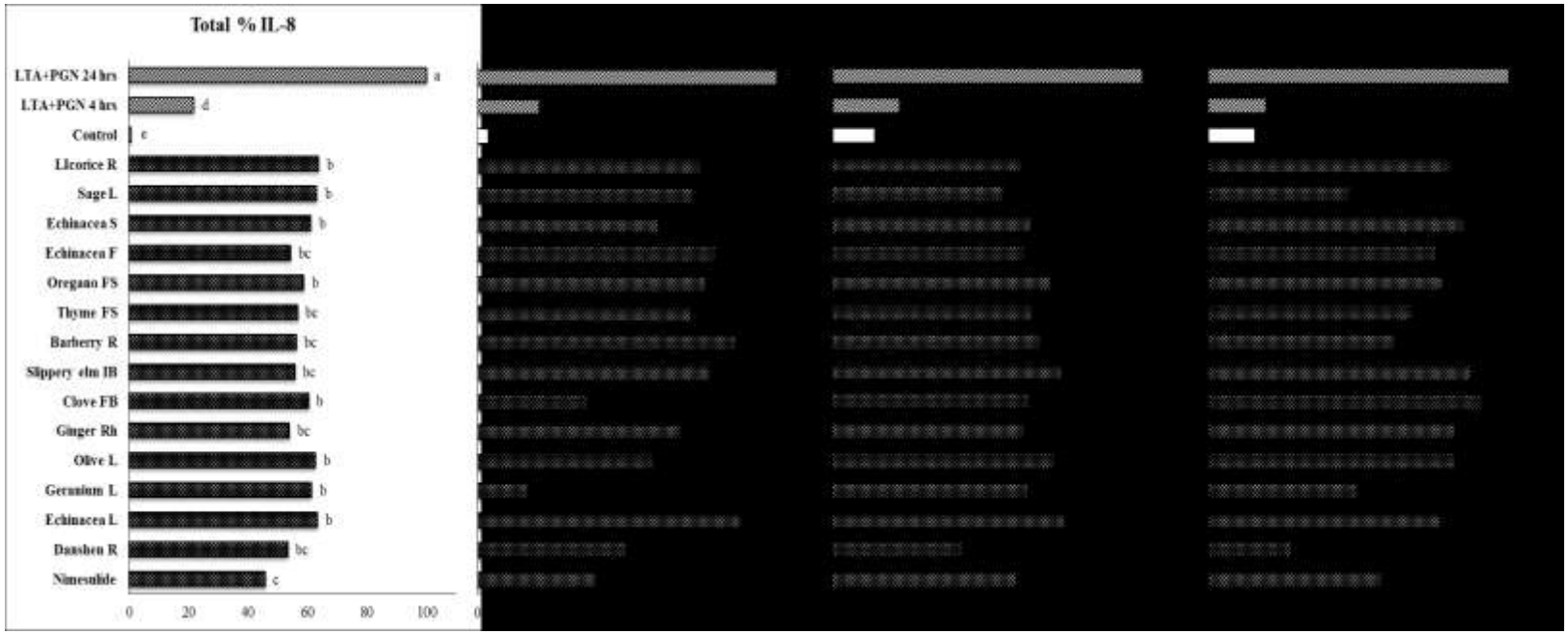

Figure 4.2: Total percentage of secreted proteins of IL-8, hBD-2 ENA-78, and GCP-2 by LTA- and PGN-stimulated human tonsil epithelial cells after incubation with selected phytochemical-rich ethanol extracts. The presented % are relative to experimental model of cells were stimulated with 10 µg/mL of LTA and PGN for 4 hr, and then post-treated with 5 µg/mL test extracts (licorice root, sage leaves, echinacea stem, echinacea flowers, thyme flowering shoots, oregano flowering shoots, barberry root, slippery elm inner bark, clove bud, ginger rhizome, olive leaves, geranium leaves, echinacea leaves and densher root for 20 hr. Data were expressed as mean±SD (n=3), Tukey's test, $p \leq 0.05$. Groups sharing different letters showed significant difference. IL-8, interleukin-8; hBD-2, human beta defensin-2; GCP-2, granulocyte chemotactic protein-2; ENA-78, epithelial-derived neutrophil activating protein-78; LTA, lipoteichoic acid; PGN, peptidoglycogen; F: flowers; FB: flower bud; FS: flowering shoots; IB: inner bark; L: leaves; Rh: rhizome; R: roots; S: stem; ▩ = 5 µg/mL; ▨= 10 µg/mL; □ = DMSO.

Figure 4.3: Total percentage of IL-8, hBD-2 ENA-78, and GCP-2 secretion in human tonsil epithelial cells after incubation with selected phytochemical-rich aqueous extracts in LTA+PGN-induced inflammation. Cells were stimulated with 10 µg/mL of LTA and PGN for 4 hr, and then post-treated with 5 µg/mL aqueous extracts (licorice root, thyme flowering shoots, oregano flowering shoots, barberry root, echinacea stem, echinacea flowers, echinacea leaves sage leaves, clove bud, ginger rhizome, olive leaves, geranium leaves and slippery elm inner bark) for 20 hr. Data were expressed as mean ± SD (n=3), Tukey's test, $p \leq 0.05$. Groups sharing different letters showed significant difference. IL-8, interleukin-8; hBD-2, human beta defensin-2; GCP-2, granulocyte chemotactic protein-2; ENA-78, epithelial-derived neutrophil activating protein-78; LTA, lipoteichoic acid; PGN, peptodoglycogen; F: flowers; FB: flower bud; FS: flowering shoots; IB: inner bark; L: leaves; Rh: rhizome; R: roots; S: stem; ; ▩ = 5 µg/mL; ▨= 10 µg/mL;□ = DMSO.

4.4.3. Effect of test extracts on morphological changes of human tonsil epithelial cells.

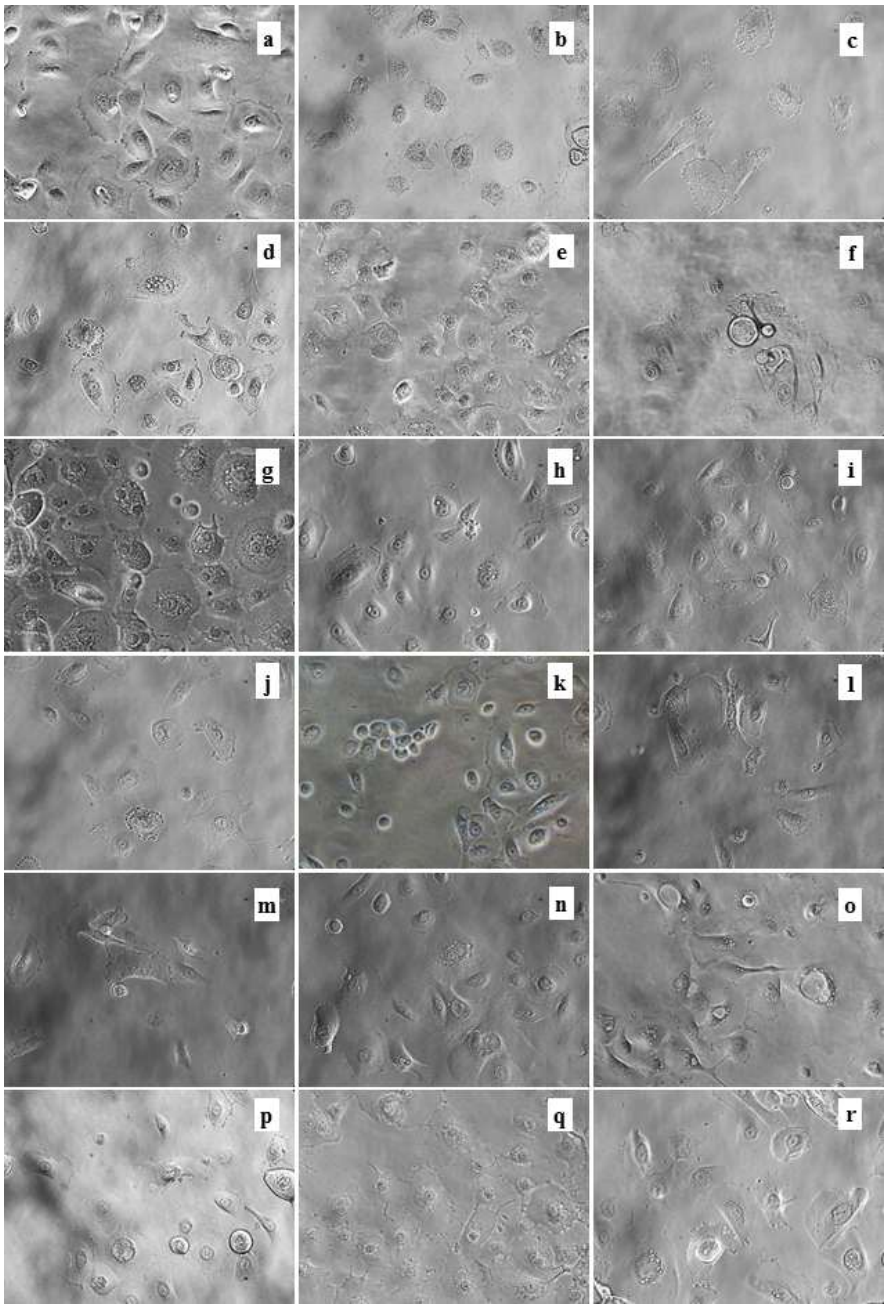

Figure 4.4: Morphological changes of human tonsil epithelial cells after incubation with ethanol extracts at the concentration of 1 µg/mL for 24 hr in LTA+PGN-induced inflammation. (a): media control; (b): 0.05% DMSO control; (c): 10 µg/mL LTA and PGN; (d): Licorice root; (e): Sage leaves; (f): Echinacea stem; (g): Echinacea flower; (h): Oregano shoots; (i): Thyme shoots; (j): Barberry root; (k): Slippery elm inner bark; (l): Clove flower bud; (m): Ginger rhizome; (n): Olive leaves; (o): Geranium leaves; (p): Echinacea leaves; (q): Danshen root and (r): Nemesulide. All images were obtained at the magnification of ×40.

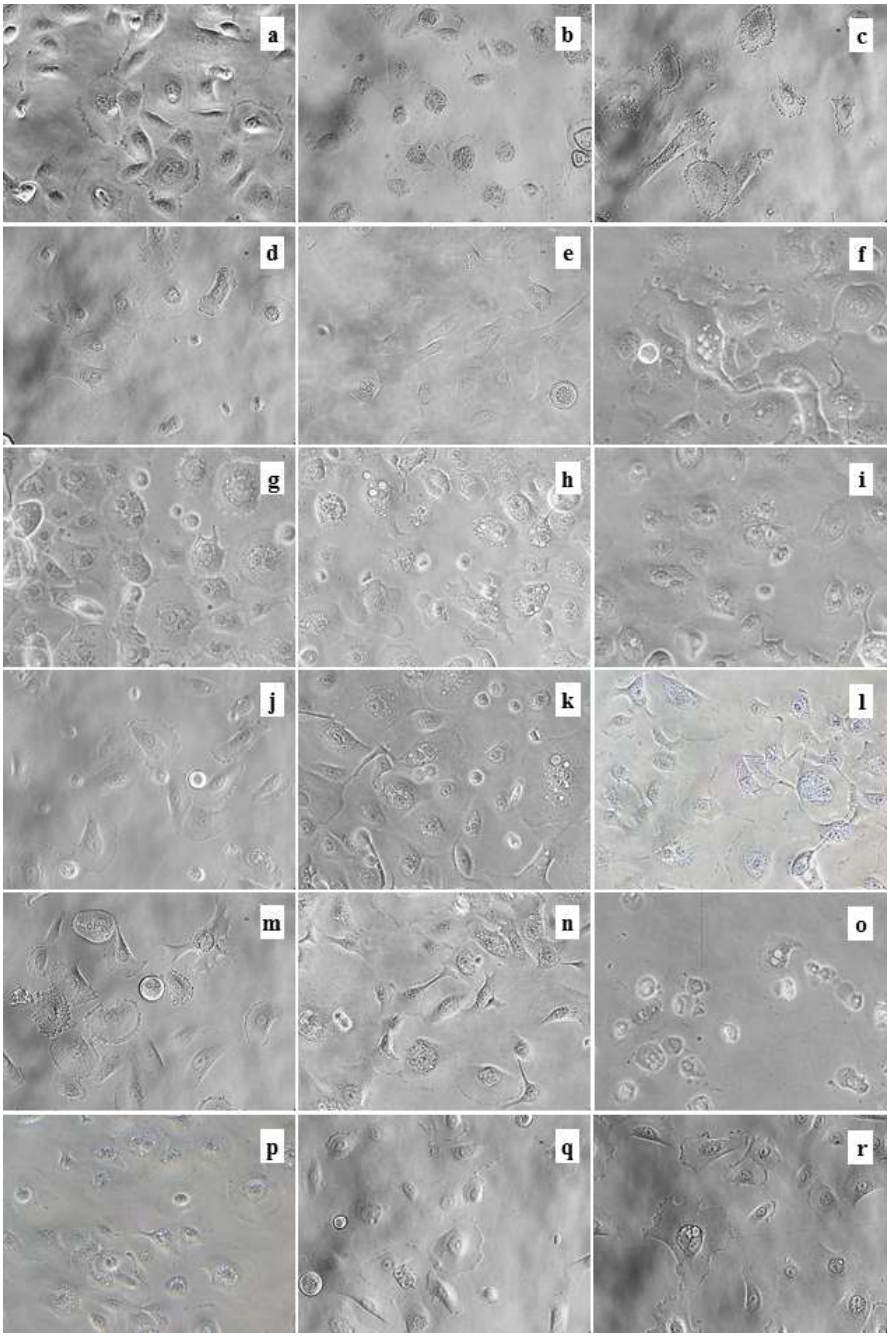

Figure 4.5: Morphological changes of human tonsil epithelial cells after treatment of ethanol extracts at the concentration of 5 µg/mL for 24 hr in LTA+PGN-induced inflammation. (a): media control; (b): 0.05% DMSO control; (c): 10 µg/mL LTA and PGN; (d): Licorice root; (e): Sage leaves; (f): Echinacea stem; (g): Echinacea flower; (h): Oregano shoots; (i): Thyme shoots; (j): Barberry root; (k): Slippery elm inner bark; (l): Clove flower bud; (m): Ginger rhizome; (n): Olive leaves; (o): Geranium leaves; (p): Echinacea leaves; (q): Danshen root and (r): Nemesulide. All images were obtained at a magnification of ×40.

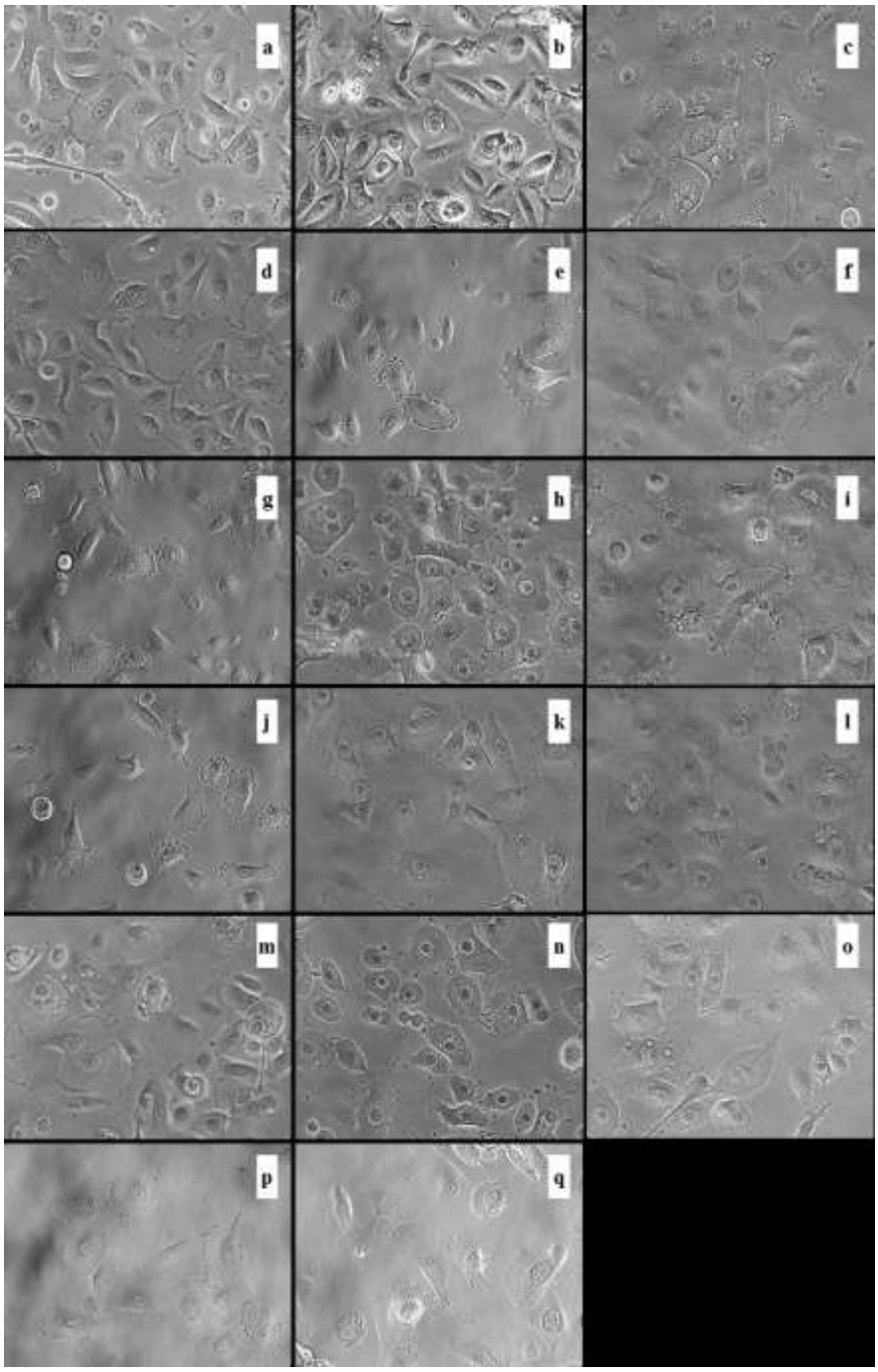

Figure 4.6: Morphological changes of human tonsil epithelial cells after treatment of aqueous extracts at the concentration of 1 µg/mL for 24 hr in LTA+PGN-induced inflammation. (a): media control; (b): 0.05% DMSO control; (c): 10 µg/mL LTA and PGN; (d): Licorice root; (e): Sage leaves; (f): Echinacea stem; (g): Echinacea flower; (h): Oregano shoots; (i): Thyme shoots; (j): Barberry root; (k): Slippery elm inner bark; (l): Clove flower bud; (m): Ginger rhizome; (n): Olive leaves; (o): Geranium leaves; (p): Echinacea leaves and (q): Nemesulide. All images were obtained at a magnification of ×40.

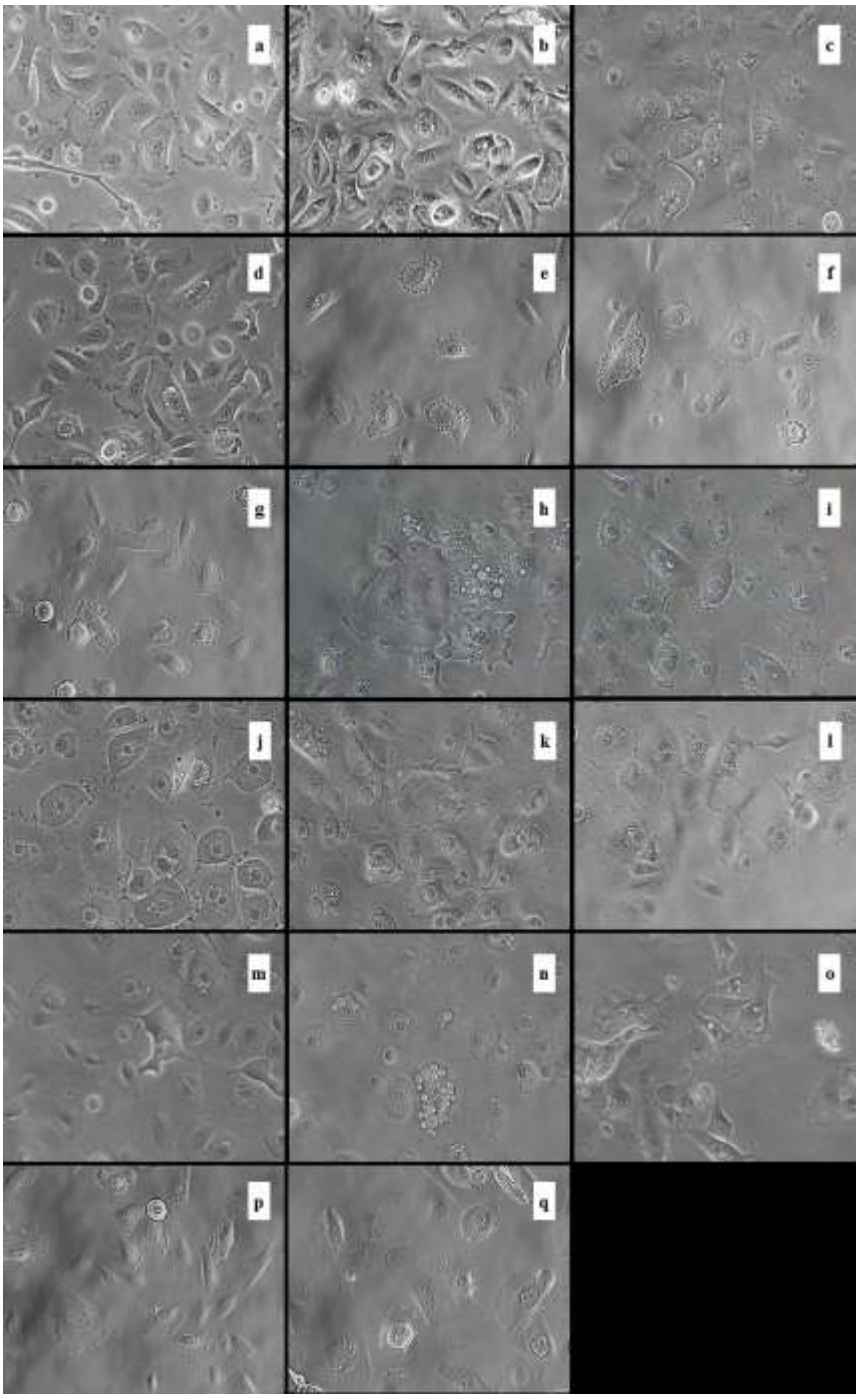

Figure 4.7: Morphological changes of human tonsil epithelial cells after treatment of aqueaous extracts at the concentration of 5 µg/mL for 24 hr in LTA+PGN-induced inflammation. (a): media control; (b): 0.05% DMSO control; (c): 10 µg/mL LTA and PGN; (d): Licorice root; (e): Sage leaves; (f): Echinacea stem; (g): Echinacea flower; (h): Oregano shoots; (i): Thyme shoots; (j): Barberry root; (k): Slippery elm inner bark; (l): Clove flower bud; (m): Ginger rhizome; (n): Olive leaves; (o): Geranium leaves; (p): Echinacea leaves and (q): Nemesulide. All images were obtained at a magnification of ×40.

4.5. DISCUSSION

Several studies have provided considerable evidence that natural compounds possess many biological activities against upper reparative infections and associated inflammations (Hostanska et al., 2011; Khouya et al., 2015; Sharma et al., 2010). The present study was conducted to evaluate the effect of Canadian medicinal plants, used for treating streptococcal pharyngitis, to down regulate secretion of chemical mediators by LTA and PGN-infected human epithelial cells. The results of the study show that secretion of tested cytokines and chemokines by tonsil epithelial cells, including IL-8, hBD-2, ENA-78 and CGP-2, were declined in the presence of the most of the extracts, except geranium EE, in a dose-dependent manner, without cytotoxic effect. Geranium leaf EE showed significantly low cell viability (41.1±2.0%) at to 5 μg/mL. Therefore, though geranium showed significant reduction of pro-inflammatory mediators, it was not considered as a potent anti-inflammatory agent. All other EEs and AEs, at concentrations up to 5 μg/mL, were not cytotoxic to human tonsil epithelial cells.

In streptococcus pharyngitis, host cells first recognize cell surface components, such as LTA and PGN and then respond to GAHBS through the innate immune system (Vroling et al., 2008). The TLRs (TLR-1, TLR-2, TLR-4) play a key role in regulating the expression of pro-inflammatory mediators that are involved in the inflammatory and immunological reactions (White, 1999). Macrophages and mast cells induce the release of inflammatory mediators, such as COX-2, iNOS, and inflammatory cytokines. Inflammatory responses have long been considered to be associated with the NF-κB signaling pathway. The NF-kB is a ubiquitous transcription factor, involved in the induction of the expression of pro-inflammatory genes, including many cytokines,

chemokines and other adhesion molecules, involved in the inflammatory response (Ghosh et al., 1998). The role of NF-κB in inflammation related to the GAS infection is mostly based on the expression of pro-inflammatory genes (Lawrence, 2009). Another study suggested that the adhesion of *S. pyogenes* alone might be sufficient to stimulate nuclear translocation of NF-κB. It is observed that inhibition of bacterial internalization resulted in lower levels of NF-κB at 30 min and 1 hr of infection (Medina et al., 2002). Interestingly, a recent study demonstrated that polyphenols isolated from *Lonicera japonica* (honeysuckle), significantly decreased lipopolysaccharide (LPS) -induced mRNA and protein expression of iNOS, COX-2, as well as mRNA expression of tumor necrosis factor-alpha (TNF-α), IL-1β, and IL-6. Aqueous extract of *Vitex trifolia* (Simpleleaf chastetree) was shown to regulate inflammatory mediators in LPS-stimulated macrophages (RAW 264.7) through inhibition of NF-κB translocation and expression (Matsui et al., 2012). Moreover, polyphenols demonstrated this anti-inflammatory effect on LPS-stimulated RAW 264.7 cells via inactivation of NF-κB by blocking κB degradation and phosphorylation (Park et al., 2012).

Mitogen-activated protein kinases (MAPKs) pathways were also reported to be involved in inflammatory response by GAS infection. The MAPKs pathway was found to stimulate by the activating NF-κB and activating protein 1 (AP-1) activity which is a transcription factor and. Similarly, MAPKs pathway modulates the balance between growth and death of cells during inflammation (Tsai et al., 2006). According to the literature, this pathway consists of three MAPKs that are regulated by phosphorylation. Induction of phosphorylation by p38 MAPKs was identified previously *in vitro,* in mouse osteoblastic cells, upon the infection by *S. pyogenes* (Okahashi et al., 2003). In the same

way, recent findings suggested the importance of p38 MAPK, as a target to upgrade acute lung injury in severe streptococcal infections (Zhang et al., 2012). A fraction of stem bark extract of entada (*Entada Africana*) suppressed LPS-induced inflammation in RAW 264.7 cells. This study was demonstrated that bark extraction fractions contributed to the significant suppression of TNF-α and IL-6, by inhibiting the activity of p38 MAPK kinase (Ayissi Owona et al., 2013).

The chemokines have different biological activities and several previous studies have emphasized the importance of the production of IL-8, ENA-78, hBD-2, and GCP-2, in relation to oral infections such as pharyngitis. Beta defensins are produced by epithelial cells, and combat infection, both through direct microbicidal action and by modulation of cell-mediated immunity (Yang et al., 1999). IL-8 is a chemokine subgroup and is the most potent neutrophil chemotactic and activating protein. Up-regulation of IL-8 production in various cells was observed in response to different stimuli, such as pro-inflammatory cytokines, microorganisms or their derivatives (Sachse et al., 2005). A study demonstrated that mRNA and protein of IL-8 were up-regulated rapidly by TNF-α within the first few hr after stimulation and onset of the inflammatory processes in epithelial cells (Sachse et al., 2005). The hBD-2, produced by inflammation-stimulated epithelium, possesses chemotactic activity for memory T cells and influences cytokine and chemokine secretin. Besides its activities related to immune system, it also has antimicrobial ability (Meyer et al., 2006). The ENA-78, a CXC chemokine, acts as a potent chemo-attractant and activator of neutrophils (Walz et al., 1997). The GCP-2 is also a CXC chemokine which is expressed by monocytes, macrophages, and epithelial cells during inflammation process (Linge et al., 2008; Wuyts et al., 2003). However, it was observed that GCP-2 protein expressed by non-

epithelial cells in human tonsils was mainly involved in acute and chronic inflammation, whereas epithelial cells were expressed to a lesser degree (Wuyts et al., 2003). The TNF-α and IL-1 are involved in up-regulating the expression of GCP-2, while gamma interferon has a down-regulating effect (Sachse et al., 2005).

The percentage suppression of IL-8 by danshen root EE (46.7%), ginger EE (46.1%), echinacea flower EE (45.8%), and slippery elm EE (44.3%) was significant ($P < 0.05$) at the concentration of 5 μg/mL, when compared to 5 μg/mL of nemesulide (54.3%) and 1 μg/mL of nemesulide (52.9%). As well, clove AE (62.1%), echinacea flower AE (59.8%), oregano flowering shoots AE (56.9%), echinacea leaves AE (56.5%) and sage leaves AE (54.8%) showed significantly higher suppression than the reference drug. These findings are in agreement with other studies which showed diminished expression of various cytokines, including IL-8 by herbal extracts (Hostanska et al., 2011; Sharma et al., 2010). For example, "echinaforce", a compound found in EE of herb and roots of *Echinacea purpurea* (echinacea), inhibited the several bacterial inductions of various cytokines, including IL-4, IL-6, IL-8, TNF-α and monocyte chemoattractant protein-1, in a human tracheo-bronchial epithelial cell line (BEAS-2B) and a human lung epithelial cell line (A-549) (Sharma et al., 2010). Possible reasons for this lower activity of danshen root EE, ginger EE, echinacea flower EE and slippery elm EE, may be because of the chemical constituents like flavonoids, quinones, alkaloids, triterpenes and polyacetylates. Therefore, further studies need to be employed on phytochemical analysis.

In the present study, clove, ginger, echinacea flower, oregano, thyme and sage as well as clove, ginger and danshen EEs which were effective inhibitors of chemokine IL-8 production, also potently inhibited hBD-2 production. A previous study has shown that the

levels of hBD-2 strongly correlated with those of increased IL-8 synthesis by lung epithelial cells *in vitro* (A549) and in human primary bronchial epithelial cell lines (Van Wetering et al., 1997). A herbal formulation (BNO 1030), which was an extract of seven different herbal drugs, was shown to possess anti-inflammatory properties. It was demonstrated that BNO 1030, at low non-cytotoxic concentrations, significantly suppressed the secretion of IL-8 and hBD-2 in cultured epithelial A549 cells (Hostanska et al., 2011). It also explained that phenolic compounds, such as flavonoids, tannins and phenolic acids present in BNO 1030, possessed cytokines suppressive capacity. The significant suppression of the production of hBD-2 and IL-8AEs of thyme, oregano, echinacea, sage, clove and ginger as well as EEs of danshen root, ginger and clove may also explained due to their phenolic compounds. Interestingly, above mentioned extracts showed significantly higher suppression of hBD-2 than nemesulide should consider in commercial application. However, this correlation between hBD-2 and IL-8 is biologically relevant because both hBD-2 and IL-8 inhibit cytokine produced by T helper 2 cells (Nomura et al., 2003). This finding agreed with synergistic support of hBD-2 and IL-8 expression for the T helper 1 system, as described by (Meyer et al., 2006). Another finding suggested that bacteria-induced epithelial inflammatory responses through NF-κB/MAPK signaling pathways (Tsai et al., 2006). The activation of NF-κB and AP-1 is a compulsory prerequisite for increased IL-8 and IL-6 expression in epithelial cells, in response to *S. pyogenes* infection and inflammation responses. Therefore, additional studies on NF-κB pathways are necessary to clarify the effect in inflammatory processes of efficacious extracts.

Examining the percentage expression of pro-inflammatory cytokines of GCP-2, it was found that EEs of danshen root, clove, ginger, thyme, oregano, sage, licorice, echinacea flower and stem, as well as all the AEs, account for significant reduction of LTA- and PGN-induced HTonEpiCs. Furthermore, suppression of ENA-78 by 5 μg/mL sage root EE (59.72%) was shown to be significantly higher than that of 5 μg/mL nemesulide (41.60%). Nevertheless, it should be noted that there was a significant reduction of ENA-78 by EE 5 μg/mL of clove, 5 μg/mL sage leaves, 5 μg/mL licorice root, 5 μg/mL of ginger, 5 μg/mL of Echinacea flower and 5 μg/mL thyme, as 37.73%, 46.16%, 39.66%, 39.26%, 39.03% and 36.74%, respectively. Among AEs, clove, echinacea leaves and ginger showed the highest suppression ($P < 0.05$) of LTA and PGN on ENA-78 secretion. Although GCP-2 is structurally most closely related to ENA-78, functionally, it can be used by both GCP-2 and IL-8 receptors to chemo-attract neutrophils (Mittal et al., 2008; Wuyts et al., 2003). Therefore, GCP-2 was shown to have higher antibacterial activity against *S. pyogenes,* compared with ENA-78 and other chemokines. The angiogenic properties of GCP-2, along with its chemotactic property, cause this enhanced antibacterial activity (Sachse et al., 2005). There was abundant expression of IL-8 and GCP-2 in the surface epithelium of an acute type of tonsillitis and ENA-78 was almost undetectable (Sachse et al., 2005). This is contrary to the present results, where compared to GCP-2, pronounced ENA-78 protein was expressed by phytochemical-rich extracts. One reason may be the difference in the cell models used for the studies.

Conventional therapy, steroidal and NSAIDs are used to treat acute inflammation. However, some of those drugs exhibit several undesired side effects and also are unsuccessful at curing chronic inflammatory diseases, such as rheumatoid arthritis or

osteoarthritis (Kim et al., 2013). Therefore, alternative treatments, with safer compounds, have still to be discovered. Phytochemicals, which are the secondary metabolites of plants, have long been of medicinal interest to man for treating different diseases and infections. Plant compounds have been used in the pharmaceutical industry either directly or as precursor compounds.

Numerous studies have demonstrated that crude plant extracts, specific plant-derived compounds and fractions of extracts exhibit an inhibitory effect on inflammation activity, by limiting the production of pro-inflammatory mediators. Combined treatment of thyme and oregano essential oils, has been shown to limit the production of pro-inflammatory cytokines, and reduce 2,4,6-trinitrobenzene sulphonic acid-induced colitis in mice (Bukovska et al., 2007). The thyme aromatic oil contained about 48% *p*-cymene and 24% of thymol, while oregano aromatic oil, which contained about 55% of carvacrol, was the major active compound of these extracts (Bukovska et al., 2007). An EE of a Chinese herb *Tripterygium wilfordii* (thunder duke vine) exhibited potent immunosuppressive and anti-inflammatory properties in human bronchial epithelial cells. Furthermore, triptolide is an active compound in extracts which inhibit the expression of inflammatory genes, including cytokines, chemokines, and adhesion molecules associated with inflammation, by inhibiting NF-κB transcriptional activation (Zhao et al., 2000). Similarly, *in vivo* anti-inflammatory activities of EE *of Polygonum hydropiper* (water pepper) (Yang et al., 2012) and EEs of *Cinnamomum cassia* (Chinese cinnamon) (Yu et al., 2012) suppressed the production of nitric oxide, TNF-α, and PG E2, in LPS-activated RAW264.7 cells, along with peritoneal macrophages, in a dose-dependent manner.

Among the tested herbs, ethanolic extracts of danshen root, ginger, clove, echinacea flower and AEs of clove, ginger, echinacea flower significantly ($p \leq 0.05$) diminished the LTA- and PGN-induced pro-inflammatory cytokines secretion. Danshen root extract was the most potent inhibitor of IL-8, hBD-2, ENA-78 and GCP-2 production. These results were consistent with the previous reports which demonstrate that there are active anti-inflammatory phytochemicals of different plant extracts. For example, a previous study reported that [6]-gingerol, a pungent constituent of ginger, also possessed analgesic and anti-inflammatory activities (Young et al., 2005). Another species of echinacea, *Echinacea angustifolia,* showed suppression of the transcription of pro-inflammatory mediators induction and possess anti-inflammatory activities in H_2O_2-stimulated macrophages (Pomari et al., 2014). Carnosic acid and carnosol, present in sage (*Salvia officinalis*) extracts, improved the anti-inflammatory profile of the secreted cytokines and inhibited the expression of iNOS (Poeckel et al., 2008).

Although both EE and AE of licorice root and barberry suppressed the production of all the tested cytokines in the present study, the percentage reductions of some cytokines were significantly lower than that of ginger, clove and echinacea extracts. However, there has been reported anti-inflammatory effects of licorice root extracts and its phytochemicals, such as glycerrhitinic acid, glycyrrhizin and glycerol. Anti-inflammatory effects were observed in glycerol isolated from *Glycyrrhiza uralensis* in LPS-stimulated RAW264.7 macrophages (Shin et al., 2008). As well, anti-inflammatory activities of both glycerrhitinic acid and the aqueous licorice extract were reported, in comparison with diclofenac sodium, using the carrageenan-induced paw edema model in male albino rats (Aly et al., 2005). However, further studies on other inflammatory markers are required

before recommending licorice and barberry, which are well-established anti-inflammatory agents for pharyngitis treatments.

The anti-inflammatory activities of the major compounds presents in the clove, ginger, sage as well as echinacea extracts (Chaieb et al., 2007; Chu et al., 2012; Fachini-Queiroz et al., 2012; Kondo et al., 2010; Kumar and Ramaiah, 2011; Rani et al., 2012) were reported earlier and these may explain the present results. However, the activities of this efficacious extracts could be even caused by the minor compounds presence in the extracts. Therefore, it is important and still requires the identification of phytochemical profiles which may account for the anti-inflammatory activities and to establish the mechanism of action of its extract before reaching a final conclusion.

In recent years, much effort has been made to identify the potential anti-inflammatory herbal extracts in several medicinal plant extracts and to account for their *in vitro* immune-stimulatory effects using animal models (Da Silva et al., 2011; Masresha et al., 2012; Woldesellassie et al., 2011). It is also reported that *in vitro* anti-inflammatory and analgesic effects of specific phytochemicals, such as carvacrol (Arigesavan and Sudhandiran, 2015; Kara et al., 2015), eugenol (Magalhaes et al., 2010), thymol (Nieddu et al., 2014; Petrujkic et al., 2013) using mice, rats or piglets models. Therefore, *in vivo* efficacy of most efficacious extracts, explored in the present study, can further evaluate in animal models, such as mice, rats or piglets for their *in vivo* efficacy. The effects of the pro-inflammatory cytokine profiles and inhibition of their secretions, indicated by the present study, suggest that a diet-rich in herbs and spices may contribute to the reduction of inflammation against streptococcal pharyngitis.

4.6. CONCLUSION

This study demonstrated that anti-inflammatory properties of danshen root EE, clove AE and EE, ginger AE and EE, echinacea flower AE and EE, oregano leaves AE, sage leaves EE or thyme flowering shoot AE by suppressing of pro-inflammatory mediators, such as IL-8, hBD-2, CGP-2 and ENA-78 protein production in an experimental model system of LTA- and PGN-stimulated HTonEpiC. Ginger AE and EE, clove EE and AE, sage root EE as well as echinacea AE and EE extracts were the most efficacious extracts and could be used to treat pharyngitis, along with antibiotic treatments, with the aim of relieving the complications associated with inflammatory conditions. Furthermore, these efficacious extracts could be used to develop a throat lozenges or herbal teas designed for streptococcal pharyngeal management. However, underlying the mechanism of inhibiting inflammatory mediators is still to be understood. Thus, further studies should be performed to investigate the mechanisms and signaling pathways involved in suppression by these extracts.

CHAPTER 5. CONCLUSION

S. pyogenes is exclusively a human pathogen, causing a wide range of infections, including pharyngitis. Streptococcal pharyngitis is a common upper respiratory tract infection with systemic symptoms, such as sore throat, fever, inflammation and pain. Antibiotic therapy is recommended to eradicate bacteria from the throat and to prevent bacterial transmission. However, treatment challenges, such as therapeutic failures, poor patient compliance and considerable adverse side effects due to the use of antibiotics, are widely reported. The administration of anti-inflammatory drugs, along with the antibiotics, is also a common disease management practise, which aims to reduce symptomatic pain. Therefore, there is an interest in investigating alternative and complementary therapeutic agents which are efficacious and safer. Plant-derived polyphenols and isoprenoids have been reported to possess a wide range of medicinal properties which can used as effective potential sources for novel drugs. A number of studies have described the development of plant extracts-based alternative antimicrobial drugs. Thus, the present study was conducted with the aim of screening for inhibition of growth and formation of biofilm of *S. pyogenes* and anti-inflammatory effects of phytochemical-rich herbal extracts derived from 12 different medicinal plants.

This present study showed that: crude ethanolic extracts of licorice root, echinacea flower and sage leaves; essential oils of oregano flowering shoots and sage leaves and; aqueous extracts of licorice root, oregano and thyme flowering shoots, can inhibit the growth of the planktonic *S. pyogenes* and prevent the formation of biofilm of the bacteria. However, future investigations are needed to explore the mode of action of inhibitory

activity of phytochemicals present in those extracts. As well, it is suggested that an examination of the role of herbal extracts on beneficial bacteria, including gastrointestinal species such as *Lactobacillus* species. Several scientific evidences exist that herbal extracts stimulate the growth of beneficial bacteria. However, since most of herbal extracts and their phytochemicals possess a wide range of anti-microbial activity, it may be useful to determine whether efficacious extracts identified in this study are pathogen specific.

Due to their diverse chemical composition, plant extracts and their components have diverse and wide-ranging applications in medicinal therapy. This underlies the necessity of continuing further investigations to discover major polyphenols, before addressing their modes of action. Therefore, it is necessary to identify the active compounds are present in the efficacious ethanol and aqueous extracts by using UPLC-MS/MS. Essential oils were tested for their major polyphenols using GC-FID; however, chromatographic analysis showed that there are some unknown compounds which should to be identified. Thus, several other standards, such as zingiberene, geranial, geraniol, β-caryophyllene, 1-methyl-3-(1-methylethyl) –benzene, camphor and 1, 8 cineole, could be quantified. The anti-adhesive mechanism of phytochemicals may contribute to the initial stage of control of bacterial infection. The current research did not provide any evidence related to the adhesion of *S. pyogenes*. Therefore, the potential for anti-adhesion activity of the extracts need to be determined and the potential anti-adhesion phytochemicals remain to be identified.

The present study used three different strains of *S. pyogenes*, however, their serotypes were unknown. In general, invasion by different *S. pyogenes* serotypes may show different patterns and their actions inside the human epithelial cells may have variations. It

was observed that three strains of the present study was demonstrated different concentrations required to inhibit the growth and/or to form biofilms. Accordingly, the identification of their serotypes is important and yet to be revealed. The present study used crude extracts to evaluate the anti-bacterial and anti-biofilm activities. If the extracts are fractioned, the concentrated phytochemical amount and efficacy are expected to be greatly enhanced. Therefore, further studies need to be conducted on activity-guided fractionation of the selected extracts.

Most of the reported studies have focused on the observation of antibacterial or anti-inflammatory activity of herbal extracts evaluated as a single source, but not in combinations of extracts as in traditional herbal recipes. Therefore, efficacious compounds or extracts of different plant species can be combined in appropriate ratios and these herbal recipes may also be employed for their anti-bacterial properties.

In the present study, LTA- and PGN-induced human epithelial cells showed significant *in vitro* anti-inflammatory activities for sage root AE, clove AE, ginger AE, echinacea flower AE, clove EE, ginger EE by supressing the secretion of pro-inflammatory cytokines, including IL-8, hBD-2, CGP-2 and ENA-78 at non-cytotoxic concentrations. However, secretion of other common pro-inflammatory cytokines, including IL-6, TNF-α, and PGE2 as well as COX-2 enzyme, may give further understanding of the inflammation process. This study also reveals the ferric reducing antioxidant potential of extracts. However, it is recommended that other types of antioxidant capacity assays, such as oxygen radical absorbance capacity and (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging activity, be employed, to reconfirm their total anti-oxidant capacity.

In conclusion, significant growth and biofilm inhibitory activity against *S. pyogenes* and anti-inflammatory activities of some plant extract among tested crude extracts were revealed. Oregano essential oil, sage essential oils and ethanol extracts, licorice ethanol extract and echinacea flower ethanol extract were the most efficacious extracts against inhibition of *S. pyogenes*. The most effective anti-inflammatory extracts were either ethanol or aqueous extracts of ginger, clove, oregano, thyme; ethanol extract of danshen root and aqueous extracts of echinacea and sage. Considering both targeted activities of anti-bacterial and anti-inflammatory, oregano, sage, echinacea and thyme were the most efficacious plant sources. Therefore, either crude extracts or isolated compounds from these efficacious extracts could be used as bioactive agents to incorporate into natural health products such as honey lozenges intended to soothe the sore throat and relieve from associated pain. The processes of incorporating the most effective extracts into the honey lozenge, and assessing the safety and sensory attributes of the new products must be accomplished, before industrial application and commercialisation can occur. Furthermore, the anti-inflammatory effects of efficacious extracts needs to be validated in animal models of *S. pyogenes* or bacterial cell wall compounds-induced inflammation to further confirm their claim in treatment of pharyngitis.

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