CHARACTERIZATION OF ANTI-INFLAMMATORY, ANTI-*STREPTOCOCCUS PYOGENES* AND ANTI-BIOFILM ACTIVITIES OF SUMMER SAVORY PHYTOCHEMICALS

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ABSTRACT

Streptococcus pyogenes is a common cause of sore throat that requires clinical intervention. Antibiotics and non-steroidal anti-inflammatory drugs are often prescribed to inhibit the growth of bacteria and relieve pain, respectively. However, the side effects of using those drugs have been reported, thus, alternative treatments are required. Summer savory (*Satureja hortensis* L.) has been used as a traditional medicinal herb. In this study, fifteen summer savory phytochemicals including polyphenols, isoprenoids, and extracts were investigated for anti-inflammatory, anti-*S. pyogenes* and anti-biofilm properties. Rosmarinic acid and apigenin were the major phytochemicals in the ethanol extract. The ethanol extract from leaves, apigenin and carvacrol, and essential oil from whole plant showed the greatest anti-inflammatory activities *in vitro* in THP-1 differentiated macrophages and human tonsil epithelial cells (HTonEpiC). The above preparations and phytochemicals also exhibited strong anti-*S. pyogenes* activity towards three strains of *S. pyogenes*.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ADM	Agar dilution method
ANOVA	Analysis of variance
AP	Activation protein
ATCC	American Type Culture Collection
α-Ter	Alpha-terpinene
BDM	Broth dilution method
BHI	Brain heart infusion
BMDM	Broth micro dilution
β-My	Beta-myrcene
Carva	Carvacrol
Cary	Caryophyllene
CFU	Colony forming unit
COX-2	Cyclooxygenase-2
Cont.	Control
CO_2	Carbon dioxide
DDM	Disc diffusion method
D-GalN	D-galactosamine
Diclo	Diclofenac
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECG	Epicatechin-3-gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamic acid-leucine-arginine
ENA-78	Epithelial-derived neutrophil activating protein-78
EO	Essential oil
EOC	Commercial essential oil
EOL	Essential oil extracted from leaves
EOP	Essential oil extracted from fresh whole plant
EOS	Essential oil extracted from stems

EPS	Extracellular polymeric substances
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FBS	Fetal bovine serum
GAS	Group A *Streptococci*
GCP-2	Granulocyte chemotactic protein
GRO-α	Growth-regulated oncogene alpha
γ-Ter	Gamma-terpinene
hBD-2	Human beta defensin-2
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HTonEpiC	Human tonsil epithelial cells
ICAM-1	Intercellular adhesion molecule-1
IκB	Inhibitor of kappa B
IκK	IκB kinase
IL	Interleukin
INF-α	Interferon-alpha
iNOS	Inducible nitric oxide synthase
JNK	C-jun N-terminal kinase
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinase
MBC	Minimum Bactericide Concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MIC	Minimum Inhibitory Concentration
MRM	Multiple reaction-monitoring
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
Nime	Nimesulide
NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drugs

PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
p-Cy	p-Cymene
PGE_2	Prostaglandin E_2
PGN	Peptidoglycan
PMA	Phorbol myristate acetate
PMS	Phenazine methosulfate
PZ-DHA	Docosahexaenoic acid ester of phloridzin
Q-3-G	Quercetin-3-O-glycosides
Ros.acid	Rosmarinic acid
TBM	Tube broth method
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TNS	Trypsin neutralization solution
UPLC-MS	Ultra-pressure liquid chromatography coupled with tandem mass spectrometry

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CHAPTER 1 INTRODUCTION

Acute pharyngitis or sore throat is one of the most common clinical problems in medicine all over the world, and accounts for 1% to 2% of all visits to outpatient departments, physician's offices, and emergency departments (*1*). Children are the most frequently susceptible group for sore throat and have been estimated a total of 7 million diagnosed cases in U.S.A. annually (*2*).

Various pathogens may cause sore throat such as viruses and bacteria. Even though most cases are viral in origin, *Streptococcus pyogenes* (Group A *Streptococci*) is the most important causative agent requiring etiologic diagnosis and specific therapy (*2, 3*). An array of diseases related to *S. pyogenes* infections have been indicated ranging from non-invasive symptoms (pharyngitis and impetigo) to severe scarlet fever, necrotizing fasciitis and toxic shock syndrome resulting in high morbidity and mortality (*4, 5*).

Although most of *S. pyogenes* infections are self-limiting, antibiotics are frequently prescribed to relieve pain, minimize transmission, and decrease complications (*6*). Penicillin remains the first choice of antibiotic for the treatment of streptococcal pharyngitis because of its desirable features of low cost, high efficacy and safety, and narrow antibacterial spectrum (*7*). Erythromycin belonging to macrolide group is often used as an alternative therapy for the patients allergic to penicillin. However,

erythromycin-resistant *S. pyogenes* strains have been increasingly noticed in different areas of the world, particularly in Asian and European countries (*7-9*). Erythromycin resistance might result from efflux encoded by *mefA* gene (*8-10*). The modification of target site is also associated with the resistance due to ribosomal methylation by blocking the antibiotic from its ribosomal target (*8-10*).

A biofilm is a community of microbial sessile cells strongly adhering to a substrate and generating extracellular polymeric substances (EPS) (*11, 12*). EPS may comprise exopolysaccharides, proteins and DNA (*12*). *S. pyogenes* has been indicated to form biofilm leading to the antibiotic therapeutic failure (*6, 13, 14*). Biofilm creates a barrier and allows bacteria to survive and proliferate in the presence of antibiotics (*6, 15*). Numerous studies have demonstrated that higher concentration of antibiotics are needed to achieve anti-biofilm activity than to inhibit the growth of corresponding planktonic cells (*15, 16*).

Humans possess the ability to recognize the invading bacterial pathogens and mount a significant innate response to control infection (*4, 17*). The cellular recognition is mediated by the interaction between pathogen-associated molecular patterns (PAMPs) and Toll-like receptors (TLRs) (*17, 18*). Most of PAMPs are molecules from cell-wall and can be found in both Gram-negative and Gram-positive bacteria including lipopolysaccharide

(LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA). TLRs are a group of leucine-rich repeat proteins related to the recognition of bacterial PAMPs. TLR2 and TLR4 are involved in LPS recognition, while PGN and LTA are specifically recognized by TLR2. The recognition of PAMPs by TLRs activates signaling cascades that result in inflammation (*17, 19*). Inflammation is a protective immune response to various stimuli including foreign pathogenic challenge and tissue injury (*20*). Epithelial cells in the upper respiratory tract are the major targets of *S. pyogenes* colonization and act as a barrier protecting the internal milieu from invasion by external pathogen (*19, 21*). Various pro-inflammatory cytokines (Interleukin [IL]-1β, IL-6, IL-8, interferon-α [INF-α] and tumor necrosis factor-α [TNF-α]) and chemokines (IL-17, IL-23, granulocyte chemotactic protein [GCP-2], and growth-regulated oncogene alpha [GRO-α]) are associated with triggering a cascade of inflammatory responses in epithelial cells (*5, 17, 18, 22, 23*).

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for the therapy of inflammation. The mode of action is based on the down regulation of prostaglandin production through reversibly or irreversibly inhibiting cyclooxygenase 2 (COX-2) formation (*24, 25*). Side effects of NASIDs use include increased hazard for cardiovascular death and gastrointestinal damage and have been widely studied and are associated with the use of NSAIDs (*26, 27*).

Due to the high incidence of antibiotic treatment failure and the increased risk of using NSAIDs for inflammation therapy, effective solutions are necessary to control streptococcal throat. A preponderance of evidence suggests that specific plant secondary metabolites (phytochemicals) could provide an alternative treatment as therapeutic agents reviewed in Cushinie et al, (2011) (*28*). Polyphenol-rich extracts from herbs and fruits have shown profound anti-bacterial, anti-biofilm and anti-inflammatory activities (*29, 30*). Essential oils, aromatic oily substances from various plants, also exert pharmacologically significant capacity against *S. pyogenes* and its biofilm formation, as well as suppression of inflammation (*14, 31*). Natural compounds such as carvacrol and rosmarinic acid isolated from specific plants have been suggested to suppress inflammation using rat models and to inhibit both planktonic bacteria and biofilm growth *in vitro* (*32-35*).

Summer savory (*Satureja hortensis*), is a species of the *Satureja* genus and is traditionally used as a culinary herb. It is recognized that phytochemicals mainly as the essential oils, rosmarinic acid and apigenin derived from aerial parts of the plant, have been used as folk medicinal treatments for various ailments including cold, cramps, muscle pain, stomachache, intestinal disorders, diarrhea, cholera, and other infectious diseases (*36, 37*). With an abundance of phytochemicals associated with pharmacological activity, summer savory may provide an attractive and potentially new option for incorporation into

dehydrated honey products intended for use in pain relief and the treatment of upper respiratory indications.

The research hypothesis of this study is that bioactive phytochemicals of summer savory can reduce inflammatory conditions *in vitro* and also suppress the growth of *Streptococcus pyogenes* and its associated biofilm under experimental conditions. The overall objective of this research was to identify bioactive phytochemical rich essential oils, extracts and major components of summer savory, grown in Nova Scotia, that exhibitanti-inflammatory, anti-*Streptococcus pyogenes*, and anti-biofilm properties. To investigate anti-inflammatory benefits, phorbol myristate acetate (PMA)-differentiated LPS-induced THP-1 macrophages and human tonsil epithelial cells (HTonEpiC) were used as cell model systems. Investigation of the anti-*Streptococcus pyogenes* and anti-biofilm activities, were assessed based on testing of three strains of *S. pyogenes* (ATCC 19615, ATCC 49399, and a clinical isolate).

CHAPTER 2 LITERATURE REVIEW

2.1 SORE THROAT

Sore throat, or pharyngitis, is an acute inflammatory syndrome of the pharynx and/or tonsils. As one of the most common clinical problems in medicine all over the world, in 2007, acute pharyngitis accounted for over 15 million patients seeking healthcare in U.S.A. (*38, 39*). Numerous factors may contribute to sore throat, including both infectious and noninfectious causes (Table 2.1). Sometimes these causes sometimes overlap. Most cases are infectious with more than 50% are caused by viruses and approximately 20% caused by bacteria (*40-42*). Rhinovirus, coronavirus and influenza are the most frequently reported viral agents associated with sore throat (*43*). Epstein-barr virus is another viral cause that is often associated with infectious mononucleosis (*44*). *S. pyogenes* is the major cause of bacterial pharyngitis accounting for 15% of all episodic pharyngitis cases warranting routine treatment with antibiotics (*42*). On rare occasions, fungi and parasites have also been reported to cause sore throat (*45*). A proportion of noninfectious agents may be capable of producing throat irritation, including different physio-chemical factors such as cigarette smoking, shouting, allergic rhinitis and environmental factors such as dry hot air, hot food, and liquids (*42, 45*).

Table 2.1 Various causes of sore throat

Category	Organism/ Factor	References
Viruses	*Rhinovirus*	(*44*); (*46*)
	Adenovirus	
	Epstein-barr virus	
	Influenza A and B virus	
	Coronavirus	
	Herpes simplex virus types 1	
	Parainfluenza virus	
	Human immunodeficiency virus (HIV)	
Bacteria	*Streptococcus pyogenes* (group A)	(*42*); (*43*); (*47*);
	Streptococci of serogroup G and C	(*48*)
	Staphylococcus aureus	
	Arcanobacterium haemolyticum	
	Corynebacterium diphtheriae	
	Neisseria gonorrhoea	
	Yersinia enterocolitica	
	Haemophilus influenzae	
Fungi	*Candida species*	(*46*); (*48*)
	Histoplasmosis capsulatum	
	Cryptococcosis neoformans	
	Blastomyces dermatitidis	
	Paracoccidoides brasiliensis	
Parasites	*Toxoplasma gondii*	(*48*)
Physio-chemical factors	Cigarette smoking	(*41*); (*42*); (*45*)
	Allergies and allergic rhinitis	
	Shouting	
	Gastrointestinal reflux	
	Post-nasal drip and mouth breathing	
Environmental factors	Dry, polluted air	(*42*); (*45*)
	Temperature and humidity	
	Hot food, liquids	
	Smoke	

Category	Organism/ Factor	References
	Chemical toxins	
	Inhaled irritants	

2.1.1. *Streptococcus pyogenes*

S. pyogenes (Group A *Streptococci* [GAS]) is the most critical causative agent for sore throat due to subsequently potential severe complications arising such as acute rheumatic fever, rheumatic heart disease, and acute post-streptococcal glomerulonephritis (*16, 49, 50*). Worldwide, *S. pyogenes* is responsible for an minimal estimated 600 million cases of throat infection per year and the human body is its only known natural reservoir (*4*).

Streptococcal pharyngitis occurs in all age groups, and is most often observed in school-age children and adolescents accounting for 15%-30% of overall sore throat cases in children, and an incidence of 5%-10% is observed among adults (*43, 51*). As a human-restricted pathogen, *S. pyogenes* is transmitted by person-person contact, probably via nasal droplets or oral transmission from infected individuals. As such, the highest incidence of streptococcal pharyngitis results from crowded places. For example, outbreak of streptococcal pharyngitis has been reported among university students in a judo club, wherein, one out of nine asymptomatic students showed GAS-positive culture (*50*). Finally, epidemiological evidence of food transmission of *S. pyogenes* has also been documented

in several studies (*52, 53*).

2.1.2 Treatments for streptococcal pharyngitis

Although streptococcal pharyngitis is usually self-limiting, antibiotics are often prescribed to minimize transmission, to relieve the pain, and to prevent development of potential complications (*6, 54*). In recent trials, it has been indicated that prescription of antibiotic decreases the risk of complications of acute pharyngitis by a minimum of 50% (*55*).

Generally, two groups of antibiotics such as β-lactams and macrolides are involved in the therapy (Table 2.2.). β-Lactam antibiotics possess bactericidal activity by specifically inhibiting the synthesis of peptidoglycan in the bacterial cell wall. This process results from the inactivation of penicillin-binding proteins (*56*). Penicillin, or its derivatives, remain the first-line choice for the treatment of streptococcal pharyngitis due to low cost, proven efficacy and safety, and a narrow spectrum of activity (*57, 58*). In spite of increasing cases of resistant respiratory bacterial pathogens, *S. pyogenes* is uniformly susceptible to penicillin on a worldwide basis (*59, 60*). However, more recently, penicillin treatment failure has been suggested in up to 5-30% of children experiencing streptococcal pharyngitis (*61*). Various explanations may contribute to the failure of penicillin as a therapeutic and mainly be due to non-compliance with the prescribed antibiotic regime,

recurrent exposure to bacteria, copathogenicity of β-lactamase-producing bacteria, and bacterial coaggregation, biofilm formation or internalization into epithelial cells (*51, 62-64*).

Macrolides, as an alternative therapy, are prescribed for patients with streptococcal pharyngitis who are allergic to β-lactam antibiotics (*8*). Macrolides act as bactericidal agents through inhibiting protein synthesis by reversibly binding to 50S subunit of the bacterial ribosome (*65*). However, erythromycin-resistant *S. pyogenes* particularly macrolide-resistant strains has appeared globally. In a study performed in Argentina, 8.2% of clinical *S. pyogenes* isolates mostly from paediatric pharyngeal swabs were revealed to show resistance to erythromycin (*66*). During the period 1999-2000, erythromycin-resistant *S. pyogenes* strains were shown to exist at low rates of prevalence in U.S.A (5.2%) and in Mexico (4.9%) (*67*). Moreover, it was reported in Italy that 38.3% of *S. pyogenes* isolates from children with streptococcal pharyngitis were resistant to erythromycin (*68*). A high level of erythromycin resistance was recorded in Korea and Taiwan with the ratio of 41.3% in 1998 and 61.1% in 2011, respectively (*68-70*). Two principle mechanisms are related to acquiring erythromycin resistance in *S. pyogenes* including target site modification and active efflux pump (*10*). Target site modification results from methylases (encoded by *erm* genes) by modifying an adenine residue in the 23S rRNA preventing

antibiotic binding to its 50S ribosomal target (*8, 10, 71*). Active efflux system (mediated by *mef* genes) is associated with a membrane protein leading to an energy-dependent efflux (*10, 66, 71*).

Table 2.2. Current antibiotics prescription and doses for the treatment of streptococcal pharyngitis

Class of antibiotics	Antibiotic	Daily dose	Duration of therapy
β-Lactams	Penicillin VK or Amoxicillin	≤ 27 kg : 250 mg, 2-3 times > 27 kg : 500 mg, 2 to 3 times	10 days
	Penicillin G benzathine	≤ 27 kg : single dose of 600,000 units IM > 27 kg : single dose of 1.2 million units IM	NM
	Cephalexin or Cefadroxil	30 mg/kg, 4 divided doses	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	500 mg, single dose	5 days
	Erythromycin esotlate	20-40 mg/kg	10 days
	Erythromycin Ethylsuccinate	40 mg/kg, 2-4 divided doses	10 days

***NM: not mentioned**

***Modified from* (*51*) and (*57*).**

2.2 BIOFILM

A biofilm is a community of sessile microbial cells surrounded by extracellular

polymeric substance (EPS) consisting of exopolysaccharides, proteins and DNA (*12, 72*). As sessile cells exhibiting a modified phenotype compared to homologous planktonic cells, a biofilm represents a protection mode allowing microorganisms to survive and proliferate in a hostile environment (*11, 15, 72*).

2.2.1 Biofilm formation

Bacterial biofilms arise from the adhesion of planktonic cells to surfaces. Non-biological and biological surfaces are involved in the architectural attachment of bacteria (*73*). In the case of streptococcal pharyngitis, *S. pyogenes* successfully invades the host followed by adherence to the mucosal surface of pharynx cavity (*74*). If environmental conditions are favorable, bacteria might start proliferating and forming a society. Simultaneously, bacteria produce EPS stabilizing the biofilm structure (*75*). It has been reported that glucose levels play an essential role in stimulating biofilm formation of *S. pyogenes* (*76, 77*). Within the society, cell-cell signaling, the production of extra-cellular hydrolytic enzymes and polysaccharide formation create a specific metabolic network and contribute to the formation of communities (*75*).

M-protein encoded by *emm* gene is one of the essential components of *S. pyogenes* virulence factors. This protein is exposed on the surface of bacteria and involved in adhesion to the epithelium (*78*). A significant number of reports have emphasized that

biofilm formations vary among different M types of *S. pyogenes* (*13, 79*).

Antibiotic treatment failure has been indicated to be related to biofilm formation in streptococcal pharyngitis (*79*). Pooled studies have demonstrated that dramatically higher concentration of antibiotics are needed for the removal of biofilm than that of corresponding planktonic cells (*15, 16*).

2.3 INFLAMMATION

Inflammation is a protectively physiological host response to external stimuli, tissue injury and infections (*20, 80, 81*). Redness, swelling, pain, and heat are the typical symptoms of the inflammatory response (*82*). Numerous studies have shown that several chronic diseases such as chronic asthma, arthritis, obesity, and diabetes can be indicated resulting from excessive and chronic inflammation (*83-85*).

Macrophages, originating from circulating blood monocytes, are primary pro-inflammatory cells and play a significant role in defending against harmful stimuli (*86, 87*). Activated macrophages secrete pro-inflammatory mediators including TNF-α, IL-1β, and IL-6. COX-2 is also produced by macrophages for the synthesis of prostaglandin E-2 (PGE_2) causing vasodilation and pain (*86, 88*).

2.3.1 Streptococcal pharyngitis and inflammation

Epithelial cells of the upper respiratory tract are the main target for *S. pyogenes*

colonization causing streptococcal pharyngitis. Further, epithelial cells provide the first line of protection from invasion of external pathogens to the internal milieu (*19, 89*). *S. pyogenes* can be recognized by the host innate immune system and a robust inflammatory response which has been triggered (*90*). The recognition is initiated by pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs) (*17, 18*). Pepetidoglycan (PGN) and lipoteichoic acid (LTA) are the major PAMPs of *S. pyogenes* and are associated with inducing a pro-inflammatory response (*19*). PGN is an elementary component of *S. pyogenes* bacterial cell wall, while LTA functions to maintain the structure of bacterium by spanning and linking the carbohydrate network of the cell wall. Further, LTA also contributes to hydrophobicity and mediates *S. pyogenes* adhesion to host cells (*79, 91*). The innate immune response to *S. pyogenes* is based on the activation of TLR2 and TLR13. Specifically, *S. pyogenes* is initially recognized by TLR2 followed by TLR13 upon internalization (*92*). TLR9, the receptor for unmethylated CpG sequences, facilitates eradication of *S. pyogenes* by increasing macrophage hypoxia-inducible factor-1α levels, oxidative burst and nitric oxide production (*90*). All TLRs, except TLR3, activate by central adaptor protein myeloid differentiation factor 88 (MyD88). MyD88 is essential for transcription factor NF-κB activation which stimulates the expression of pro-inflammatory cytokines and chemokines (*90, 93, 94*).

2.3.2 Pro-inflammatory cytokines and chemokines

Inflammation cascades result in the production of various pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, interferon-alpha (INF-α), tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), and prostaglandin E_2 (PGE_2) and pro-inflammatory chemokines such as IL-8, IL-17, IL-23, granulocyte chemotactic protein-2 (GCP-2), and growth-regulated oncogene-alpha (GRO-α) (*18, 22, 23, 95*). Thus, those pro-inflammatory mediators are often used as inflammatory biomarkers.

IL-6 is secreted by a wide range of cells including epithelial cells and macrophages and contributes to the initiation and development of bacteria-stimulated inflammatory response (*96-100*). Likewise, TNF-α production can be induced by bacterial PAMPs such as LPS or PGN. Previous studies have suggested that TNF-α exerts the ability to promote recruitment of macrophages to the infection site and thereby provide protection to the epithelium cells during mucosal inflammation (*99, 101, 102*). COX-2 is a rate-limiting enzyme in the synthesis of prostaglandins (*103-105*). An inducible COX isoform, COX-2 can be stimulated in monocytes, macrophages, endothelial cells, and fibroblast cells in response to various pro-inflammatory factors including cytokines, endotoxin, mitogen, and other stimuli (*106-109*). PGE_2 is a classic lipid mediator that arises from COX-2 involving increase of vasodilatation and local blood flow that results in typical symptoms of

inflammation (pain, swelling and redness) (*110, 111*).

Chemokines (chemotactic cytokines) are functionally categorized into four groups, CC, XC, CXC, and CX_3C, based on the position of conserved cysteine residues. The presence or absence of a glutamic acid-leucine-arginine (ELR) motif further divides CXC group into ELR+ and ELR subfamilies. GCP-2, IL-8, and ENA-α all belong to ELR+ CXC chemokines (*23, 112*). GCP-2, initially isolated from cytokine-stimulated human osteosarcoma cells, displays in different cells, namely neutrophils, epithelial cells, endothelial cells and fibroblasts after induction of bacterial components or other pro-inflammatory cytokines (*23, 113-117*). In addition, *in vivo* and *in vitro* studies have suggested that GCP-2 production is associated with tumor development and tumor invasion by neutrophil recruitment (*118, 119*). ENA-78 stimulates chemotactic recruitment and activates neutrophils. It has been shown that ENA-78 secretion increased in the presence of IL-1β, or of TNF-α plus interferon-γ (*120, 121*). Similarly, IL-8 acts as a neutrophil chemoattractant and also shows angiogenetic activity (*122*).

2.3.3 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs are commonly used for the treatment of inflammatory diseases. All drugs in this class work by selectively or non-selectively inhibiting COX enzyme activity. COX enzymes are associated with the production of

prostaglandins. Selective drugs only block rate-limiting enzyme COX-2 activity, while non-selective drugs also function as COX-1 inhibitor, where COX-1 is a "house-keeping enzyme" expressed in most tissues (*123, 124*). However, a significant number of reports have identified various side effects of NSAIDs prescription ranging from gastrointestinal damage to cardiovascular death (*26, 27, 124*). Gastrointestinal damage is the most common NSAID-induced adverse effect resulting from the suppression of cell migration (*125*). High incidence of NSAID-stimulated gastrointestinal injury has been reported in elderly people (*126*). Likewise, in a study of outpatients with stable atherothrombotic disease, elevated cardiovascular events were most frequently observed in older female white individuals following prolonged usage over a period of 4 years (4,420/44,095) (*26*).

2.4 BIOACTIVE PHYTOCHEMICALS AS NOVEL THERAPEUITIC AGENTS

In some cases of pharyngitis, patients are treated with two or three antibiotics due to the constraints in diagnosis or multiple infections. However, bacterial pathogen *S. pyogenes* has developed antibiotic-resistance owing to the worldwide misuse of antibiotics (*4, 127-130*). Patients, who are allergic to treatment with antibiotics, could develop serious undesirable side effects once used. Further, biofilm has been examined in its role leading to the failure of antibiotic therapy. Though in various of research, serious side effects of NSAIDs have been determined for the treatment of inflammatory diseases (*25, 26*). Thus,

there is an urgent and constant need to discover new and potent antibacterial, anti-biofilm and anti-inflammatory agents for the substitution of antibiotic and NSAIDs therapy. In attempt to find alternatives, various natural products have been investigated over past several decades (*131*). In general, medicine derived from plants dates back to prehistory and people from all continents have benefited from plant-based traditional medicine (*132*).

Major groups of natural antimicrobial, anti-biofilm and anti-inflammatory agents include plant secondary metabolites such as phenolic acids, quinones, flavonoids, tannins, coumarins, terpenoids and alkaloids. Plant secondary metabolites serve as defense mechanisms against microorganisms, insects and herbivores or are responsible as signal molecules to interact with the environment such as releasing odors compounds or making colors to attract pollinating insects (*133*).

2.4.1 Anti-bacterial assays

Several parameters, like Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), are typically investigated in assessing the antimicrobial properties of a novel compound and to enable comparison with products of known antimicrobial activity. MIC is defined as the lowest concentration of an antimicrobial agent that can inhibit the visible growth of a microorganism after overnight incubation. MBC is the lowest concentration of an antimicrobial compound that is able to

kill a bacterium. Viability of the bacterial inoculum should be reduced by ≥ 99.9 %. These methods are commonly used to diagnose antibiotic bacterial resistance and also to determine *in vitro* antimicrobial activity of a new compound (*134*). Agar or broth dilution methods, including the tube broth method and the micro-dilution method, can be used and adapted to determine both parameters. The plate hole well diffusion assay and the disc diffusion method can also be used to determine the antibacterial activity of a compound by measuring the diameter of the inhibition zone. Various antibacterial testing methods and their advantages and disadvantages are summarized in Table 2.3.

Antimicrobial activity can also be tested by using mixed bacterial culture assays in order to mimic the microbial ecosystem. For example, *in vitro* fermentation assays which reproduce the active microbiota in chicken gut, was used to determine inhibition of *Campylobacter jejuni* by terpenoid compounds (*135*). To further characterize the antimicrobial properties, a compound can also be tested *in vitro* using mouse or human macrophages and *in vivo* using experimental mouse models of infection (*136, 137*). *Ex-vivo* testing, using freshly excised dog and cat skin samples maintained under viable conditions *(*for a short period of time) has been used to test the antimicrobial efficiency of some chemicals and biocides (*138*).

2.4.2 Anti-biofilm assays

Anti-biofilm activity of phytochemicals can be investigated using different parameters such as percentage of inhibition, minimum biofilm inhibitory concentration (MBIC), and minimum biofilm eradication concentration (MBEC) (*143*). Percentage of inhibition is often calculated based on the reduction as compared to a positive control (biofilm, no treatment) (*144*). MBIC is defined as the minimum phytochemical concentration where no biofilm could form. MBEC is the lowest phytochemical concentration causing a significant decrease in sessile cells in comparison to positive control ($p \leq 0.05$) (*145*). Previous studies have addressed various methods on anti-biofilm activity of phytochemicals (*12, 146, 147*). Colorimetric methods, including crystal violate staining and MTT reduction assays are used in numerous studies. CFU counting is also involved in anti-biofilm assays to enumerate biofilm. In Table. 2.4, different anti-biofilm testing methods with their advantages and limitations are listed.

2.4.3 Anti-*Streptococcus pyogenes* activity of bioactive phytochemicals

A diverse range of phytochemical antibacterial agents has been reported to suppress the growth of *S. pyogenes*, including polyphenols, isoprenoids and plant extracts. In the category of polyphenols, epicatechin, catechin, gallic acid, 3-*O*-methyl gallic acid, and caffeic acid extracted from tea possessed antibacterial capacities (*148*). Among these five

Table. 2.3 Description and comment of different antibacterial methods

Method	Short description	Advantage	Disadvantage	References
Broth Dilution Method (BDM): -Tube Broth Method (TBM)	TBM: Serial dilutions of antibacterial agents in broth tubes, then addition of the adjusted bacterial inoculum and incubation.	Quantitative, simultaneous and easy	Poor growth of many anaerobic microorganis	(*139*)
-Broth Micro-Dilution Method (BMDM)	BMDM: Serial dilutions of antibacterial agents in 96- well plates.	Use of absorbance and indicator dyes	Inaccurate if antimicrobial causes clumping of cells	
Agar Dilution Method (ADM)	Serial dilutions of antibacterial agents in agar medium, followed by inoculation of adjusted bacterial inoculum on the surface of the medium and incubation.	Simultaneous and quantitative	Time-consuming ; labor- intensive	(*139*)
Disc Diffusion Method (DDM)	Bacterial inoculum adjusted to certain concentration and spread on agar media. Filter paper discs (6mm diameter) loaded with antibacterial agents are placed on the surface of agar and plates are incubated.	Inexpensive and simple	Inaccurate ; time-consuming ; labor- intensive	(*140*); (*141*); (*142*)

compounds, the most potent phytochemicals against *S. pyogenes* were catechin, gallic acid and epicatechin, with a proportion of growth against control of 0% for catechin and gallic acid and 5% for epicatechin (*148*). Another effective phenolic compound extracted from green tea was epigallocatechin gallate (EGCG). A MIC of 100 μg/mL for the inhibition of *S. pyogenes* was shown using green tea extract containing 94% of EGCG. In the same study, the MIC of tested probiotics strains *Lactobacillus* sp. tested was much higher (> 800 μg/mL), indicating that EGCG would not interfere with the function of potential beneficial bacteria. Moreover, depending on the probiotic strain used and the tea concentration, the combination of probiotic strains and green tea extract showed a 3- to 30- fold reduction in the viability of *S. pyogenes*, compared to green tea extract alone. Besides, the combination of this green tea extract and probiotic strains demonstrated enhancement of the suppression efficiency, in, demonstrated a synergic effect and a potential combined therapy for streptococcal pharyngitis (*152*). Chamuangone is a polyisoprenylated benzophenone isolated from the leaves of *Garcinia cowa*, a plant commonly consumed in southern Thailand. This compound exhibited antibacterial activity against several bacteria including *S. pyogenes* and *S. aureus (153)*.

Medicinal and edible plant extracts play a significant role as the source of antibacterial agents. For example, an ethaolic extract from the brown cortex of a

predominant plant from Iran, Oak (*Quercus brantii*) fruits showed high antibacterial effect on *S. pyogenes*. In addition, tannin and phenolic compounds were thought to contribute to the suppressive capacity (*154*). In a previous study, all the ethanol extracts of three medicinal plants of Khuzestan (Iran) had an inhibitory effect on *S. pyogenes* (*155*). Antibacterial activities of 51 different extracts prepared with three types of solvents (water, ethanol and methanol) of 16 different Turkish medicinal plant species were tested against 10 bacteria. Among them, 11 plant extracts, from 7 different plants were active against *S. pyogenes*, especially ethanol extract of *P. pungens* (*156*). Several water extracts of plant species have also been tested. For example, aqueous extracts of leaves of *Schinus lentiscifolius* demonstrated antimicrobial activity against *S. pyogenes* (MIC=125 μg/mL) (*157*). Antimicrobial activities of several leaves and inter-nodal callus extracts of *Mentha arvensis* using different type of solvents, has also been demonstrated (*158*). For the leaf extract, an ethanol and water extract provided the best antimicrobial effect against *S. pyogenes* while ethanol and ethyl acetate was most effective for inner nodal segment extracts.

Essential oils carry the fragrance of plants and are often highly enriched in terpenes which can provide antimicrobial activity against bacteria and other microorganisms (*133*). Essential oils of six species of *Eucalptus*, revealed certain antibacterial ability. Among

these, *Eucalptus odorata* essential oils possessed the best activity against *S. pyogenes,* but also presented a significant cytotoxicity on eukaryotic *in vitro* cell lines (*159*). Olbas® Tropfen is a complex commercially available essential oil distillate (peppermint oil, eucalyptus oil, cajuput oil, juniper berry oil and wintergreen oil), and is traditionally used to treat headaches, colds and cough. Its antimicrobial activity, and those of its individual essential oil components, especially peppermint EO and Olbas®, have been shown to display inhibition of *S. pyogenes* strains. Moreover, these last two exhibited bactericidal activity against the tested strain after 24 h of incubation with a concentration of 5 mg/mL for Olbas® and 2.5 mg/mL for peppermint EO, respectively (*160*). Carvacrol, a major component of essential oils of *Origanum* and *Thymus* plants, has been tested for anti-*Streptococcus pyogenes* activity against 32 erythromycin-resistant strains. The results revealed that carvacrol functions as a promising alternative therapy in the presence or absence of erythromycin (*161*).

2.4.4 Anti-biofilm activity of bioactive phytochemicals

A wide spectrum of phytochemicals have also shown anti-biofilm activity. For example, polyphenol-rich wine and barley coffee were found to display anti-biofilm activity against *Streptococcus mutants* (*162, 163*). In a study of medicinal plants from Caatingga, Brazil, of 45 aqueous extracts from different parts of 24 Caatingga regional

Table 2.4. Various anti-biofilm methods and their advantages and disadvantages

Method	Short description	Advantages	Disadvantages	References
Crystal violet staining	Serial dilutions of phytochemical treatment before (MBIC) or after (MBEC) biofilm formation followed by staining biomass with crystal violet.	Cheap and quantifying the biofilm biomass.	Undistinguishable for live or dead cells.	(*12*); (*143*)
MTT reduction assay	Serial dilutions of phytochemicals exposure before or after biofilm formation followed by MTT biomass metabolic reduction.	Distinguishable for live or dead cells; easy and fast.	Susceptible to respiration rate of bacteria and biofilm thickness.	(*149*); (*150*)
Colony counting method	Pre- or post-treated biofilm with serial diluted phytochemicals scraped from wells. Well contents removed and centrifuged. Supernatant resuspended and plated on agar plate.	Numerable for active biofilm cells.	Time-consuming and labor-intensive; error-prone.	(*151*); (*147*)

plant species, stem bark of *Commiphara leptophloeos* exerted the strongest anti-biofilm effect on *Staphylococcus epidermidis*. Inhibitory activity (67.3 ± 8.5%) was reported at the concentration of 0.4 mg/mL (*164*). Anti-biofilm potential was also highlighted in crude extracts of Italian plants, for example, *Quercus cerris*, a plant commonly used as a traditional ethnotherapy of diarrhea, leucorrhea, hemorrhoids, and rheumatism. The butanol extracts of leaf and stem/fruit parts of *Quercus cerris*, were found to be the most active against *Staphylococcus aureus* at a test dose of 200 μg/mL, with 63 ± 10% and 74 ± 4% inhibition, respectively (*165*). Ethanol extract of *Rhodomyrtus tomentosa*, and its main component rhodomyrtone, also showed markedly anti-staphylococcal activity and was demonstrated to exhibit greater capacity compared to vancomycin (*34*). Further, the improvement of anti-biofilm efficiency of ciprofloxacin, together with plant extract (Zingerone), suggested presence of a synergistic effect and a potential adjunct therapy for biofilm-related diseases attributed to *Pseudomonas aeruginosa* (*166*).

Anti-biofilm activity is observed in essential oils as well. For example, cinnamon bark oil and its major constituent, cinnamaldehyde, significantly decreased biofilm formation of human pathogen *Pseudomonas aeruginosa* at 0.05%, while lower concentration (0.01%) of essential oils (cinnamon bark oil, cinnamaldehyde, and eugenol) was indicated in the suppression of *Escherichia coli* O157:H7 biofilm formation (*167*). In

another study, lemongrass essential oil was shown to be superior to grape fruit essential oil in suppressing the biofilm formation of *S. aureus*, whereas, neither essential oil showed capacity to eradicate formed biofilms (*143*). As similar phenomenon can be found in the study of cranberry extracts against *E. coli* and *Staphylococcus* species (*168*). Though cranberry extracts inhibited biofilm production, no eradicative ability was illustrated in established biofilm. Other studies have indicated that biofilm of oral pathogens were also susceptible to impact by essential oils. As reported by Ciandrini *et al*. (2014), in addition to significant anti-bacterial activity, carvacrol was shown to possess a greater inhibitory property than chlorhexidine (a cationic polybiguanide antibacterial compound) in single or multiple biofilm formation of *Streptococcus mutants*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* (*169*).

It is noted that studies on phytochemicals against biofilm of *S. pyogenes* are limited. Essential oils from *Pogostemon* has been investigated as potentially promising anti-biofilm agents. A potent biofilm suppression of 35-40% was noticed for 5% essential oils from *Pogostemon* and the highest concentration of essential oils (15%) were demonstrated to possess 60-70% inhibition (*14*). Besides, the extracts of three Thai plant species (*Boesenbergia pandurate* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk.) also demonstrated significant inhibition of biofilm formation at

a concentration of 0.24-125 μg/mL (*170*).

2.4.5 Anti-inflammatory activity of bioactive phytochemicals

Phytochemicals offer a great potential as an alternative therapy for inflammatory disorders. Various *in vitro* and *in vivo* studies have claimed the inhibitory activity of phytochemicals related to inflammation. Polyphenols, including apigenin and rosmarinic acid, were reported to possess anti-inflammatory activity. As previously illustrated (*80*), apigenin suppresses phorbol myristate acetate (PMA)-induced expression of pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, and TNF-α) and activator protein-1 (AP-1) factors, leading to the inhibition of inflammation in A549 cells (lung epithelial cells). AP-1 belongs to a family of transcription factor and contributed to inflammation. In another study, rosmarinic acid was tested in rat models of local (carrageenan-induced rat paw oedema) and systemic inflammation (liver ischaemia-reperfusion and thermal injury models). Both inhibitory and protective effects of this polyphenol were observed, respectively (*33*). The same animal oedema model was used in several other anti-inflammatory studies. *Carlina acanthifolia* root essential oil and thymol both demonstrated a weight drop response of rat oedema in a dose-dependent manner (*20, 171*). In addition to the weight reduction, wound healing was also indicated in treatment with thymol.

Carvacrol, a phenolic monoterpene, was shown to suppress inflammation by

decreasing the mRNA and protein secretion of pro-inflammatory markers such as COX-2, TNF-α, IL-6, inducible nitric oxide synthase (iNOS) and NF-κB in D-galactosamine induced hepatotoxic rats (*35*). Different studies have emphasized the importance of NF-κB in the inflammation process. It is recognized that phytochemicals act as anti-inflammatory agents by down-regulating the expression of pro-inflammatory mediators through attenuating NF-κB activation (*172, 173*).

2.4.6 Potential medicinal activities of summer savory

Summer savory (*Satureja hortensis* L.) is a well-known aromatic herb widely distributed in different areas of Iran, Anatolia region of Turkey and southern Europe (*36, 174, 175*). Summer savory is an annual plant (height: 10-35 cm) with purplish or white flowers and linear leaves (*176*). Despite its culinary function, summer savory has been used as a folk medicinal therapy for cardiovascular disease, various ailments including cramps, muscle pains, nausea, indigestion, diarrhea, and infectious diseases (*177, 178*).

Recently, numerous studies have addressed the anti-bacterial, anti-fungal, anti-oxidant and anti-inflammatory activities of summer savory. Those pharmacological characteristics mainly reside in the bioactive phytochemicals from aerial parts of plants. Polyphenol-rich extracts and essential oils have been investigated as the major components of summer savory phytochemicals (Fig. 2.1). Low concentration of essential oils (17-130

μL/L) were indicated to inhibit the growth of Gram-positive bacteria *Staphylococcus aureus* (*179*). Another study also confirmed the anti-bacterial effect of its essential oil on different pathogens including *S. aureus* and *S. pyogenes*. Carvacrol, comprising 67% of essential oil component, was regarded as the major contributor to bacterial suppression (*178*). Anti-microbial activity of summer savory essential oils is also observed in fungi. Strong inhibitory effects of carvacol- and thymol- rich essential oil on growth and production of aflatoxin by *Aspergillus parasiticus* were observed (*175*). The major polyphenol constituent, rosmarinic acid, was found to be predominant in providing high anti-oxidant activity of summer savory extracts. A high correlation coefficient of 0.85 was examined between rosmarinic acid concentration and anti-oxidant activity in the same study. The therapeutic potential of summer savory phytochemicals as anti-inflammatory agent have been suggested in several studies. As illustrated in Uslu *et al*. (2003), rhinosinusitis-treated rabbit exposed to summer savory aqueous extract showed a significant decrease in $NO\cdot$ metabolites and edema formation (*180*). Reduced edema was also indicated in a carrageenan-induced rat model after the administration of summer savory hydroalcoholic extract and essential oil (*181*).

In Canada, summer savory is an adventive herb brought by early settlers and widely used in cookery. Sufficient in phytochemicals associated with therapeutic activity,

summer savory may offer a promising novel choice for incorporation into dehydrated honey products intended for use in pain relief and the antimicrobial treatment of upper respiratory indications including sore throat.

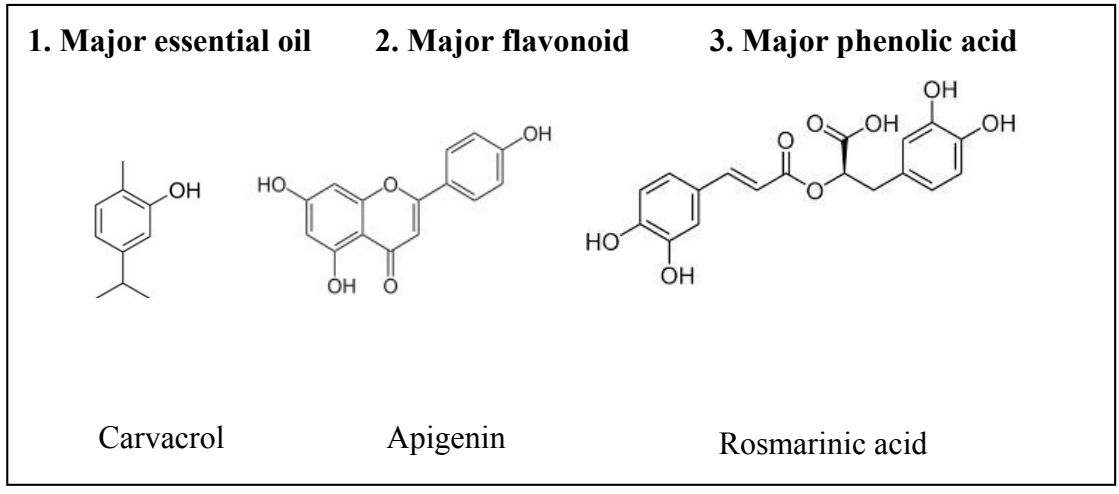

Fig. 2.1 Chemical structures of selected bioactive components of summer savory

CHAPTER 3 ANTI-INFLAMMATORY ACTIVITY OF SUMMER SAVORY PHYTOCHEMICALS ON LIPOPOLYSACCHARIDE (LPS)-INDUCED INFLAMMATION IN THP-1 DERIVED MACROPHAGES

3.1 ABSTRACT

Inflammation is a pathophysiological process mediated by various signaling molecules produced by leukocytes, macrophages and mast cells. Summer savory (*Satureja hortensis* L.) has been reported to possess anti-inflammatory activity. Essential oils extracted from stems, leaves, and fresh whole plant and commercial essential oil in addition to their major constituents including carvacrol, p-cymene, α-terpenine, γ-terpenine, β-myrcene and caryophyllene were involved in this study. In addtion, water and ethanol extracts containing polyphenols such as apigenin and rosmarinic acid were also tested. Polyphenols present in crude extracts were identified and quantified using UPLC-MS. Rosmarinic acid and apigenin were found as the major constituents. All summer savory phytochemical components, except essential oils extracted from fresh whole plant and commercial essential oil, showed no cytotoxicity to THP-1 macrophages at concentrations of 50 μg/mL and below in the 3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) - 2H - tetrazolium (MTS) assay. Isoprenoids, such as commercially available essential oil, essential oil extracted from leaves, p-cymene and carvacrol, and polyphenols present in ethanol extract from top-cut leaves, apigenin and

rosmarinic acid showed the greatest anti-inflammatory activity ($p \leq 0.05$) by down-regulating the protein secretion of cyclooxygenase-2 (COX-2), prostaglandin-2 (PGE_2), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in THP-1 differentiated macrophages stimulated using lipopolysaccharide (LPS).

Key words: Summer savory phytochemicals, inflammation, COX-2, PGE_2, IL-6, TNF-α

3.2 INTRODUCTION

Inflammation is a defense process of the host to the stimuli such as irritation, toxins, and pathogens (*182*). The inflammatory response is characterized by redness, swelling, pain and heat sensation at the site of infection (*82*). Secretion of pro-inflammatory mediators from macrophages such as cyclooxygenase-2 (COX-2), prostaglandin-E_2 (PGE_2), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) plays an important role in the inflammatory response (*82, 183-185*). However, over production of pro-inflammatory mediators may cause inflammatory diseases such as rheumatoid arthritis and ulcerative colitis (*185*) or tissue injury (*185, 186*).

Non-steroidal anti-inflammatory drugs (NSAIDs) are often used as inhibitors of the COX-2 pathway which blocks COX-2 derived prostaglandins (*187*). Among NSAIDs, a specific drug like nimesulide only inhibits isoenzyme COX-2 which is expressed only when inflammation is induced. Whereas, a non-specific drug, like diclofenac, inhibits both

COX-1 and COX-2, where COX-1 is a constitutive isoenzyme in most tissues (*26, 188*). However, gastrointestinal disorders and increase of cardiovascular risk have been reported as common side effects of NSAIDs (*26, 188*).

Natural products provide new and potentially potent accessories to anti-inflammatory therapy for inflammatory disorders. Summer savory (*Satureja hortensis* L.), is an established species of the *Satureja* genus, and has been widely used as a culinary herb (*37*). The most abundant polyphenols in summer savory such as apigenin and rosmarinic acid, have been shown in different cell models to possess anti-inflammatory capacity by decreasing the expression of inflammatory factors including IL-6, IL-8, COX-2, nitric oxide (NO), and TNF-α (*189-191*). In addition, phenolic fractions and essential oils of summer savory, have also been reported by reducing carrageenan-stimulated paw edema in rats to possess anti-inflammatory activity (*192*).

The present study aimed to identify specific summer savory phytochemicals as potential anti-inflammatory agents using an *in vitro* experimental model of THP-1 differentiated macrophages with LPS-induced inflammation. The specific objectives were to: (1) extract and quantify summer savory phytochemicals; and (2) examine anti-inflammatory capacity of summer savory phytochemicals by measuring the protein secretion of pro-inflammatory cytokines (COX-2, PGE_2, TNF-α and IL-6) as compared to

two commonly prescribed anti-inflammatory drugs (nimesulide and diclofenac).

3.3 MATERIALS AND METHODS

3.3.1 Preparation of summer savory phytochemicals

Summer savory plants were collected from Farmer John's Herbs located at Canning, Nova Scotia. Essential oil constituents such as carvacrol, p-cymene, α-terpenine and γ-terpenine, β-myrcene and caryophyllene and polyphenols including apigenin and rosmarinic acid were purchased from Sigma-Aldrich (Oakville, ON, Canada). Commercial summer savory essential oil was purchased from Liberty Natural Products Inc. (Oregon City, OR, USA). Essential oils from stems, leaves, fresh whole plants of summer savory were extracted by steam distillation method as described by Annan *et al*. (2013) (*37*). Briefly, 50 g of milled, dried stem or leaf or fresh whole plant of summer savory were added into a Clevenger apparatus with 500 mL of distilled water and hydro-distilled for 3 h. The essential oils were collected followed by drying over anhydrous Na_2SO_4 and stored in dark bottles at 4ºC. Summer savory extracts were prepared by solvent-based extraction methods (*193*). Water and ethanol were used as solvents to obtain polyphenol-rich phytochemicals. In brief, ethanol (95%, 50 mL) was added to the dried and milled top-cut leaves (leaves of top section of the plant) or oven dried fresh top-cut plants (leaves, flowers, and stems of top section of the plant) of summer savory (5.0 g) in flasks. The mixtures

were then subjected to ultra-sonication in the ultrasonic bath of 20 kHz/1000 Watts (model 750D, VWR, West Chester, PA, USA) at temperature between 20 and 28ºC for 60 min (3 times for 15 min each with 10 min interval in between). Ethanol extracts were then concentrated using a rotary evaporator (Rotavapor, R-200, Buchi, Flawil, Switzerland) at 30ºC followed by nitrogen drying to evaporate ethanol. Top-cut leaves (commercial powder) of summer savory were extracted by water (100%, 100 mL) in the water bath (BVS Hetomix, Heto-Holten, Allerød, Denmark) at the temperature of 80 to 90ºC with shaking speed of 40% for 20 min. Freeze drying in an FTS Dura-Stop kinetics freeze dryer (Kinetics, FTS Systems Inc, Stone Ridge, NY, USA) was employed to remove water for 29 h. Extracts were prepared in triplicate and stored at -80ºC for further use.

3.4.2 UPLC-MS analysis of summer savory phytochemicals

Analyses of phenolic compounds present in summer savory extracts were performed according to the methods described by Xie *et al*. (2011) and Rupasinghe *et al*. (2010) (*194, 195*). All analyses were performed using an ultra pressure liquid chromatography unit (H-class, Waters, Milford, MA, USA) equipped with Micromass Quattro micro API MS/MS system and controlled with Masslynx V4.0 data analysis system (Micromass, Cary, NC, USA). Separation of samples was carried out using Aquity BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) (Waters, Milford, MA, USA). The binary

gradient of mobile phase was consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A linear gradient profile was used as follows: Solvent A applied at time t (min); (t, A%): (0, 94%), (2, 83.5%), (2.61, 83%), (2.17, 82.5%), (3.63, 82.5%), (4.08, 81.5%), (4.76, 80%), (6.75, 20%), (8.75, 94%), (12, 94%).

Electrospray ionization in negative ion mode (ESI-) was employed for the analysis of polyphenols. The following ionization conditions were applied: capillary voltage of 3000V, nebulizer gas (N_2) temperature of 375 ^{o}C, and a flow rate of 0.35 mL/min. Quantification was conducted using single ion-monitoring (SIM) mode using specific precursor/product ion transitions as compared to standards: m/z 359.1 for rosmarinic acid, m/z 269.0 for apigenin, m/z 301 for quercetin (Q), m/z 609 for Q-3-*O*-rutinoside, m/z 463 for Q-3-*O*-glucoside and Q-3-*O*-galactoside, m/z 448 for Q-3-*O*-rhamnoside, m/z 594.75 for Q-3-*O*-arabinogluside, m/z 273 for phloritin, m/z 435 for phloridzin, m/z 353 for chlorogenic acid, m/z 179 for caffeic acid, m/z 193 for ferulic acid and isoferulic acid, m/z 289 for catechin, m/z 290 for epicatechin, m/z 305 for epigallocatechin, m/z 457 for epigallocatechin gallate, and m/z 441 for epicatechin gallate.

3.3.3 Cell culture

THP-1 human monocytes (ATCC®TIB202^{TM}) were obtained from Cedarlane (Burlington, ON, Canada) and maintained according to the manufacturer's instructions.

The cells were cultured in RPMI-1640 medium with 10% fetal calf serum and 0.05 mM 2-mercaptoethanol at 37 ºC in humidified 5% CO_2 (CO_2 incubator, Model 3074, VWR International, West Chester, PA, USA). Sub-culturing at the cell density of 1×10^6 cells/mL was performed after the initial culture period of 6 to 8 days. The medium was removed after 2-3 days and the subculture cells were preserved in the fresh medium supplemented with 5% (w/v) DMSO. THP-1 monocytes (5×10^5 cells/well) were seeded into a 24-well plate and differentiation to macrophages (THP-1/M cells) was stimulated by incubating the cells with 0.1 μg/mL phorbol myristate acetate (PMA) for 2 days. The differentiated cells were washed by pre-warmed Hanks buffer (Cedarlane, Burlington, ON, Canada) and incubated in fresh serum free medium overnight for further use.

3.3.4 Cell viability assay

MTS (3- (4,5- dimethylthiazol-2-yl) -5- (3- carboxymethoxyphenyl) -2- (4-sulfophenyl) - 2H - tetrazolium)assay was employed to measure cell viability, which is based on the color-reduction of tetrazolium salts from pale yellow to dark brown due to formation of the water soluble formazan pigment resulting from the action of mitochondrial dehydrogenases in live cells, while dead cells show pale yellow color (*196*). Macrophages were plated in 96-well plate at the density of 2.5×10^4 cells per well. The cells were then treated with 10, 25, 50 or 100 μg/mL summer savory phytochemicals and

two non-steroidal drugs nimesulide and diclofenac. After 24 h incubation, the medium was removed and 100 µL of fresh medium (serum free) was added to each well. Then, MTS reagent (20 µL, freshly prepared by mixing 2 mL MTS and 100 µL PMS) was added to each well and incubated at 37 ºC for 4 h. Absorbance was then measured at 490 nm by using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The percentage of viable cells was calculated as follows:

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ the\ treated\ wells - Absorbance\ of\ Blank}{Absorbance\ of\ the\ control\ wells - Absorbance\ of\ Blank} \times 100\%$$

Where, the treated wells contained the cells incubated with test compounds, the control wells contained the cells incubated in media without test compounds, and the Blank wells contained culture medium only with MTS reagent.

3.3.5 Summer savory phytochemical treatment for inflammation

Differentiated THP-1 cells were treated with non-cytotoxic concentrations of 25 µg/mL and 50 µg/mL of 14 summer savory phytochemicals for 4 h followed by LPS-induced inflammation for 18 h. Two non-steroidal anti-inflammatory drugs nimesulide and diclofenac were used as positive control. DMSO (0.05% (v/v)) was used as the negative control.

3.3.6 COX-2 assay

The human COX-2 ELISA kit (Enzo Life Sciences Inc. Faringdale, NY, USA) was

employed for the quantitative determination of COX-2 in cell culture lysates. After inflammation, the cells were washed with PBS to remove the test compounds. Trypsin EDTA was added into each well to create a suspension of cells. The cells were then centrifuged at 1000 rpm for 4 min followed by washing with PBS. Cell culture lysates were prepared by macerating cells in TNE buffer (10 mM Tris, pH 8.0, 0.15 M NaCl, 1% NP-40, 1mM EDTA) on ice using a smasher. Standards provided with the kit and test samples were added into appropriate wells. The plate was sealed and incubated at 37ºC for 1 h. During the incubation period, the wash solution was prepared by diluting 25 mL of the supplied concentrate with 975 mL of deionized water. The wells were emptied and washed with 200 µL wash solution for 7 times. The labeled antibody was then pipetted into each well, except the blank. After incubating at 4ºC for 30 min, the plate was decanted and rinsed 9 times with 200 µL wash solution. Then, 3, 3', 5, 5'- tetramethyl benzidine (TMB) substrate (100 µL) was added into each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 100 µL of stop solution. The absorbance was detected at 450 nm by a microplate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The COX-2 concentration in each well was calculated in ng/mL.

3.3.7 PGE_2 assay

The PGE_2 Express EIA kit, obtained by Cayman Chemical Company (Ann Arbor,

MI, USA) was used to determine the protein concentration of PGE_2 in the cell culture supernatant. Enzyme immunoassay buffer, standards, test samples, PGE_2-ACE tracer and PGE_2-monoclonal antibody were added into appropriate wells as described in the protocol provided by the manufacturer. The plate was covered and incubated for 18 h at 4 ºC. Subsequently, the wells were emptied and rinsed with wash buffer for 3 times followed by the addition of substrate. The covered plate was incubated for 1 h at 37ºC. Then stop solution (50 µL) was added into each well and the absorbance was measured at 590 nm using a micro-plate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The PGE_2 concentration in each well was calculated in pg/mL.

3.3.8 TNF-α assay

The commercial ELISA kit supplied by BD Biosciences (Mississauga, ON, Canada) was used to determine the protein secretion of TNF-α in the cell culture supernatant. Standards were prepared according to the instructions provided with the kit. Test samples were added into designated wells, and then the plates were incubated for 2 h at room temperature with sealer covered. Each well was aspirated and washed 3 times with wash buffer. Then, 100 µL of freshly prepared working detector (mixing enzyme concentrate with detection antibody = 1:250) was added into each well followed by 1 h incubation period at room temperature. Wash steps were performed by aspirating and washing with

wash buffer for 7 times. After adding 100 μL of substrate reagent, the plates were incubated for 30 min at room temperature in the dark. Then, 50 μL of the stop solution was added into each well and the absorbance was read at 450 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtech, Burham, NC, USA). The TNF-α concentration in each well was calculated in ng/mL.

3.3.9 IL-6 assay

The protein concentration of IL-6 was measured by ELISA kit provided by BD Biosciences (Mississauga, ON, Canada). The ELISA procedure was the same as illustrated in 3.3.8.

3.3.10 Statistical analysis

Completely randomized design was used for all experiments, which were conducted in triplicate and twice, independently. The normal distribution of the data was tested using Anderson-Darling test. The statistical analyses were carried out by using one-way ANOVA in Minitab 17 statistical software and multiple comparison of means was conducted using Tukey's test at $p \leq 0.05$. In addition, transforming PGE_2 level with the treatment of summer savory isoprenoids to reciprocal of square root was needed to satisfy the assumptions of ANOVA as described by Montgomery (2013) (*197*).

3.4 RESULTS

3.4.1 Quantification of summer savory polyphenols

The distribution of phenolic compounds in summer savory crude extracts was determined using methods previously described (*194, 195*). Rosmarinic acid and apigenin were found to be the most predominant components in water and ethanol extracts (Table 3.1). Specifically, rosmarinic acid ranked the highest amount in ethanol and water extracts from top-cut leaves and while apigenin was found to be the highest concentration in ethanol extract from oven dried whole plants.

3.4.2 Cell viability

The cytotoxicity of summer savory phytochemicals were determined using MTS assay in THP-1 differentiated macrophages. A concentration range of 10, 25, 50, and 100 μg/mL were used in the experiment for the treatment of summer savory phytochemicals for 24 h. Cell viability showed a dose-dependent response in most of testing compounds (Table 3.2). Almost all summer savory phytochemicals below 50 μg/mL showed low cytotoxicity on the macrophages, except essential oil extracted from fresh whole plant (EOP) and commercial essential oil (EOC), which demonstrated significant cytotoxic towards cells at this concentration with cell viability of 12.8% and 26.2%, respectively. Cells were less viable at high concentration of 100 μg/mL in most of the test compounds.

LPS and two non-steroidal drugs (nimesulide and diclofenac) were non-cytotoxic at test concentrations between 1 μg/mL and ≤ 50 μg/mL, respectively.

3.4.3 Protein secretion of inflammatory biomarkers

Summer savory polyphenols showed a dose-dependent inhibitory effect on inflammatory biomarkers. With the increase of summer savory polyphenol concentration, the protein level of all four inflammatory biomarkers (COX-2, PGE_2, TNF-α and IL-6) decreased. As shown in Fig. 3.1, apigenin and rosmarinic acid possessed the most effective anti-inflammatory potential as compared to other summer savory polyphenols ($p \leq 0.05$). By contrast, the water extract showed no inhibitory activity of the production of inflammatory biomarkers. Specifically, the COX-2 and TNF-α protein concentrations were significantly lowered ($p \leq 0.05$) with the treatment of rosmarinic acid, apigenin, and ethanol extract at the concentration of 50 μg/mL, whereas rosmarinic acid showed equal inhibitory activity to the anti-inflammatory drug diclofenac. Apigenin strongly decreased the IL-6 concentration and similar anti-inflammatory activity to that of the drug nimesulide which showed the greatest inhibitory activity. The water extract showed no suppression ability towards TNF-α or IL-6 levels; however, it significantly decreased the secretion of COX-2 and PGE_2 at concentrations of both 25 μg/mL and 50 μg/mL. Similarly, summer savory isoprenoids decreased the production of inflammatory biomarkers in a dose-

dependent manner (Fig. 3.2). Commercial essential oil, essential oil extracted from leaves, carvacrol and p-cymene effectively reduced the concentration of inflammatory biomarkers ($p \leq 0.05$). By contrast, essential oil extracted from stems showed the lowest effectiveness (or no effectiveness) in inhibiting the secretion of inflammatory biomarkers such as TNF-α, IL-6, and PGE_2. However, commercial essential oil possessed the greatest inhibitory ability causing reduction levels of COX-2 at 25 μg/mL as equal to nimesulide and diclofenac at 50 μg/mL, followed by essential oil extracted from leaves, carvacrol and p-cymene. In addition, TNF-α and IL-6 levels were significantly impaired to nearly 50% by p-cymene at a concentration of 50 μg/mL. Moreover, carvacrol and commercial essential oil provided approximately 25% and 43% reduction in TNF-α and IL-6 productions at the concentration of 25 μg/mL, respectively and in comparison to LPS control. All isoprenoids demonstrated identical suppressive activity in reducing PGE_2 concentration. Essential oil extracted from stems significantly lowered the production of IL-6 while at the same time demonstrating no reducing ability in the production of COX-2, TNF-α, and PGE_2.

3.5 DISCUSSION

Summer savory (*S. hortensis* L.) has been used in traditional medicine for various ailments such as cold, cramps, muscle pain, stomach ache and intestinal disorders, cholera,

Table. 3.1 Concentration of polyphenols (mg/100 g of dried extract) in summer savory crude extracts as determined by UPLC-MS

Category	Test compounds	Water extract	Ethanol extract	
		Top-cut leaves	Top-cut leaves	Top-cut whole plants
Flavonoid	Apigenin	5.4±2.1	47.0±1.7	140.6±3.5
	Quercetin (Q)	8.8±6.3	16.5±1.4	2.3±0.6
	Q-3-*O*-glucoside	11.1±0.3	3.6±0.4	0.1±0.1
	Q-3-*O*-rhamnoside	0.8±0.1	5.1±0.01	1.0±0.02
	Q-3-*O*-galactoside	8.7±0.5	18.0±1.4	0.4±0.1
	Q-3-*O*-rutinoside	15.4±0.4	31.6±1.1	0.1±0.04
	Q-3-*O*-arabinogluside	0.3±0.2	0.1±0.07	0.03±0.01
	Luteolin	18.3±7.6	26.2±0.2	49.6±2.7
Phenolic acid	Rosmarinic acid	410.4±8.07	1404.6±10.0	21.2±6.0
	Phloridzin	5.2±0.3	4.7±0.4	0.6±0.3
	Phloritin	0.4±0.2	4.6±0.3	2.1±0.04
	Chlorogenic acid	6.8±0.7	12.3±1.7	0.9±0.8
	Isoferulic acid	41.5±4.8	29.0±2.0	23.8±1.6
	Cafeic acid	105.6±6.9	45.6±1.7	9.4±0.2
	Ferulic acid	1.4±0.1	1.7±0.3	0.7±0.04
Caffeic acid	EGC	1.5±0.5	2.6±0.4	3.1±0.4
	Catechin	0.4±0.4	0.5±0.3	0.5±0.3
	Epicatechin	0.7±0.06	0.2±0.05	0.6±0.04
	EGCG	0.5±0.1	0.8±0.5	0.8±0.3
	ECG	0.4±0.06	1.2±1.1	0.4±0.04
Total phenolics (mg/100 g dried extract)		643.6±31.5	1655.6±3.3	256.9±9.6

***Data are presented as mean ± SD. Q, quercetin; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate.**

diarrhea and cardiovascular diseases (*180, 198*). It has been revealed that essential oils, flavonoids such as apigenin and apigenin-4'-methyl ether and phenolic acids such as labiatic acid, also known as rosmarinic acid, were the main components of the aerial parts

Table 3.2 Percentage cell viability after exposure of THP-1 differentiated macrophages to summer savory phytochemicals

Test compounds	Concentration (μg/mL)					
	1	10	25	50	100	0.05% DMSO
Cont.	ND	ND	ND	ND	ND	100^{a-b}
LPS	$93.3\pm8.7^{a-c}$	ND	ND	ND	ND	ND
EE	ND	ND	109.7 ± 5.7^{a}	$89.6\pm12.3^{a-d}$	12.2 ± 4.1^{h}	ND
WE	ND	ND	$97.7\pm5.7^{a-b}$	$110.6\pm18.9^{a-b}$	$91.7\pm24.5^{a-d}$	ND
Api	ND	ND	$86.0\pm3.3^{a-d}$	$91.5\pm7.8^{a-d}$	$88.7\pm7.1^{a-d}$	ND
Ros.acid	ND	$99.9\pm0.5^{a-b}$	$93.4\pm5.7^{a-c}$	$93.3\pm8.7^{a-c}$	ND	ND
EOC	ND	$85.7\pm3.9^{a-d}$	$84.9\pm4.7^{a-e}$	$26.2\pm6.0^{g-h}$	0.6 ± 0.9^{h}	ND
EOL	ND	109.1 ± 2.1^{a}	$95.0\pm0.9^{a-d}$	$101.1\pm10.0^{a-b}$	$86.1\pm2.9^{a-d}$	ND
EOS	ND	$88.7\pm0.6^{a-d}$	$90.0\pm6.2^{a-d}$	$85.8\pm0.2^{a-d}$	$84.2\pm1.9^{a-e}$	ND
EOP	ND	$87.1\pm3.8^{a-d}$	$85.8\pm0.03^{a-d}$	12.8 ± 13.1^{h}	1.7 ± 2.4^{h}	ND
α-Ter	ND	$87.1\pm8.5^{a-d}$	$91.6\pm7.6^{a-d}$	$84.0\pm4.4^{a-e}$	$52.7\pm0.2^{f-g}$	ND
γ-Ter	ND	$101.3\pm4.1^{a-b}$	$107.5\pm4.1^{a-b}$	$83.0\pm0.4^{a-e}$	$80.6\pm11.3^{b-e}$	ND
Cary	ND	$96.3\pm10.7^{a-b}$	$87.2\pm3.7^{a-d}$	$88.0\pm4.3^{a-d}$	ND	ND
Carva	ND	$92.3\pm1.0^{a-d}$	$85.8\pm0.6^{a-d}$	$82.8\pm2.9^{a-d}$	$57.4\pm1.1^{e-f}$	ND
p-Cy	ND	$90.5\pm3.1^{a-d}$	$84.1\pm2.9^{a-d}$	$80.4\pm0.5^{a-e}$	$51.0\pm0.3^{f-g}$	ND
β-My	ND	$93.6\pm2.7^{a-c}$	$95.3\pm21.4^{a-c}$	$83.7\pm1.4^{a-e}$	ND	ND
Diclo	ND	ND	$87.9\pm2.0^{a-e}$	$81.2\pm0.01^{a-e}$	$67.5\pm10.6^{d-f}$	ND
Nime	ND	ND	$91.8\pm2.3^{a-d}$	$82.9\pm1.9^{a-e}$	$70.5\pm5.4^{c-f}$	ND

*** Macrophages were incubated with 10, 25, 50, and 100 μg/mL of test compounds (apigenin, rosmarinic acid, SSO, SSC, S2, L2, carvacrol, α-terpinene, p-cymene, γ-terpinene, caryophellene, β-myrcene) for 4 h. Cell viability is presented as the percentage compared to the control. Data were shown as mean ± SD (n=3). Cont.: Control (0.05% DMSO); LPS: 1 μg/mL lipopolysaccharide; EE: ethanol extract from top-cut leaves; WE: water extract; Api: apigenin; Ros.acid: rosmarinic acid; EOC: commercial essential oil; EOS: essential oil from stems; EOL: essential oil from leaves; EOP: essential oil from fresh whole plants;α-ter: α-terpinene; γ-ter: γ-terpinene; Cary: Caryophyllene; Carva: Carvacrol; p-Cy: p-Cymene; β-My: β-myrcene; Diclo: Diclofenac; Nime: Nimesulide; ND: Not detected.**

of summer savory (*192*). The present study also confirmed this observation, where rosmarinic acid and apigenin were quantified to be the dominant constituents in crude water and ethanol extracts. However, the concentration of rosmarinic and apigenin showed significant difference as compared to a previous study. As reported in Krasniewska *et al*. (2014), markedly higher concentration of rosmarinic acid was reported in water extract than in ethanol extract while both extracts exhibited similar amount of apigenin (*199*). In this study, the ethanol extract from top-cut leaves demonstrated the highest amount of rosmarinic acid while the greatest concentration of apigenin was observed in ethanol extract from plants. This might result from different extraction procedures and origin of the raw plant materials. Essential oil composition is generally constant for different parts of summer savory, however, the concentration shows an area-dependent characteristic. Carvacrol is the major component of summer savory essential oils, consisting of highest amounts of 25-45% as reported in Portugal and Canada (*37, 200*). This isoprenoid even occurs at the concentration of up to 87% in summer savory essential oil sourced from Turkey (*201*). γ-Terpinene, p-cymene, α-terpinene, β-myrcene and caryophyllene were also found as the main constituents in summer savory essential oils (*37, 200*). Further gas chromatography studies would be necessary to quantify the isoprenoids-based bioactive components in summer savory essential oils.

Inflammation is a complex pathophysiological process, where following activation, macrophages play an essential role in phagocytosis of cellular and extracellular debris during inflammation and healing (*88, 202*). THP-1 differentiated macrophages, a widely used cell model for human inflammatory disease, is involved in inflammatory responses by the stimulation of LPS which is a constituent of the outer membrane of Gram-negative bacteria (*183, 202*). Recently, several attempts have been made to identify natural alternative for anti-inflammatory agents due to the side effects of using non-steroidal anti-inflammatory drugs. Plant-sourced chemicals, such as essential oils and polyphenols, have received growing attention and have been investigated to possess anti-inflammatory capacity (*88, 203, 204*). A limited number of studies have focused on the anti-inflammatory activity of summer savory phytochemicals. This study provided *in vitro* evidence of the inhibitory effect of summer savory phytochemicals in acute inflammation using the experimental model of THP-1 differentiated macrophages.Numerous studies have suggested that phytochemicals exhibit inhibitory effect on inflammation activity by limiting the expression of pro-inflammatory mediators such as COX-2, PGE_2, IL-6 and TNF-α (*88, 205*). COX-2 is an important enzyme in the biosynthetic pathway of PGE_2 which belongs to the panel of eicosanoids. PGE_2 is a lipid mediator produced from arachidonic acid and exerts pro-inflammatory effect via increasing the vasodilation and

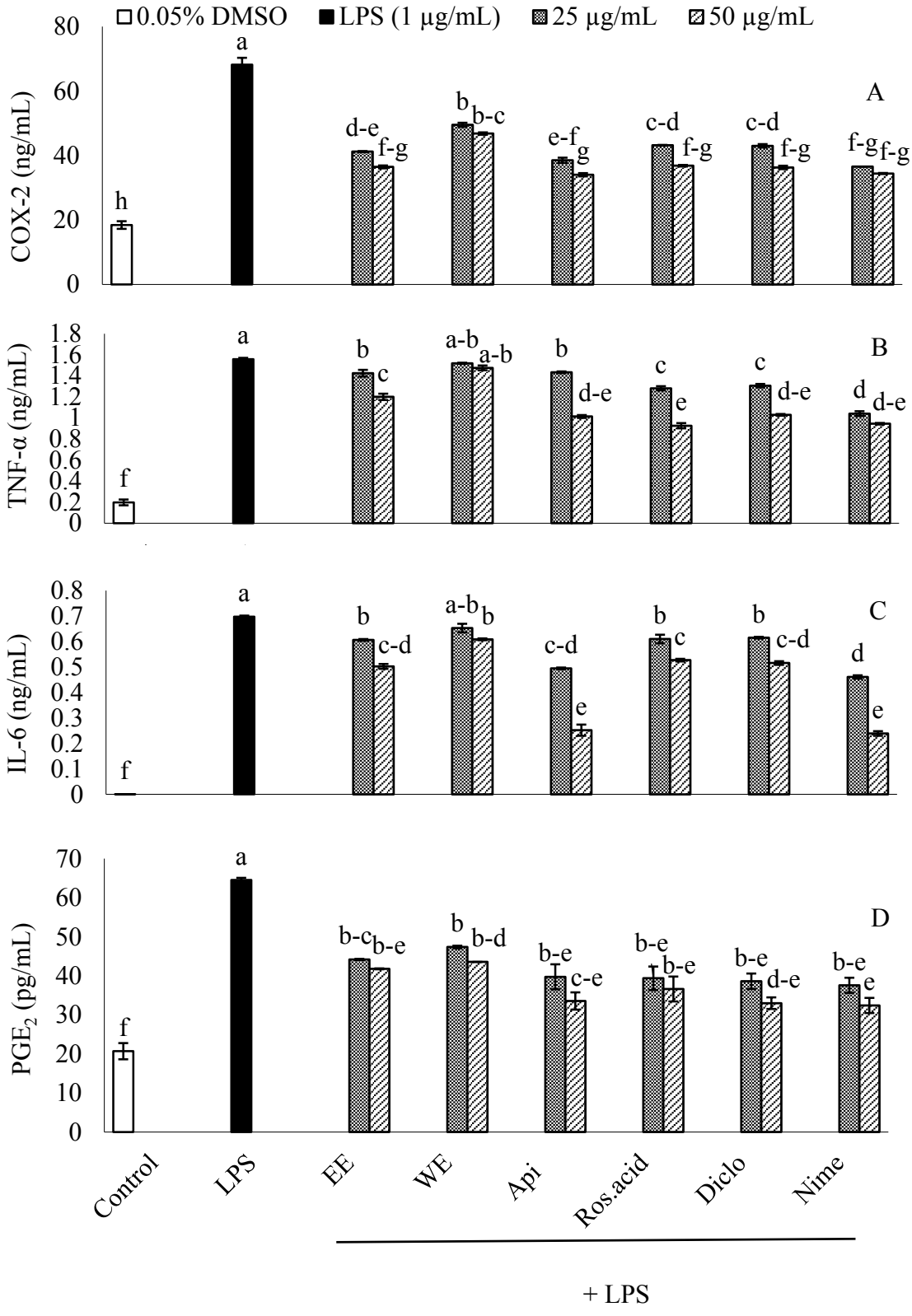
0.05% DMSO
LPS (1 µg/mL)
25 µg/mL
50 µg/mL
COX-2 (ng/mL)
TNF-α (ng/mL)
IL-6 (ng/mL)
PGE2 (pg/mL)
A
B
C
D
Control
LPS
EE
WE
Api
Ros.acid
Diclo
Nime
+ LPS

Fig. 3.1 Protein secretion of inflammatory biomarkers (COX-2 (A), TNF-α (B), IL-6 (C) and PGE_2 (D)) after pre-treatment with summer savory polyphenols in LPS-activated THP-1 differentiated macrophages *in vitro*. THP-1 monocytes were differentiated into macrophages with PMA for 2 days. Cells were pre-treated with 25 µg/mL and 50 µg/mL summer savory polyphenols (ethanol extract from top-cut leaves, water extract from top-cut leaves, apigenin, rosmarinic acid) for 4 h, followed by LPS-induced inflammation for 18 h. Data were presented as mean ± SD (n=3), Tukey's test, $p \leq 0.05$. Means sharing the same letter are not significantly different. Control: 0.05% DMSO; LPS: 1 µg/mL lipopolysaccharide; COX-2: cyclooxygenase-2; PGE_2: prostaglandin-E_2; IL-6: interleukin-6; TNF-α: tumor necrosis factor-alpha; EE: ethanol extract from top-cut leaves; WE: water extract from top-cut leaves; Api: apigenin; Ros.acid: rosmarinic acid; Diclo: diclofenac; Nime: nimesulide.

local blood flow and cell proliferation (*106, 111*). A 10 to 80 fold increase in the production of COX-2 has been reported in various cells including macrophages in response to the stimulation of inflammatory cytokines and endotoxins (*104*). The expression of COX-2 and PGE_2 has been indicated to be correlated with tumor development and different cancers (*206-208*). Down-regulating the production of these mediators represents a promising strategy for therapy of inflammation (*209, 210*).

In this study, the loss of cell viability was not observed in all test phytochemicals below 50 µg/mL. The exception was observed in commercial summer savory essential oil and essential oil extracted from fresh whole plant. Based on the previous anti-inflammatory investigation of *Rosmarinus officinalis*, a culinary herb, up 200 µg/mL of ethanol extract showed no cytotoxicity in THP-1 cells (*211*). However, as reported in Ocana-Fuentes *et al*.

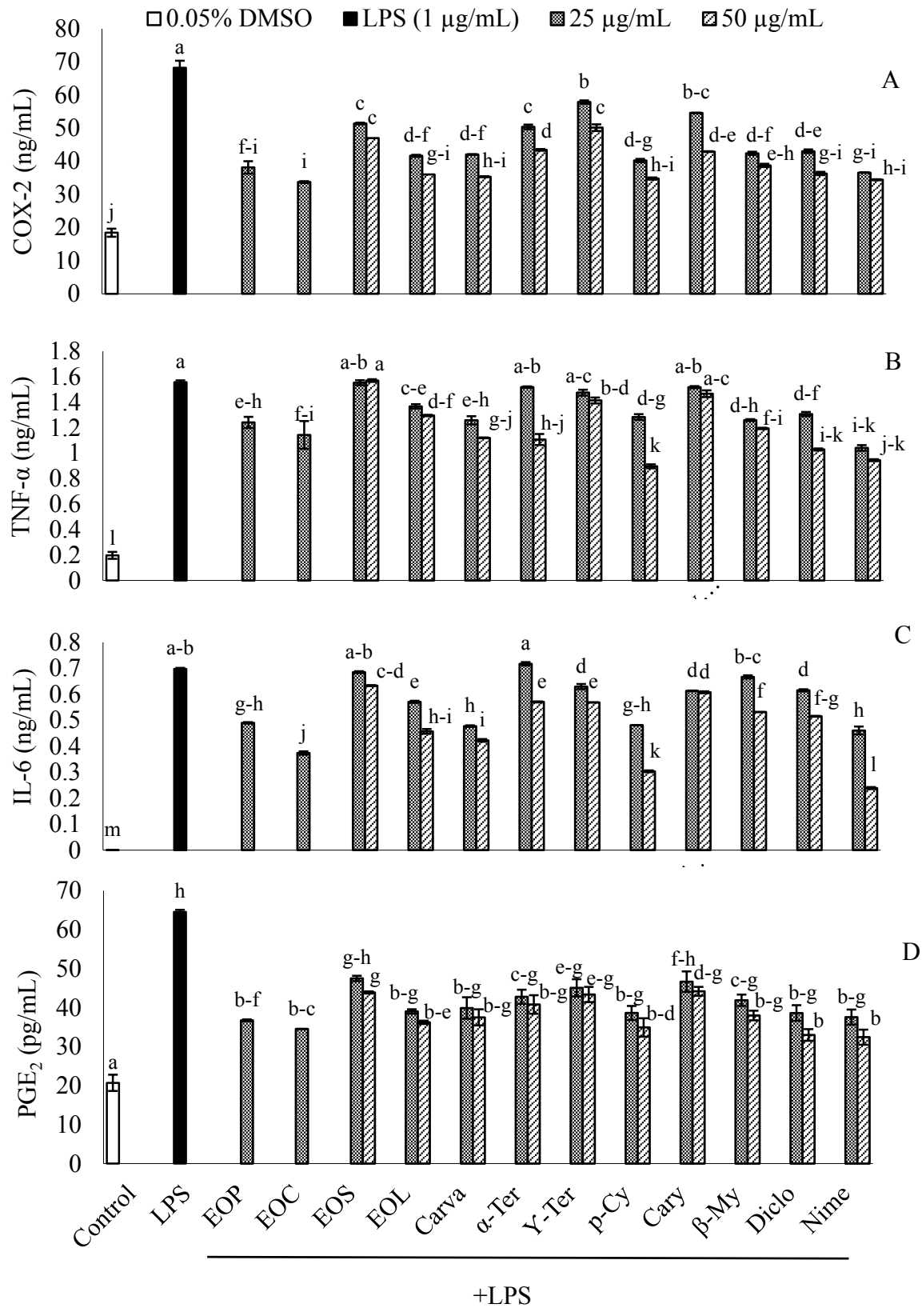
0.05% DMSO
LPS (1 µg/mL)
25 µg/mL
50 µg/mL
A
COX-2 (ng/mL)
B
TNF-α (ng/mL)
C
IL-6 (ng/mL)
D
PGE2 (pg/mL)
Control
LPS
EOP
EOC
EOS
EOL
Carva
α-Ter
Y-Ter
p-Cy
Cary
β-My
Diclo
Nime
+LPS

Fig. 3.2 Concentration of inflammatory biomarkers (COX-2 (A), TNF-α (B), IL-6 (C) and PGE_2 (D)) released by THP-1 differentiated macrophages treated with LPS and summer savory isoprenoids. THP-1 monocytes were induced to differentiation in the presence of PMA for 2 days. Cells were then pre-treated with summer savory isoprenoids (essential oil extracted from stems (EOS), essential oil extracted from leaves (EOL), carvacrol (carva), p-cymene (p-Cy), α-terpenine (α-Ter) and γ-terpenine (γ-Ter), β-myrcene (β-My) and caryophyllene (Cary)) at the concentration of 25 µg/mL and 50 µg/mL and essential oil from fresh whole plant (EOP) and commercial essential oil (EOC) at 25 µg/mL for 4 h, followed by LPS-stimulated inflammation for 24 h. Data are presented as mean ± SD (n=3), Tukey's test, $p \leq 0.05$. Means expressing different letters are significantly different.

(2010), essential oil rich in caryophellene and carvacrol extracted by supercritical fluids from oregano (*Origanum vulgare*) leaves caused a reduction in cell viability at concentrations higher than 30 µg/mL in THP-1 cell model (*212*).

The present study also showed that apigenin, rosmarinic acid, ethanol extract, commercial essential oil, essential oil extracted from leaves, carvacrol and p-cymene markedly suppressed LPS-induced protein expression of COX-2, PGE_2, TNF-α and IL-6 in THP-1 differentiated macrophages. The anti-inflammatory ability of essential oils might be due to the synergistic effect of isoprenoid constituents, since carvacrol and p-cymene both showed pronounced suppressive effect on decreasing the protein production of inflammatory biomarkers. However, as reported in Tung *et al*. (2008), minor constituents or synergic activity of constituents of essential oils from indigenous cinnamon (*Cinamomum osmophloeum*) twigs was suggested to contribute to the anti-inflammatory

activity in PGE_2 production since major components such as caryophyllene oxide and L-bornyl acetate showed no suppressive effects (*205*). Carvacrol, as the major component of summer savory essential oil has been reported as an anti-inflammatory agent in many studies (*35, 213*). In the research of LPS-induced inflammation of the porcine alveolar macrophages, carvacrol exhibited dose-dependent inhibitory activity resulting from the decrease of the protein secretion of TNF-α, and which is accordance with our study (*214*).

Similarly, synergic activity of rosmarinic acid and apigenin may contribute to the inhibitory capacity of ethanol extract. There is abundant evidence that rosmarinic acid and apigenin are involved in attenuating various inflammatory mediators and thus, inhibit inflammation (*189, 191, 215, 216*). It has been suggested that apigenin showed strong anti-inflammatory activity in the LPS-stimulated RAW 264.7 cells by suppressing NO production and inducible nitric oxide synthase (iNOS) and COX-2 expression at the concentration of 15-30 μM (*189*). Besides, apigenin at the concentration of 9.3 to 74 μM decreased the inflammatory factors including COX-2, intercellular adhesion molecule-1 (ICAM-1), reactive oxygen species (ROS), IL-6, and IL-8 through the suppression of nuclear factor kappa B (NF-κB) activation in *Helicobacter pylori*-infected gastric adenocarcinoma cells (*191, 215*). In the present study, significant inhibitory effect of apigenin was observed at the concentration of 92.5-185 μM. The probable reason for varied

effective concentration of apigenin would be due to different cells used and culture conditions in those studies. Hot water extract of dried leaves from *Artemisia* (A.) *annua* L., an annual wormwood whose major component was rosmarinic acid, showed a dose-dependent decrease of IL-8 and IL-6 secretion and achieved its maximal effect at 3300 μg/mL (*216*). However, in this study, hot water extract of summer savory expressed its suppressive effect on IL-6 secretion at markedly lower concentration of 25-50 μg/mL. This might be due to the different amount of rosmarinic acid or other different phytochemicals compositions and different cell types.

NF-κB is a transcription factor that plays a central role in inflammatory responses by regulating the expressions of numerous genes that code pro-inflammatory adhesion molecules such as E-selectin, cytokines including TNF-α, and enzymes (i.e.COX-2) (*35, 82, 190*). Under quiescent conditions, NF-κB is sequestered in the cytoplasm bniding with inhibitors of kappa B (I κB). Upon exposure to extracellular stimuli, NF-κB is activated and translocated into the nucleus and serves to up-regulate the expression of target genes including COX-2, TNF-α and IL-6 (*35, 217*). Inhibition of the NF-κB pathway has been documented as a mode of action of anti-inflammatory activity of phytochemicals (*82, 109, 218*). Hexane extracted fraction from *Laminaria japonica* (kelp) was shown to exert its anti-inflammatory effect on LPS-induced RAW 264.7 macrophages through the NF-κB

pathway through detection of the degradation of I κB determined by RT-PCR and western blot methods (*218*). Down-regulation of NF-κB signaling in the same cell model has also been observed in researching the anti-inflammatory ability of α-cyperone extracted from the rhizomes of *Cypeus rotundus* (nutgrass) (*82*). Sekhon-Loodu *et al*. (2015) illustrated the inhibitory effect of docosahexaenoic acid ester of phloridzin (PZ-DHA) on the production of pro-inflammatory biomarkers (COX-2, PGE_2, TNF-α and IL-6) through the suppression of NF-κB translocation in THP-1 differentiated macrophages (*109*). The NF-κB signal pathway provides a powerful illustration of mode of action, further study involving measuring the transfection of NF-κB is warranted.

3.6 CONCLUSION

Rosmarinic acid and apigenin were found as the main constituents of crude summer savory extract. Summer savory phytochemicals including polyphenols and isoprenoids exerted anti-inflammatory capacity using LPS-induced THP-1 differentiated macrophages. Cell viability was not influenced by summer savory phytochemicals below 50 μg/mL except commercial essential oil and essential oil extracted from fresh whole plant. Among the tested phytochemicals and extracts, commercial essential oil, essential oil extracted from leaves, carvacrol, p-cymene, ethanol extract from top-cut leaves, apigenin and rosmarinic acid were found to be the most effective in reducing the protein secretion of

pro-inflammatory cytokines such as COX-2, PGE_2, TNF-α and IL-6 ($p \leq 0.05$). Commercial essential oil showed greater inhibition of COX-2 than anti-inflammatory drug nimesulide at the concentration of 25 μg/mL. Rosmarinic acid (50 μg/mL) possessed equal inhibitory activity as anti-inflammatory drug diclofenac (50 μg/mL) in COX-2 and TNF-α assay. Thus, essential oils, ethanol extract from top-cut leaves and specific summer savory phytochemicals such as carvacrol, p-cymene, apigenin and rosmarinic acid offer potential alternatives for inhibiting inflammation. Further *in vivo* studies of mice model are warranted to confirm the present findings.

CHAPTER 4 EFFECT OF SELECTED SUMMER SAVORY PHYTOCHEMICALS AGAINST LIPOTEICHOIC ACID OR PEPTIDOGLYCAN-INDUCED INFLAMMATION IN HUMAN TONSIL EPITHELIAL CELLS

4.1 ABSTRACT

Streptococcal pharyngitis, one of the most common upper respiratory tract diseases around the world, is characterized by *Streptococcus pyogenes*-triggered inflammation in the throat. Tonsil epithelial cells are the main targets of *S. pyogenes* infection. Summer savory is a folk medicinal herb exerting anti-inflammatory activity. In this study, selected summer savory phytochemical components including polyphenols (ethanol extract from top-cut leaves, apigenin, and rosmarinic acid) and isoprenoids (essential oil extracted from leaves, commercially extracted essential oil, carvacrol, p-cymene and β-myrcene) were tested for their inhibitory activity against lipoteichoic acid (LTA) or peptidoglycan (PGN)-stimulated inflammation in human tonsil epithelial cells (HTonEpiC). The cells were activated by LTA/PGN for 4 h, followed by post-treatment with selected summer savory phytochemicals for 20 h. The cell viability was measured using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) method by measuring the differentiated color of live and dead cells indicated the non-cytotoxic effects below the concentration of 10 μg/mL of all tested phytochemicals. With the post-treatment of phytochemicals at 10 μg/mL, morphological changes of the cells

were observed in response to apigenin, rosmarinic acid and diclofenac. All phytochemicals showed significant reduction ($p \leq 0.05$) in the protein production of pro-inflammatory mediators such as interleukin-8 (IL-8), epithelial-derived neutrophil activating protein-78 (ENA-78), human beta defensin-2 (hBD-2), and granulocyte chemotactic protein-2 (GCP-2) in both LTA and PGN- simulated inflammation. By contrast, none of the phytochemicals demonstrated marked inhibition of LTA-induced IL-8 secretion. Apigenin, ethanol extract and β-myrcene were shown to be the most effective phytochemicals suggesting their potential to be used as novel therapeutic agents in treatment of streptococcal pharyngitis. Further studies are required to fully understand the anti-inflammatory capacity of these phytochemicals.

Key words: summer savory phytochemicals, human tonsil epithelial cells, streptococcal pharyngitis, inflammation, pro-inflammatory mediators

4.2 INTRODUCTION

Throat pain due to acute pharyngitis is a very common medical condition all over the world. Acute pharyngitis is characterized by the inflammation of the pharynx and manifest as pharyngitis or tonsillitis (*2*). Bacterial pharyngitis is often caused by Gram-positive bacteria *Streptococcus pyogenes* and accounting for 5% to 30% of acute pharyngitis. *S pyogenes* is responsible for 15%-30% of overall pharyngitis causes in

children, and 5%-10% among adults (*2, 43, 51*). School-aged children and adolescents are the most vulnerable groups to *S. pyogenes* (*2, 219*).

The mucosal layer of upper respiratory tract is the first line of colonization of *S. pyogenes* (*17, 18*). Upon recognition of this pathogenic bacteria, the host immune system is triggered and generates a robust inflammatory response (*220*). Cellular surveillance and recognition of the innate immune system is mediated by the recognition of nonself agents known as pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLRs) (*89*). Lipoteichoic acid (LTA) and peptidoglycan (PGN) are the major constituents of the cell wall of Gram-positive bacteria (*221*). These molecules are considered as the main PAMPs of *S. pyogenes* contributing to host inflammatory response (*221-223*). It has been widely reported that TLR4 or TLR2 is associated with *S. pyogenes*-induced inflammation (*220, 224*). These TLRs function as regulators in inducing different pro-inflammatory cytokines, chemokines and other molecules (*18, 101, 221*). Interleukin-8 (IL-8), epithelial-derived neutrophil activating protein-78 (ENA-78), human beta defensin-2 (hBD-2), and granulocyte chemotactic protein-2 (GCP-2) are major chemokines that are secrected by epithelial cells in response to *S. pyogenes* PAMPs (*23, 225-227*).

Diclofenac, belongs to the non-steroidal anti-inflammatory drugs (NSAIDs) and could be used for pain relief due to streptococcal pharyngitis (*228*). This non-specific drug

down-regulates inflammatory response by blocking inflammatory prostaglandin synthesis through inhibiting the activation of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes (*25*). Moreover, a wealth of data has demonstrated that phytochemicals present in herbs and fruits also suppress inflammation. These phytochemicals consist of polyphenols, isoprenoids and alkaloids, which have been shown to contribute to anti-inflammatory activity (*171, 229, 230*). Summer savory (*Satureja hortensis* L.), is a folk medicinal herb in Mediterranean areas and is recognized to exhibit anti-inflammatory activities (*180, 181*). In this study, it was hypothesized that specific phytochemical components of summer savory can provide an inhibitory effect on inflammatory biomarkers in the experimental mode of tonsil cells. Specifically, this research was designed to evaluate selected summer savory phytochemicals against LTA/PGN-induced inflammation by determining cell viability and protein secretion of pro-inflammatory biomarkers in a human tonsil epithelial cell culture model system.

4.3 MATERIALS AND METHODS

4.3.1 Chemicals and reagents

LTA, 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), carvacrol (≥ 98%, food grade (FG)),

apigenin (≥ 97%, thin-layer chromatography (TLC)), rosmarinic acid (≥ 98%, high performance liquid chromatography (HPLC)), p-cymene (99%),β-myrcene (≥ 90%, FG) 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, poly-L-lysine stock solution (10 mg/mL), trypsin neutralization solution (TNS), and tonsil epithelium cell growth supplement were purchased from ScienCell Research Laboratory (San Diego, CA, USA). Fetal bovine serum (FBS) was purchased from ATCC (Manassas, VA, USA). IL-8 ELISA kit was purchased from BD Biosciences (Mississauga, ON, Canada); human ENA-78 and GCP-2 ELISA kits from Ray Biotech, Inc. (Norcross, GA, USA); human BD-2 ELISA kit from PromoCell GmbH (Sickingenstraße, Heidelberg, Germany).

4.3.2 Human tonsil epithelial culture

HTonEpiC cells were maintained according to the manufacturer's guidelines. Poly-L-lysine coated flask (2 μg/cm^2 T-75 flask) was prepared before culturing the cells. Sterile water (10 mL) was added into the T-75 flask followed by addition of 15 μL of poly-L-lysine stock solution (10 mg/mL). After overnight incubation at 37 ºC with 5% CO_2 (Model 3074, VWR International, West Chester, PA, USA), the T-75 flask was washed with sterile water twice and replaced with 20 mL of complete medium (500 mL of tonsil

epithelium cell medium mixed with 5 mL of tonsil epithelium cell growth supplement). The cryopreserved cells were completely thawed in a 37 ºC water bath (ISOTEMP^{TM} Digital-Control Water Baths: Model 205, Fisher Scientific Company, Ottawa, ON, Canada). Subsequently, re-suspension of cells was carried out by dispensing the contents of the vial into the equilibrated, poly-L-lysine coated culture flask. After gently rocking the flask to distribute the cells evenly, the cell culture was initiated by an incubation period of 16-24 h. Medium was removed and fresh supplemented medium was added the next day after establishing the cells. Every three days, the medium was changed with fresh supplemented medium until the cells were approximately 70% confluent. The cells were sub-cultured when the culture reaches almost a confluence level of 90%.

Sub-culturing

The first, medium was aspirated and discarded followed by rinsing the cell layer with Dulbecco's phosphate buffered saline (DPBS) (Ca^{2+} and Mg^{2+} free). Then, 5 mL of DPBS and 5 mL of trypsin/EDTA were added to the flask. After gently rocking, the flask was incubated at 37ºC for 3-5 min. The trypsin/EDTA solution was subsequently transferred from the flask to a 50 mL centrifuge tube supplemented with 5 mL of fetal bovine serum. The empty flask was then continuously incubated at 37 ºC for 1 to 2 min. The detachment of cells was monitored under inverted microscope (ECLIPSE TS 100/TS

100-F, Nikon Instruments Inc., Melville, NY, USA). Trypsin neutralization solution (TNS) (5 mL) was then added and detached cells were transferred from the flask to the 50 mL centrifuge tube. Afterwards, additional 5 mL of TNS was added to harvest the residual cells. The harvested cells were then centrifuged at 1,000 rpm for 5 min. Following this procedure, the cells were re-suspended in growth medium. After counting under the inverted microscope using a haemocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA), the cells were plated in a second poly-L-lysine coated 96-well plate with a cell density of 5, 000 cells/well for further use.

4.3.3 Cell viability assay

Cells were cultured in 96-well plates at a density of 5,000 cells/well and treated with different concentrations of selected summer savory phytochemical components: ethanol extract, rosmarinic acid, apigenin, essential oil extracted from leaves, commercial summer savory essential oil, carvacrol, p-cymene, and β-myrcene in 5, 10, 25, 50 and 100 µg/mL at 37 ºC for 24 h. The cells in the solvent control group contained 0.05% DMSO without any phytochemical treatments. LTA and PGN with concentrations of 5, 20, and 50 µg/mL were used as bacterial antigen controls. Diclofenac (25, 50 and 100 µg/mL) was used as a positive control. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was employed to determine cell viability.

After 24 h incubation, the cells were refreshed by adding 100 μL of fresh medium followed by 4 h incubation in the presence of 20 μL of MTS mix (MTS:phenazine methosulfate (PMS)=20:1). The absorbance was measured at 570 nm by using a FLUOstar OPTIMA micro-plate reader (BMG Babtech, Durham, NC, USA). Cell viability was calculated as follows:

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ the\ treated\ wells - Absorbance\ of\ Blank}{Absorbance\ of\ the\ control\ wells - Absorbance\ of\ Blank} \times 100\%$$

Where, the treated wells contained the cells incubated with test compounds, the control wells contained the cells without compound treatments, and the Blank wells contained culture medium only.

4.3.4 Phytochemical treatment

The cells were seeded in 24-well plates (35,000 cells/well) and treated with 10 μg/mL of LTA/PGN for 4 h, followed by exposure to various 10 μg/mL of selected summer savory phytochemical components based on the cell viability results at 37 ºC for 20 h. Wells containing 4 h and 24 h stimulated LTA/PGN were used as controls for inflammation. Diclofenac was used as the positive control and 0.05% of DMSO in the absence of test phytochemicals was used as the negative control. Cell culture supernatants were collected and stored at -20 ºC for further determination of pro-inflammatory biomarkers.

4.3.5 Cell morphological assessment

After the treatment with 10 μg/mL selected summer savory phytochemicals, the cells were examined under inverted microscope (ECLIPSE TS 100/TS 100-F, Nikon Instruments Inc., Melville, NY, USA) with 40 x magnification. The images were captured and saved using a Lumenara Infinity camera (1-2 USB, 2.9 Megapixel, Lumenara Corporation, Ottawa, ON, Canada) coupled with capture and analyzing software (Infinity Analyze, Lumenara Corporation, Ottawa, ON, Canada).

4.3.6 IL-8 assay

The concentration of pro-inflammatory chemokine IL-8 was measured by using a commercial ELISA kit. The 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 appropriated wells. After gentle shaking for 5 s, the plates were covered with plate sealer and incubated for 2 h at room temperature. The wells were decanted and washed with 300 μL wash buffer (diluting 20 × wash concentrate with deionized water) for five repeat cycles. The detecting antibody (100 μL) was added into each well. The covered plates were incubated for 1 h at room temperature followed by rinsing steps with wash buffer. Then, 100 μL of TMB One-Step Substrate Reagent was pipetted into each well and incubated for

30 min at room temperature in the dark. Stop solution (50 μL) was added into each well before absorbance reading at 450 nm using a micro-plate reader (FLUOstar OPTIMA, BMG Babtech, Durham, NC, USA). The concentration of IL-8 in each sample was expressed in pg/mL using the standard curve.

4.3.7 ENA-78 assay

The Human ENA-78 ELISA Kit was used to detect the secretion of ENA-78 protein in the cell culture supernatant. This assay employed specific antibody for human ENA-78 coated on 96-well plates. After adding 100 μL standards and samples into appropriate wells, the plates were incubated for overnight at 4ºC after gentle shaking. The solution was then discarded and the plates were washed 4 times with wash solution. Then biotinylated antibody (100 μL) was pipetted into each well and incubated for 1 h at room temperature with gentle shaking. Washing steps were performed before the addition of Streptavidin solution (to provide enzyme activity) 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 addition of 100 μL of TMB One-Step Substrate Reagent into each well and incubated for an additional 30 min at room temperature with gentle shaking and protected from light. The reaction was terminated by adding 50 μL of the stop solution. The absorbance was measured at 450 nm. The ENA-78 concentration was calculated using a standard curve and

the data is expressed as pg/mL.

4.3.8 Human BD-2 assay

The content of human BD-2 in cell supernatants was determined by Human BD-2 ELISA kit. Anti-human BD-2 antibody coated plates were prepared by using detection antibody and avidin-horseradish peroxidase (HRP) conjugate according to the manufacturer's protocol. All incubations involved in this assay were carried out in room temperature. Briefly, the 96-well plates were initially coated with capture antibody and incubated overnight with plate sealing. After washing and blocking, standards and samples (100 μL) provided with the kit were added into appropriate wells and incubated for at least 2 h. The plates were washed with washing buffer followed by addition of detection antibody and incubated for 2 h. Subsequently, avidin-HRP conjugate was pipetted into each well and incubated for 30 min. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) liquid substrate was added into each well for color development. The plates were read at the absorbance of 405 nm.

4.3.9 GCP-2 assay

The human GCP-2 ELISA kit was used to measure the protein production of GCP-2. The ELISA procedure was similar as described in 4.3.7.

4.3.10 Statistical analysis

Statistical analysis was performed by using one-way ANOVA including multiple mean comparison by Tukey's test at $p \leq 0.05$ (*197*). All the experiments were conducted in triplicate and independently twice. The transformation of the amount of GCP-2 in PGN-induced inflammation to reciprocal was carried out to meet the requirement of normal distribution for ANOVA test.

4.4 RESULTS

4.4.1 Cytotoxic effect of selected summer savory phytochemical components on human tonsil epithelial cells

To determine the cytotoxicity of ethanol extract extracted from top-cut leaves, rosmarinic acid, apigenin, essential oil extracted from leaves, commercial summer savory essential oil, carvacrol, p-cymene, β-myrcene, commercial anti-inflammatory drug (diclofenac), and bacterial antigens (LTA and PGN) on the viability of human tonsil epithelial cells, cells were treated with 5, 10, 20, 25, 50, or 100 μg/mL of these compounds for 24 h. MTS assay was carried out and all cell viability percentages were calculated based on the untreated control. Cell viability showed a dose-dependent response after phytochemical treatments (Table 4.1). With the exception of ethanol extract from top-cut leaves, carvacrol, β-myrcene, and commercial essential oil, all the testing phytochemical

components showed slight/ no cytotoxicity on cells at the concentration of 10 and 25 μg/mL. The phytochemicals were cytotoxic at a concentration of 50 μg/mL and above. Bacterial antigen PGN demonstrated no cytotoxicity over all testing concentrations (10-100 μg/mL), while cell viability was reduced following treatment with 50 μg/mL of LTA. Diclofenac showed low cytotoxicity to the cells at all concentrations except 100 μg/mL.

4.4.2 Effect of test compounds on morphological changes of human tonsil epithelial cells

The human tonsil epithelial cells were examined under an inverted microscope after the treatment of the test compounds at concentration of 10 μg/mL for 24 h in LTA/PGN-triggered inflammation. The images are shown in Fig. 4.1 and Fig. 4.2, separately. The morphology between control and LTA or PGN-induced cells was similar. Both of them displayed polygonal shaped cells. Whereas, the significant morphological changes can be observed in the presence of some phytochemicals as well as in the response for diclofenac. The cells became round shaped after exposure to apigenin, carvacrol and diclofenac.

4.4.3 Inhibitory effects of selected summer savory phytochemicals on the secretion of pro-inflammatory biomarkers

Cells were induced with LTA or PGN for 4 h and then post-treated with selected summer savory phytochemicals at the concentration of 10 μg/mL for 20 h (Fig. 4.3 & Fig.

4.4). The results illustrated that polyphenols (ethanol extract from top-cut leaves, apigenin, and rosmarinic acid) and isoprenoids (essential oil extracted from leaves, commercial essential oil, carvacrol, β-myrcene, and p-cymene) possessed different inhibitory ability against the protein secretion of different pro-inflammatory biomarkers by human tonsil epithelial cells. In the study of LTA-induced inflammation, generally, polyphenolic phytochemicals were superior to isoprenoids in suppressing the production of all biomarkers except in the production of GCP-2, where commercial essential oil (51.1%) and β-myrcene (44.0%) showed similar and greatest activity to apigenin (46.2%). None of isoprenoids inhibited the induction of IL-8 while ethanol extract from top-cut leaves significantly reduced the secretion of GCP-2 by 34.2%. All test phytochemicals significantly decreased ENA-78 production ($p \leq 0.05$). All polyphenols, commercial essential oil and β-myrcene showed comparable inhibitory activity to diclofenac at the concentration of 10 μg/mL. Further, apigenin (50.1%) exerted greater suppressive effect on human BD-2 secretion compared with diclofenac (36.0%). The inflammation stimulated by PGN was similar to that of LTA (Fig. 4.2). Apigenin showed the greatest inhibitory activity for lowering all the test pro-inflammatory biomarkers. Apigenin showed greater or equal inhibitory effect on the generation of IL-8 (61.2%), ENA-78 (57.6%), hBD-2 (41.9%), GCP-2 (32.7%) compared to diclofenac where inhibitory activity of

26.0%, 48.3%, 30.9% and 33.8% was observed, respectively. All isoprenoids comparatively reduced the production of hBD-2 to a range of 22.8%-34.6%. Except carvacrol and essential oil extracted from leaves, all test phytochemicals showed similar activity in lowering the level of GCP-2. Diclofenac was comparable or inferior to tested summer savory phytochemicals in decreasing IL-8 secretion, while this drug was more effective than most of the isoprenoids including essential oil extracted from leaves, carvacrol, and β-myrcene in inhibiting the production of ENA-78.

4.5 DISCUSSION

Streptococcal pharyngitis is initiated from *Streptococcus pyogenes* adherence to tonsillar epithelial cells (*231*). Human as the only host for *S. pyogenes*, recognize this foreign agent via the innate immune system and a cascade of inflammatory responses including the expression of various cytokines and chemokines is triggered in its presence (*89, 122*). The interaction between TLRs and PAMPs plays an essential role in activating this robust inflammatory response (*17, 101*). LTA and PGN derived from Gram-positive bacterial cell wall are reported as common PAMPs in *in vitro* bacterial infection studies although many studies focused on using the bacterium itself as inflammatory inducer (*17, 23, 232-234*).

In this study, LTA and PGN succeeded in inducing inflammation in tonsil epithelial

cells through remarkable secretion of pro-inflammatory biomarkers IL-8, ENA-78, hBD-2, and GCP-2. Generally, more IL-8 and hBD-2 were produced by stimulation of PGN than LTA. A similar observations has been reported in other cell types (*232, 235, 236*).

Previous reports have emphasized the importance of the production of IL-8, ENA-78, hBD-2, and GCP-2 in relation to respiratory infections. IL-8 is also known as neutrophil activating peptide-1 and functions as a potent neutrophil chemoattractant. 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. Thus, IL-8 has been considered to be triggered by considerable inflammatory responses including bacterial infections (*122, 233, 237*). Likewise, ENA-78 and GCP-2 activate chemotactic recruitment as well as neutrophils (*115, 238*). In addition, enhanced angiogenesis and tumor development have also been reported as a result of the overexpression of GCP-2 (*118*). In addition to neutrophil chemotactic activation, hBD-2 serves as an anti-microbial peptide secreted due to external stimuli including bacterial infection or pro-inflammatory agents (*227, 239*). For example, IL-8 and GCP-2 were quantitatively detected by performing reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) in stimulated human basal epithelial cells (A549) and inflamed tonsillar tissues from different patients (*233*). ENA-78 showed low concentration

in tonsillitis in this study (*233*), which is contrary to our result where pronounced ENA-78 protein was expressed. This might be attributed to use of different test models. As reported by Hostanska *et al.* (2011), IL-8 and hBD-2 are secreted by a similar epithelial cell, A549, in response to respiratory infections. Studies conducted using bronchial epithelial cells have also confirmed the expression of hBD-2 in response to pathogens (*227, 240*). *S. pyogenes* infection has been demonstrated to be associated with the NF-κB signaling pathway. NF-κB is a transcription factor which plays an essential role in inflammatory responses. In resting cells, NF-κB remains inactivated and is coupled with inhibitor of κB (I-κB) in the cytoplasm. Upon stimulation of NF-κB by pro-inflammatory cytokines including TNF-α, IL-1β, bacterial antigens or oxidative stress, it translocates into the nucleus through the phosphorylation of IκBα protein by IκB kinase (IκK). The phosphorylated IκBα protein is then degraded and the activated NF-κB then contributes to regulate the transcription of target genes such as pro-inflammatory cytokines and chemokines (*241-243*). Upon internalization by *S. pyogenes* on human epithelial cells (HEp-2) translocation of NF-κB has been demonstrated (*244*). In addition to NF-κB signaling pathway, the mitogen activated protein kinase (MAPK) pathway is also involved in inflammatory response by *S. pyogenes* infection. MAPK cascades include the regulation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK, and c-jun N-

terminal kinase (JNK). Once triggered, MAPK is able to phosphorylate transcription factor like NF-κB and activator protein-1 (AP-1) and modulate different cellular activities (*241-243, 245*). As reported in Tsai *et al.* (2006), the increase in mRNA expression of IL-8 and IL-6 due to *S. pyogenes* infection was blocked by NF-κB and MAP kinase inhibitors, indicating the contribution of NF-κB and MAPK signaling pathways in modulating inflammatory response (*241*). Okahashi *et al.* (2003) indicated the phosphorylation of p38 MAPK after *S. pyogenes* infection in mouse osteoblastic cells (*246*). Similarly, in another study with pharyngeal epithelial cells, M protein on the surface of *S. pyogenes* activated a rapid phosphorylations of both NF-κB and p38 MAPK, suggesting the involvement of these pathways in *S. pyogenes* infection (*247*).

Recently, specific phytochemicals have gained increasing attention in the treatment of inflammation due to their high efficiency, low cost and relative safety with minimum side effects. Generally, those bioactive components exhibit their inhibitory activity by down-regulating pro-inflammatory biomarkers through different pathways. Phytochemical constituents from *Melastoma dodecandrum* such as ursolic acid, asiatic acid, terminolic acid, and casuarinin showed markedly anti-inflammatory activity of 35%-55% inhibition of IL-8 expression in IL-1β-activated human colonic epithelial cancer cells (HT-29) (*248*). Another study exploring the same cell line indicated that low concentration of acanthoic

Table. 4.1 Effect of test compounds on percentage cell viability using human tonsil epithelial cells

	Concentration (µg/mL)						
Test compounds	5	10	20	25	50	100	0.05% DMSO
Control	ND	ND	ND	ND	ND	ND	100^{ab}
LTA	$91\pm13^{a-h}$	$98\pm19^{a-b}$	$86\pm6^{a-h}$	ND	$66\pm10^{c-k}$	ND	ND
PGN	$96\pm3^{a-f}$	106 ± 5^{a}	$98\pm3^{a-c}$	ND	$93\pm5^{a-g}$	ND	ND
Ethanol extract	$95\pm7^{a-f}$	$95\pm11^{a-f}$	ND	53^{i-k}	4 ± 2^{n}	6 ± 4^{n}	ND
Apigenin	ND	$82\pm4^{a-i}$	ND	$82\pm1^{a-i}$	$81\pm3^{a-i}$	$41\pm0.3^{k-m}$	ND
Rosmarinic acid	ND	$93\pm1^{a-f}$	ND	$93\pm9^{a-g}$	$77\pm10^{b-i}$	$61\pm3^{h-k}$	ND
Essential oil from leaves	ND	$100\pm6^{a-b}$	ND	$83\pm0.8^{a-i}$	$74\pm9^{b-k}$	$64\pm14^{f-k}$	ND
Commercial essential oil	ND	$91\pm11^{a-g}$	ND	$16\pm4^{l-n}$	$10\pm5^{l-m}$	ND	ND
Carvacrol	$93\pm11^{a-g}$	$82\pm2^{a-i}$	ND	8 ± 3^{n}	8 ± 1^{n}	6 ± 1^{n}	ND
p-Cymene	ND	$97\pm5^{a-f}$	ND	$94\pm8^{a-c}$	$89\pm18^{a-g}$	$46\pm1^{j-l}$	ND
β-Myrcene	$93\pm8^{a-g}$	$97\pm19^{a-f}$	ND	$64\pm2^{g-k}$	4 ± 0.6^{n}	6 ± 2^{n}	ND
Diclofenac	ND	$89\pm4^{a-h}$	ND	$95\pm2^{a-c}$	$76\pm19^{b-j}$	$64\pm5^{e-k}$	ND

***Cells were treated with various concentration of test compounds for 24 h. Data were presented as cell viability (%) with mean ± SD (n=3), Tukey's test (p≤ 0.05). The value with different letters indicating the significant difference. ND: not detected.**

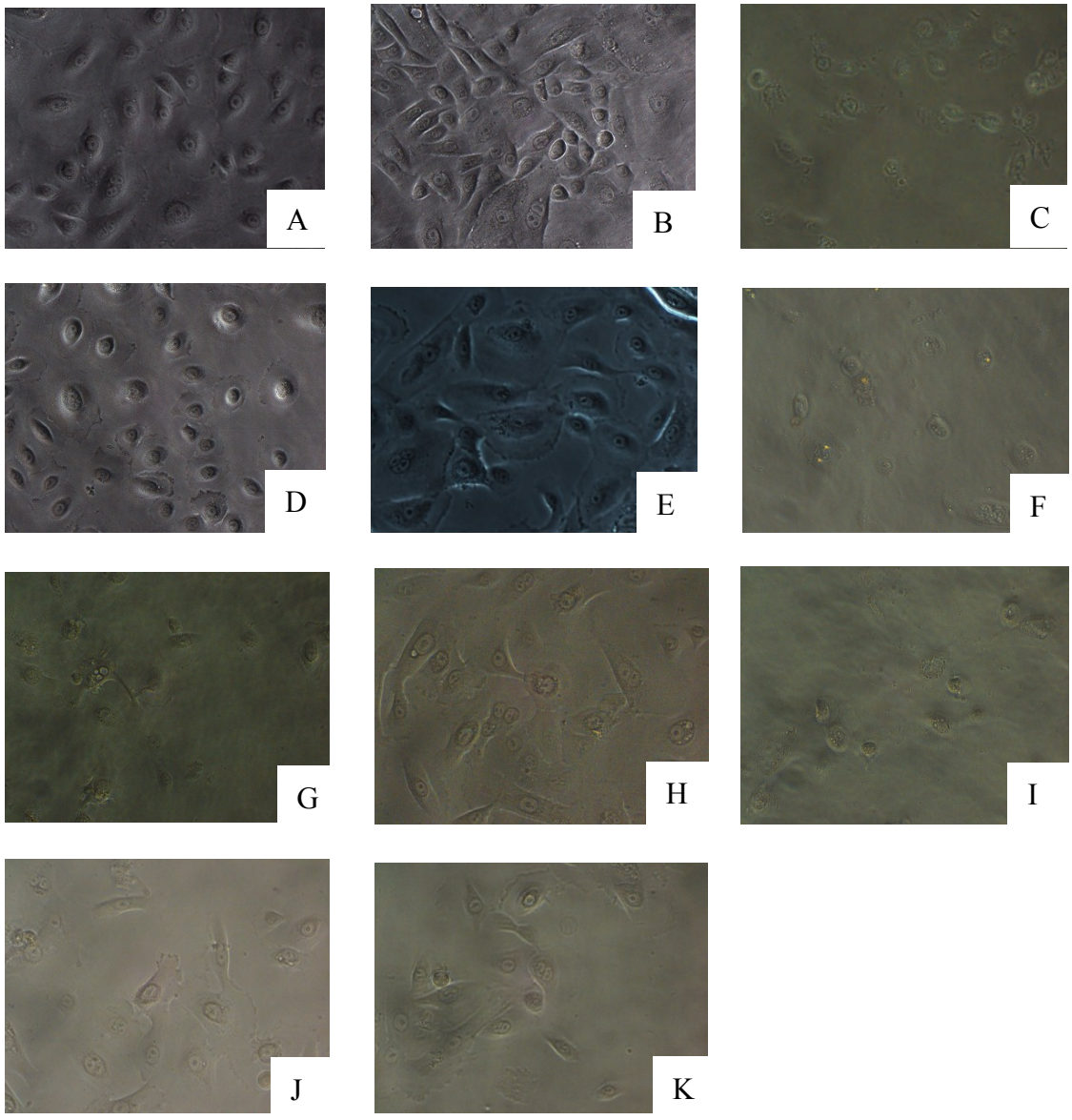

Fig. 4.1 Morphological changes of human tonsil epithelial cells after test compounds treatment at the concentration of 10 µg/mL for 24 h in LTA-induced inflammation. A): 0.05% DMSO; B): 10 µg/mL LTA; C): Diclofenac; D): Ethanol extract from top-cut leaves; E): Rosmarinic acid; F): Apigenin; G): Essential oil extracted from leaves; H): Commercial essential oil; I): Carvacrol; J): p-Cymene; K): β-Myrcene

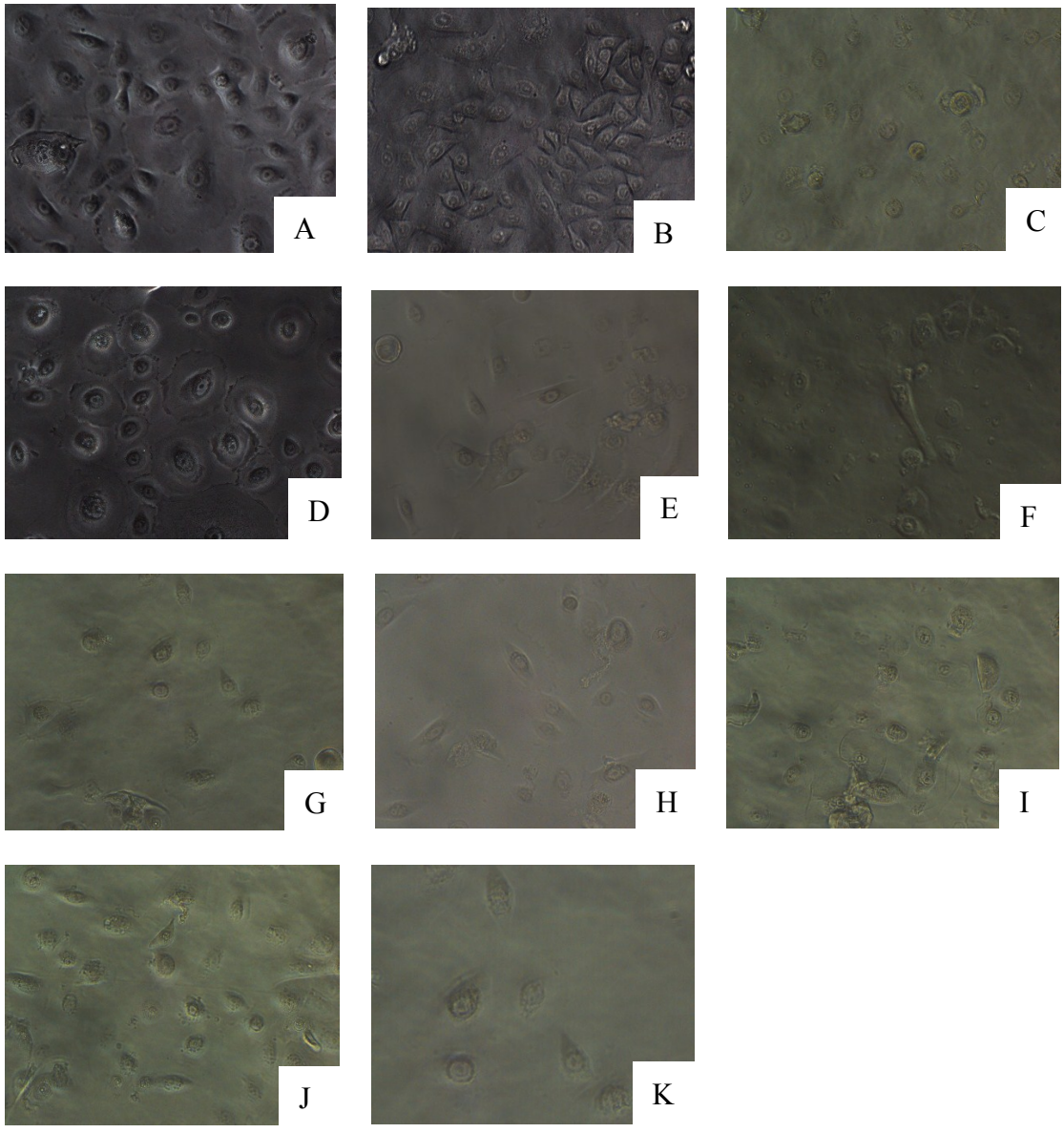

Fig. 4.2 Morphological changes of human tonsil epithelial cells after test compounds exposure (10 µg/mL) for 24 h in PGN-stimulated inflammation. A): 0.05% DMSO; B): 10 µg/mL PGN; C): Diclofenac; D): Ethanol extract from top-cut leaves; E): Rosmarinic acid; F): Apigenin; G): Essential oil extracted from leaves; H): Commercial essential oil; I): Carvacrol; J): p-Cymene; K): β-Myrcene

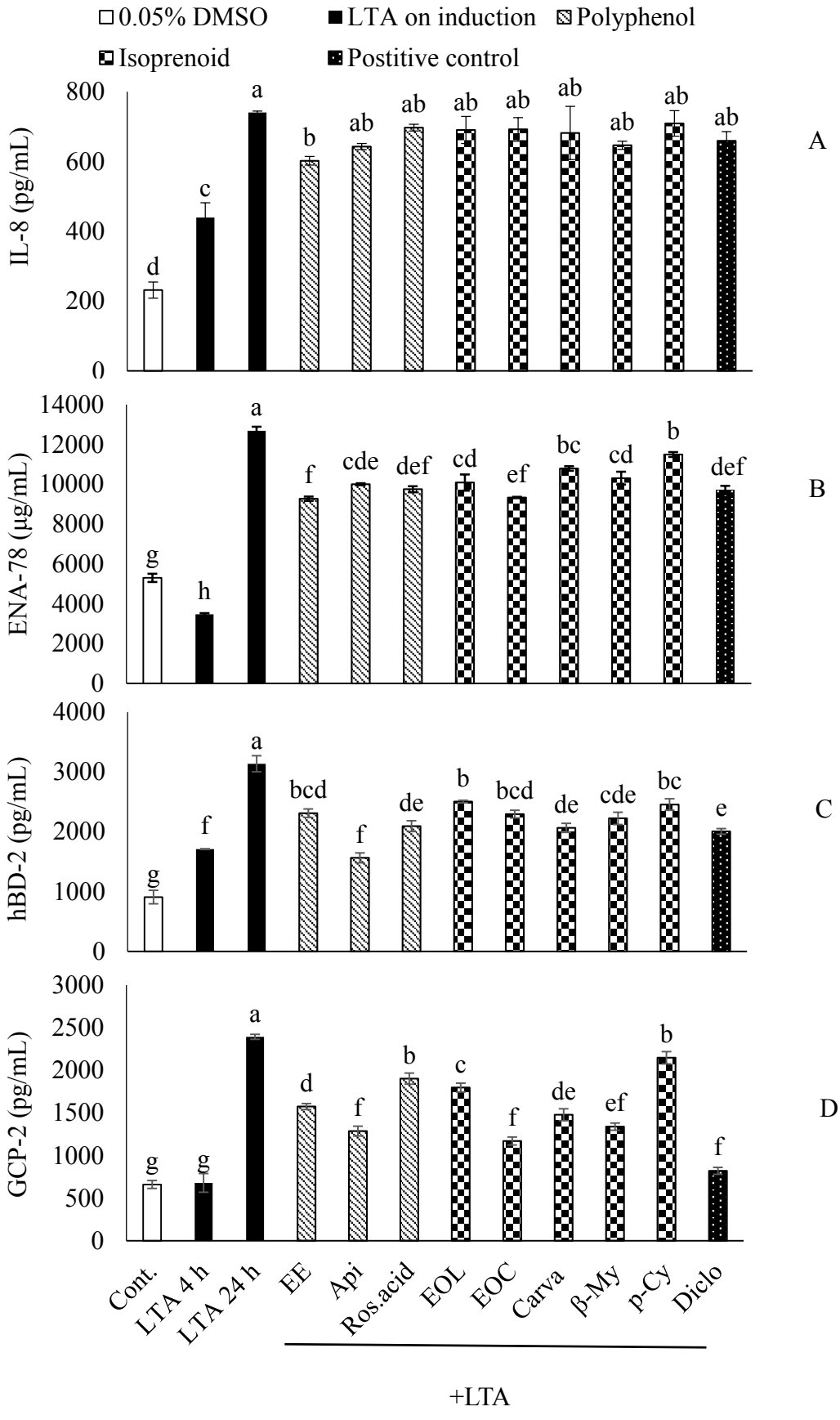
0.05% DMSO
LTA on induction
Polyphenol
Isoprenoid
Postitive control
IL-8 (pg/mL)
ENA-78 (µg/mL)
hBD-2 (pg/mL)
GCP-2 (pg/mL)
A
B
C
D
Cont.
LTA 4 h
LTA 24 h
EE
Api
Ros.acid
EOL
EOC
Carva
β-My
p-Cy
Diclo
+LTA

Fig. 4.3 Quantification of IL-8 (A), ENA-78 (B), hBD-2 (C), and GCP-2 (D) in human tonsil epithelial cells after incubation with selected summer savory phytochemicals in LTA-induced inflammation. Cells were stimulated with 10 μg/mL of LTA for 4 h, and then post-treated with 10 μg/mL test compounds (ethanol extract, apigenin, rosmarinic acid, essential oil extracted from leaves, commercial essential oil, carvacrol, β-myrcene, p-cymene, and diclofenac) for 20 h. 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; ENA-78, epithelial-derived neutrophil activating protein-78; hBD-2, human beta defensin-2; GCP-2, granulocyte chemotactic protein-2; Cont., control; LTA, lipoteichoic acid; EE, ethanol extract from top-cut leaves; Api, apigenin; Ros.acid, rosmarinic acid;EOL, essential oil extracted from leaves; EOC, commercial essential oil; Carva, carvacrol; β-My, β-myrcene; p-Cy, p-cymene; Diclo, diclofenac.

acid (10 μM) from the root of *Acanthopanax koreanum* significantly inhibited TNF-α stimulated inflammation via the reduction in IL-8 production through blocking of both MAPKs (p38, JNK1/2, and ERK1/2) and NF-κB pathways (*249*). Arvelexin extracted from the root of *Brassica rapa* (Chinese cabbage) was also suggested to inhibit the expression of IL-8 in TNF-α activated HT-29 human colonic epithelial cancer cells through reducing the translocation of p65 NF-κB subunit to the nucleus and IκBα impairment (*250*). In addition, other previous research has demonstrated that arvelexin possessed anti-inflammatory activity in macrophages through preventing NF-κB activation. However, this is mainly achieved by blocking IκB-kinase (IκK) and p38 kinase elicitation (*251*). Polyphenol-rich extract has also been reported to possess anti-inflammatory activity. An ethanolic (70%)-aqueous extract product (BNO1030) containing seven traditional medicinal plant extracts markedly lowered the secretion of IL-8 and hBD-2 at 100 μg/mL in LPS/IL-1β-induced epithelial A549 cells, with 37.7 ± 4.1% and 91.8 ± 15.6% inhibition, respectively (*122*). In the present study, all the tested summer savory phytochemicals exhibited anti-inflammatory activity to some extent at the non-cytotoxic concentration of 10 μg/mL. Morphological changes have been demonstrated on the surface of the cells without influencing cell viability after the treatment of apigenin, rosmarinic acid and

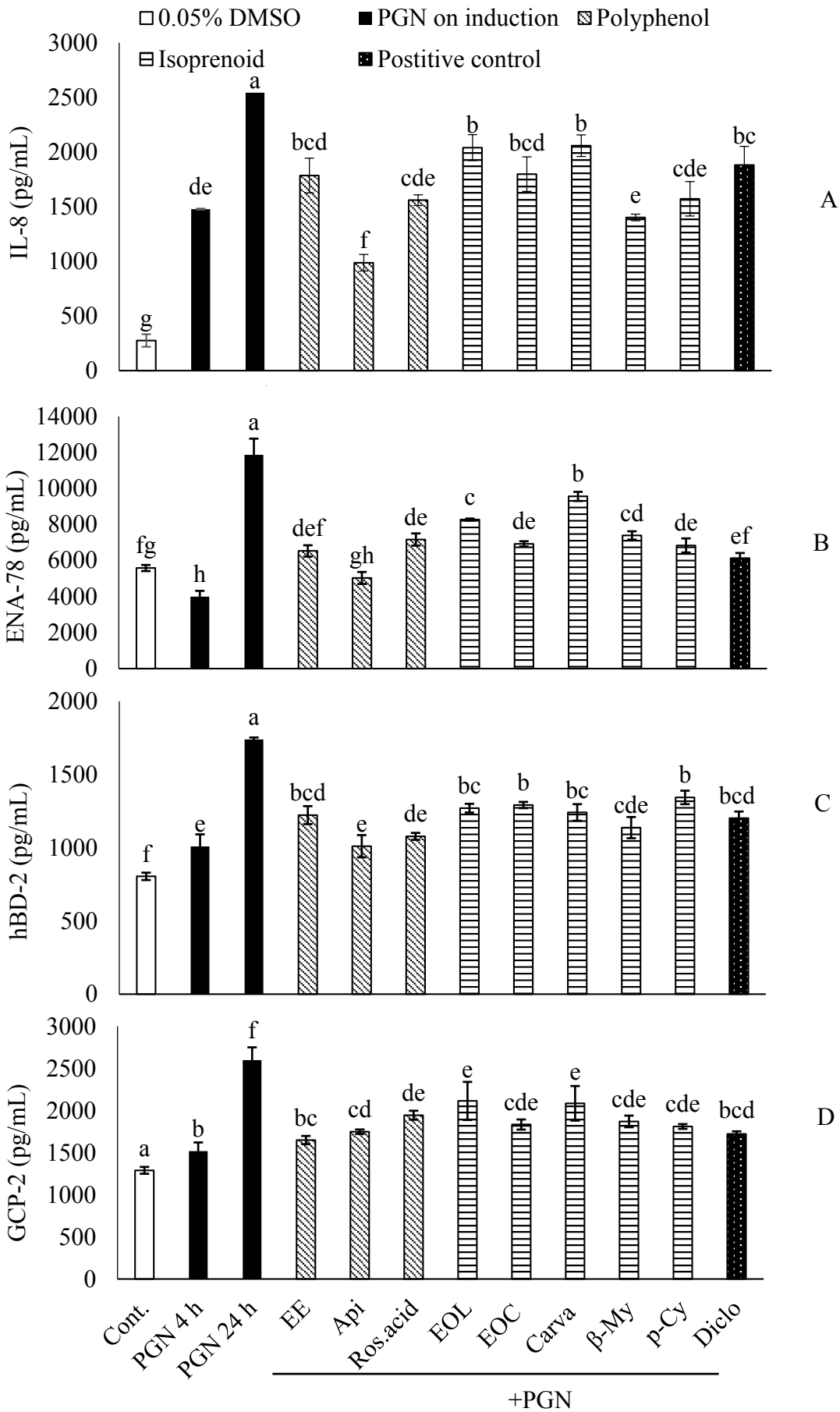
0.05% DMSO
PGN on induction
Polyphenol
Isoprenoid
Postitive control
IL-8 (pg/mL)
ENA-78 (pg/mL)
hBD-2 (pg/mL)
GCP-2 (pg/mL)
A
B
C
D
Cont.
PGN 4 h
PGN 24 h
EE
Api
Ros.acid
EOL
EOC
Carva
β-My
p-Cy
Diclo
+PGN

Fig. 4.4 Production of IL-8 (A), ENA-78 (B), hBD-2 (C), and GCP-2 (D) in human tonsil epithelial cells after incubation with selected summer savory phytochemicals in PGN-induced inflammation. Cells were post-treated by 10 µg/mL of test compounds (ethanol extract from top-cut leaves, apigenin, rosmarinic acid, essential oil extracted from leaves, commercial essential oil, carvacrol, β-myrcene, p-cymene, and diclofenac) for 20 h after 10 µg/mL of PGN stimulation for 4 h. Results were presented as mean ± SD (n=3), Tukey's test, $p \leq 0.05$. Groups sharing different letters expressed significant difference. IL-8, interleukin-8; ENA-78, epithelial-derived neutrophil activating protein-78; hBD-2, human beta defensin-2; GCP-2, granulocyte chemotactic protein-2; Cont., control; PGN, peptidoglycan; EE, ethanol extract from top-cut leaves; Api, apigenin; Ros.acid, rosmarinic acid; EOL, essential oil extracted from leaves; EOC, commercial essential oil; Carva, carvacrol; β-My, β-myrcene; p-Cy, p-cymene; Diclo, diclofenac.

diclofenac. This finding is in accordance with other reported studies. Alcoholic and aqueous extracts derived from *Terminalia arjuna* (arjuna) was reported to suppress the expression of adhesion molecule such as vascular cell adhesion and E-selectin on the surface of aortic endothelial cells (*252*). Chao *et al.* (2013) also indicated an attenuative effect of phytochemical extract of purple sweet potato leaf on adhesion molecule expression in monocyte-endothelial cell adhesion (*253*).

In general, post-treatment with apigenin was found to be the most active in LTA/PGN-stimulated human tonsil cells. The pharmacological capacity of this phytochemical in attenuating inflammation has been reported in previous study (*254*). Pre-treated apigenin (10 µM) reduced significantly iNOS and NO expression in LTA-activated embryonic mouse heart cells (H9c2) (*254*). The mode of action was shown to be blocking NF-κB translocation and IκB degradation (*254*). Further study would be necessary to fully reveal the anti-inflammatory ability of apigenin. Ethanol extract from top-cut leaves also showed considerable suppressive activity in lowering the secretion of pro-inflammatory biomarkers. This might be due to the synergic interaction between apigenin and rosmarinic acid, the most abundant two polyphenols of the extract, which have remarkably decreased the expression of pro-inflammatory mediators. The synergism among phytochemicals as

anti-inflammatory agents has been emphasized in previous studies (*255, 256*).

Carvacrol as the predominant component of summer savory essential oil has been shown to possess strong anti-inflammatory ability. As reported in Arigesavan *et al*. (2015), inflammation in the colon of Fischer 344 rats was dramatically reduced through the suppression of pro-inflammatory mediators including iNOS and IL-1β after exposure to carvacrol at 50 mg/kg body weight (o.p) (*257*). In another rat model, supplementation with different doses of carvacrol (20, 40 and 80 mg/kg) altered the secretion of pro-inflammatory cytokines such as IL-6 and TNF-α in LPS-induced inflammation (*258*). However, in this study, carvacrol showed equal or inferior inhibitory activity compared with other essential oils, suggesting the synergic activity of isoprenoids components. Likewise, commercial essential oil showed greater suppressive ability than essential oil extracted from leaves. This may be attributed to the higher amount of bioactive constituents such as carvacrol, β-myrcene, and p-cymene. Further gas chromatography analysis would be necessary to fully understand the synergism among summer savory phytochemicals. Interestingly, in this study, no inhibitory activity was found in tested phytochemicals except ethanol extract from top-cut leaves against LTA-induced IL-8 secretion while all analyzed phytochemicals showed considerable suppressive capacity in lowering PGN-stimulated IL-8 expression. This might be achieved by different inflammatory stimuli of LTA and PGN (*258*).

4.6 CONCLUSION

To our knowledge, this is the first time that anti-inflammatory capacity of summer savory phytochemicals in tonsil epithelial cells has been demonstrated. The findings of this study suggested that specific summer savory phytochemicals possess their suppressive capacity in LTA/PGN-induced inflammation through the reduction of pro-inflammatory

mediators such as IL-8, ENA-78, hBD-2, and GCP-2 protein secretion. However, further research towards understanding the signal transduction mechanism and confirmation of results using animal models are warranted to validate the anti-inflammatory capacity of these phytochemicals.

CHAPTER 5 INHIBITORY ACTIVITIES OF SUMMER SAVORY PHYTOCHEMICALS AGAINST THE PLANKTONIC GROWTH, BIOFILM FORMATION AND ERADICATION OF *STREPTOCOCCUS PYOGENES*

5.1 ABSTRACT

Streptococcus pyogenes (Group A *Streptococci*, GAS) is a major cause of bacterial pharyngitis. Antibiotics are often prescribed for the treatment of streptococcal pharyngitis. However, antibiotic resistance and biofilm interference may render the antibiotic treatment ineffective leading to therapeutic failure. Summer savory (*Satureja hortensis* L.), a traditional medicinal herb has been proven to possess anti-bacterial and anti-biofilm activities. The present study assessed inhibitory activities of fifteen summer savory phytochemical components (polyphenols, isoprenoids and extracts) against planktonic bacterial growth, biofilm formation, and established biofilm of three *S. pyogenes* strains (ATCC 19615, ATCC 49399, and a clinical isolate from a pharyngitis patient). Summer savory phytochemicals showed significant anti-*Streptococcus pyogenes* activity with minimum inhibitory concentrations (MICs) of 8-34,060 μg/mL and minimum bactericidal concentrations (MBCs) of 285-68,120 μg/mL using micro-broth dilution method. Essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, and apigenin were found to be the most active phytochemicals. The results from kinetic kill curve and agar dilution assays also supported this finding. These phytochemicals also markedly inhibited the formation of biofilm with minimum biofilm inhibitory concentrations (MBICs) between 31-1,250 μg/mL during three days incubation at 37ºC. Furthermore, one-day cultured biofilms became eradicated after the exposure to essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, or apigenin for two hours with the minimum biofilm eradication concentrations (MBECs) of 63-625 μg/mL. Penicillin G was used as the positive control and showed the most

significant inhibitory and lethal capacity against planktonic *S. pyogenes* due to the lowest MIC and MBC concentrations of 0.004 μg/mL and 0.016-0.064 μg/mL, respectively. However, penicillin G removed established biofilms at the highest tested concentration of 400 μg/mL. These results demonstrated that selected summer savory phytochemicals could be used as efficacious alternatives in the treatment of streptococcal pharyngitis.

Key words: *Streptococcus pyogenes*, pharyngitis, summer savory, phytochemicals, penicillin G, planktonic bacteria, biofilm

5.2 INTRODUCTION

Streptococcus pyogenes (Group A *Streptococci*, [GAS]) is an important human pathogen which is responsible for a broad spectrum of diseases including pharyngitis. (*5, 161, 259-261*). It has been reported that streptococcal pharyngitis results in 15 to 30% of pediatric sore throat cases and accounts for over 616 million cases annually (*262, 263*). Particularly, school-aged children are ranked as the most susceptible group to streptococcal pharyngitis (*161, 264*).

Penicillin is the first choice of antibiotic drug in the treatment of streptococcal pharyngitis due to its efficacy, safety, narrow spectrum of effect and low cost (*15, 261*). Macrolides such as erythromycin and azithromycin are prescribed as the second-line drug for the patients who are allergic to penicillin (*161*). In clinical practices, antibiotic treatment failure has been reported in up to one third of streptococcal pharyngitis patients (*15*).

A biofilm is a community of cells that is irreversibly attached to a substrate and encased in extracellular polymeric substances (EPS) (*11*). Due to biofilm formation, the cells are able to survive and proliferate in the hostile environment (*77*). *S. pyogenes* has been indicated to form biofilms and this ability is considered to be a major cause of the

antibiotic treatment failure in many pharyngitis cases. Sessile biofilms have been shown to be less susceptible to antibiotic therapy as compared to planktonic cells (*13, 15, 265*).

Owing to difficulties in eradication of *S. pyogenes* by antibiotic treatments and the recurrence of streptococcal pharyngitis, alternative therapeutics are needed. Recently, phytochemicals such as polyphenols, isoprenoids, and plant extracts have been suggested as antibacterial agents to suppress the growth of *S. pyogenes* and its biofilm formation (*12, 14, 170, 266*). Phytochemicals from summer savory have been found to show anti-bacterial and anti-biofilm effects on various bacterial pathogens (*201, 267*). Therefore, in this study, summer savory phytochemicals were investigated for their anti-*S. pyogenes* and anti-biofilm activities. The specific objectives were to: (1) identify the most effective summer savory phytochemicals against planktonic *S. pyogenes* by determining the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs); and (2) explore the inhibitory effects of selected summer savory phytochemicals on biofilm formation and eradication of established biofilms. This was achieved by examining the minimum biofilm inhibitory concentrations (MBICs), and minimum biofilm eradication concentrations (MBECs).

5.3 MATERIALS AND METHODS

5.3.1 Bacterial cultures and growth conditions

Three strains of *S. pyogenes* (ATCC 19615, ATCC 49399 and clinical isolate) were used in this study. Two were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and one clinical culture prepared from a patient with an infected sore throat was kindly provided by Dr. R. J. Davidson (Division of Infectious Diseases, Department of Medicine, Capital Health, Halifax, NS, Canada). The bacteria were cultured in Brain Heart Infusion (BHI) broth media and incubated at 37 ºC for 24 h.

5.3.2 Chemicals and reagents

BHI and bacteriological agar were purchased from Oxoid Ltd. (Nepean, ON, Canada). Sodium chloride (≥ 99.0%, ACS reagent), dimethyl sulfoxide (DMSO) (≥99.8%, gas chromatography (GC)), carvacrol (≥ 98%, food grade (FG)), apigenin (≥ 97%, thin-layer chromatography (TLC)), rosmarinic acid (≥ 98%, HPLC), p-cymene (99%), β-myrcene (≥ 90%, FG), γ-terpinene (97%), caryophyllene (98.5%, GC), α-terpinene (95%), and 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). Commercial essential oil from summer savory was purchased from Liberty Natural Products Inc., Oregon City, OR, USA.

5.3.3 Standardization of bacteria

The bacteria density was standardized to 1×10^9 CFU/mL as described by Ells and Truelstrup Hansen, (2006) with slight modifications (*268*). Briefly, overnight bacterial cultures were used as stocks and diluted two times in BHI broth. The absorbance of diluted bacterial cultures was measured at 600 nm (OD_{600}) with BHI broth as blank using a spectrophotometer (GENESYS^{TM} 20, Thermo Fisher Scientific, Waltham, MA, USA). Each bacterium (1.5 mL) were centrifuged at 13,000 rpm for 10 min. The supernatants were discarded, the pellets were resuspended in × mL of fresh BHI broth media using the following formula:

$$\times = (OD_{600} * 2 * 1.5 \text{ mL})/1$$

5.3.4 Determination of MIC and MBC

5.3.4.1 Micro-broth dilution

MIC and MBC were determined using the micro-broth dilution method as

recommended by the Clinical and Laboratory Standards Institute (*139*). The bacterial cultures were standardized using the method described in section 5.3.2, and then 10-fold diluted to 1×10^6 CFU/mL with saline water (0.85% NaCl, pH=7.0 ± 0.1). Test phytochemicals were dissolved in dimethylsulfoxide (DMSO) to produce stock solutions. The positive control, penicillin G, was dissolved in sterilized water. Serial two-fold dilutions of these samples were carried out to obtain various concentrations ranging from 0.002 to 68,120 μg/mL in total volume of 200 μL BHI with 5×10^5 CFU/mL bacteria as follows: 2-890 μg/mL for commercial essential oil, 224-114,500 μg/mL for essential oil extracted from leaves, 106-54,000 μg/mL for essential oil extracted from stems, 10-5,000 μg/mL for essential oil extracted from fresh whole plant, 644-34,060 μg/mL for α-terpinene, 21-10,900 μg/mL for γ-terpinene, 80-40,870 μg/mL for caryophyllene, 15-7,511 μg/mL for carvacrol, 131-67,110 μg/mL for p-cymene, 133-68,120 μg/mL for β-myrcene, 1-559 μg/mL for ethanol extract from top-cut plants (top-cut: top section from plants representing primary leaves, flowers, and top secondary stems (*37*)), 1-570 μg/mL for ethanol extract from top-cut leaves, 73-37,500 μg/mL for water extract from top-cut leaves, 0.02-125 μg/mL for apigenin, 5-2,400 μg/mL for rosmarinic acid and 0.002-4 μg/mL for penicillin G. *S. pyogenes* growth in the presence of corresponding concentrations of DMSO or water, and in BHI broth were used as solvent and positive controls, respectively. Sterile BHI broth with and without containing serial dilutions of summer savory phytochemicals served as negative controls. After incubation for 24 h at 37 ºC, the growth of planktonic bacteria were analyzed by measuring the absorbance at OD_{600} using a micro-plate reader (Epoch™, Biotek, Winooski, VT, USA). The MIC was defined as the lowest concentration of test compounds inhibiting bacteria growth where the absorbance showed significant change as compared to the level of positive controls ($p \leq 0.05$). The MBC was assessed by sub-

culturing 30 μL volumes from wells showing no increase in absorbance compared to negative controls (at MIC_{90} or higher) on BHI agar. After incubation for 24 h at 37 ºC, the MBC was defined as the lowest concentration of test samples at which no bacterial growth was observed on BHI agar.

5.3.4.2 Agar dilution

S. pyogenes was diluted to 1×10^7 CFU/mL using saline water. Four selected summer savory phytochemicals (carvacrol, summer savory essential oil extracted from fresh whole plant, apigenin and ethanol extract from top-cut leaves) were dissolved in DMSO (sterile water for penicillin G) and subsequently diluted twice in BHI broth to reach concentrations of 1,170-18,720 μg/mL for carvacrol, 1,563-12,500 μg/mL for summer savory essential oil extracted from fresh whole plant, 78-2,500 μg/mL for apigenin, 356-5,696 μg/mL for ethanol extract from top-cut leaves, and 0.02-3 μg/mL for penicillin G. One milliliter of each phytochemical was added to aliquots of molten, tempered (50 ºC) BHI agar (19 mL). After thoroughly mixing the agar and antimicrobial treatment, the mixtures were poured into petri dishes (9 cm-diameter) to yield final concentrations of 59-936 μg/mL for carvacrol, 78-625 μg/mL for summer savory essential oil extracted from fresh whole plant, 4-285 μg/mL for apigenin, 18-142 μg/mL for ethanol extract from top-cut leaves, and 0.001-0.128 μg/mL for penicillin G. Control (no antimicrobials) BHI agar plates containing 1.8% DMSO, 1.3% DMSO, 1.3% DMSO, 2.0% DMSO, and 0.003% sterilized water were used as solvent controls. *S. pyogenes* on BHI agar was tested as positive control and media alone was used as blank. The plates were solidified at room temperature, followed by the application of an aliquot of 2 μL of each bacteria inoculum to obtain a final concentration of 1×10^4 CFU/spot. After drying the spots, the plates were incubated at 37 ºC for 24 h. The MIC was defined as the lowest concentration of selected

summer savory phytochemicals that completely inhibited the bacteria growth that no visible colonies can be seen.

5.3.5 Kinetic kill-curve assay

S. pyogenes ATCC 19615 (type strain) was used in the kinetic kill-curve assay. The kill-curve assay was performed according to Arhin *et al*. (2009) with minor modifications (*269*). An overnight culture of *S. pyogenes* was standardized and diluted to $1x10^6$ CFU/mL as described in the section 5.3.3. After addition into BHI broth supplemented with selected summer savory phytochemicals (carvacrol, summer savory essential oil extracted from fresh whole plant, apigenin and ethanol extract from top-cut-leaves) at concentrations of 0.5 × MIC, 1 × MIC, 2 × MIC, 4 × MIC and 8 × MIC, the bacterium was incubated over a 6-h period for essential oils treatment (carvacrol and summer savory essential oil from fresh whole plant) and a 24-hour time frame for polyphenols treatment (apigenin and ethanol extract from top-cut leaves). Penicillin G was tested as positive control with the same corresponding concentrations with a 24-h exposure period. Viable bacteria plate counting was performed using the spot plating method. 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 saline water. Each dilution was spotted onto a BHI agar plate and dried before the 24 h incubation time at 37 ºC. The results were shown as mean log numbers (log CFU/mL) of bacteria counting ± standard deviation.

5.3.6 Determination of minimum biofilm inhibitory concentration (MBIC)

The biofilm formation assay was adapted from Jadhav *et al.* (*270*). Selected summer savory phytochemicals, extracts and penicillin G with a concentrations from 0.5 × MIC-8 × MIC and 0.5 × MIC-64 × MIC were added into each well followed by addition of bacteria (1×10^6 CFU/mL), respectively. After incubation for three days at 37ºC, the

plates were emptied by flipping 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 h at 37 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 were detected by measurements of the absorbance of 540 nm using a microplate reader (Epoch^{TM}, Biotek, Winooski, VT, USA).

5.3.7 Determination of minimum biofilm eradication concentration (MBEC)

The biofilm eradication assay was performed according to the protocol described by Kwiecinski *et al.* (2009) with slight modifications (*145*). *S. pyogenes* was diluted to 1 x 10^6 CFU/mL using saline water and plated in 96-well plates followed by incubation for 24 h as described in the previous sections. Then the planktonic cells were discarded and replaced with 100 μL of fresh BHI broth. Two-fold serial dilutions of penicillin G or selected summer savory phytochemicals (carvacrol: from 117 μg/mL to 3,752 μg/mL; essential oil from fresh whole plant: from 156 μg/ mL to 5,000 μg/mL; apigenin: from 8 μg/mL to 250 μg/mL; ethanol extract from top-cut leaves: from 36 μg/mL to 570 μg/ mL; penicillin G: from 25 μg/mL to 800 μg/mL) in 100 μL of BHI broth were then added into wells. Media alone was added into wells as positive control and different DMSO concentrations (lower than 2%) and 1% sterilized water were used as solvent controls. Then the plates were incubated at 37 ºC for 2 h following be determination of the viability of biofilm cells using MTT staining method (section 5.3.5). The MBEC was defined as the lowest summer savory phytochemical concentration that caused a significant ($p \leq 0.05$) decline in absorbance compared to the positive control.

5.3.8 Statistical analysis

All the experiments were performed in triplicate. Statistical analysis of differences between control and summer savory/penicillin G treatment was carried out by using Student t-test ($p \leq 0.05$).

5.4 RESULTS

5.4.1 Inhibitory effects of summer savory phytochemicals against planktonic *S. pyogenes*

A total of fifteen summer savory phytochemicals components (polyphenols, isoprenoids and extracts) were assessed for anti-bacterial activity against *S. pyogenes* (ATCC 19615 & ATCC 49399) using micro-broth dilution assay (Table 5.1). Based on the *in vitro* study, summer savory phytochemicals and extracts revealed a wide spectrum of anti-*S. pyogenes* activity. Most of those phytochemicals and extracts inhibited growth *S. pyogenes*, while bactericidal capacity was rarely observed. The MIC values ranged from 71 to 18,750 μg/mL for *S. pyogenes* ATCC 19615 and 8 to 34,060 μg/mL for *S. pyogenes* 49399, whereas, similar MBCs were demonstrated for both strains ranging from 285 to >37,500 μg/mL. Among these 15 phytochemicals and extracts, essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves and apigenin showed the most anti-*S. pyogenes* effect and were selected for further experiments. Ethanol extract from top-cut leaves possessed the strongest bactericidal activity with the lowest MBC value observed of 285 μg/mL. Apigenin was the most active phytochemical in reducing the growth of planktonic bacteria with MIC values of 16 μg/mL for *S. pyogenes* ATCC 19615 and 8 μg/mL for *S. pyogenes* ATCC 49399. Due to the limited solubility in DMSO, the MBC value of apigenin could not be determined at concentrations higher than 125 μg/mL which was not bactericidal to *S. pyogenes*. None of testing phytochemicals

possessed the equal inhibitory/bactericidal effect compared with that of penicillin G, where MIC was 0.004 μg/mL for all three *S. pyogenes* strains and a range of 0.016-0.064 μg/mL was found for MBC values.

Anti-*S. pyogenes* activity of selected summer savory phytochemicals including essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, and apigenin was also evaluated by using agar dilution method (Table 5.2). All the strains exhibited the same MIC values for each phytochemical tested ($p > 0.05$). Apigenin was the most effective phytochemical yielding the lowest MIC value of 63 μg/mL. Carvacrol displayed relatively lower activity with an MIC of 117 μg/mL. The highest MIC concentrations were detected in ethanol extract from top-cut leaves and essential oil extracted from fresh whole plant with 285 μg/mL and 313 μg/mL, respectively. Similarly, those phytochemicals were not comparable to penicillin G (MIC=0.008 μg/mL). Kinetic kill-curve assays were performed to investigate how summer savory phytochemicals suppress the planktonic cell growth of *S. pyogenes*. Selected summer savory phytochemicals (essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, and apigenin) elicited their anti-*S. pyogenes* activity in a concentration- and time-dependent manner (Fig. 5.1). The viable cells were entirely killed after carvacrol treatment for 1 h at a concentration of 4 × MIC, whilst addition of 8 × MIC of this phytochemical demonstrated bactericidal capacity within 10 min (detection limit: 10 CFU/mL). Three logs decrease in the number of viable bacteria were observed in the treatment with 2 × MIC carvacrol for 6 h (Fig. 5.1A). Similar patterns were indicated in the treatments with essential oil extracted from fresh whole plant (Fig. 5.1B). However, *S. pyogenes* was revealed to be less susceptible to this phytochemical extract than to pure carvacrol.The lethal effect of essential oil extracted from fresh whole plant at 4 × MIC

Table. 5.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of summer savory extracts and their phytochemical constituents against *Streptococcus pyogenes* strains ATCC 19615 and 49399 using micro-broth dilution method

	Test compounds	MIC (μg/mL)		MBC (μg/mL)	
		19615	49399	19615	49399
Essential oils	Commercial essential oil	890	890	>890	>890
	Essential oil from dried leaves	895	224	14,310	890
	Essential oil from dried stems	8,438	1,690	3,375	3,375
	Essential oil from fresh whole plant	313	156	1,250	1,250
Isoprenoids	α-Terpinene	4,290	2,145	>34,320	>34,320
	γ-Terpinene	2,720	5,440	>10,880	>10,880
	Caryophyllene	2,550	1,275	40,800	40,800
	Carvacrol	234	117	938	938
	p-Cymene	8,390	8,390	>67,120	>67,120
	β-Myrcene	17,030	34,060	68,120	68,120
Polyphenols	Ethanol extract from top-cut plants	559	559	>559	>559
	Ethanol extract from top-cut leaves	71	36	285	285
	Water extract	18,750	9,750	>37,500	>37,500
	Apigenin	16	8	>125	>125
	Rosmarinic acid	600	600	>2,400	>2,400
Positive control	Penicillin G	0.004	0.004	0.016	0.064

***19615: *S. pyogenes* ATCC 19615; 49399: *S. pyogenes* ATCC 49399**

on *S. pyogenes* occurred after 3 h incubation. This phytochemical extract also reduced viable bacteria by two logs at 2 × MIC within 6 h (detection limit: 33 CFU/mL). No bactericidal ability was suggested for apigenin in the highest test concentrations (Fig. 5.1C). However, the absence of exponential phase was detected after 24 h treatment of apigenin at 8 × MIC, indicating a bacteriostatic effect of the antimicrobial at that concentration. Besides, delayed exponential phase and prolonged lag phase were observed in 4 × MIC exposure for 24 h. Ethanol extract from top-cut leaves showed its bactericidal ability after 4 h treatment for 8 × MIC and 24 h incubation for 4 × MIC with the detection limit of 10 CFU/mL (Fig. 5.1D). The viable bacteria in exponential growth stage dropped by about 2

Table 5.2 Minimum inhibitory concentration (MIC) of selected summer savory phytochemicals against *Streptococcus pyogenes* using agar dilution method

Test compounds	MIC (μg/mL)		
	19615	49399	Clinical
Essential oil from fresh whole plant	313	313	313
Carvacrol	117	117	117
Ethanol extract from top-cut leaves	285	285	285
Apigenin	63	63	63
Penicillin G	0.008	0.008	0.008

***19615: *S. pyogenes* ATCC 19615; 49399: *S. pyogenes* ATCC 49399; Clinical: the clinical isolate of *S. pyogenes*.**

log CFU/mL after exposure to 2 × MIC for 10 h. Subsequently, *S. pyogenes* viable cells were suppressed by this phytochemical resulting in an approximately 2.5 log CFU/mL decrease in the stationary growth phase population at 24 h. Penicillin G exerted its bactericidal activity against *S. pyogenes* at the concentration of 4 × MIC and 8 × MIC in 24 h (detection limit of 10 CFU/mL) (Fig. 5.1E). Besides, sharp decreases in planktonic *S. pyogenes* counts were indicated at both MIC and 2 × MIC, which resulted in approximately 5.5 log CFU/mL and 3 log CFU/mL reductions, respectively.

5.4.2 Effect of selected summer savoy phytochemicals on *S. pyogenes* biofilm formation

The biofilm inhibitory activity in the metabolism of selected summer savory phytochemicals was assessed by MTT reduction method using MIC-based concentrations (0.5 × MIC-8 × MIC) of each phytochemicals and showed that the MBICs of those phytochemicals ranged from 31 to 1,250 μg/mL (Table 5.3). With the exception of ethanol extract from top-cut leaves and apigenin, all the test phytochemicals and extracts showed the same MBIC value for each of the three *S. pyogenes* strains. Similarly to the MIC results, apigenin exhibited the most pronounced activity in inhibiting biofilm formation (MBICs=31-63 μg/mL) among four selected summer savory phytochemicals. Ethanol

extract from top-cut leaves was comparable to apigenin in reducing the biofilm formation of *S. pyogenes* clinical isolate with an MBIC value of 36 μg/mL. However, when considering the corresponding MIC values, all four phytochemicals except ethanol extract from top-cut leaves exerted similar anti-biofilm activity in the presence of 4 × MIC for *S. pyogenes* ATCC 19615. The same result were found after treatment with apigenin, where 4 x MIC was required to completely inhibit biofilm formation of *S. pyogenes* ATCC 49399. Relatively higher concentrations of phytochemicals (8 × MIC for essential oil extracted from fresh whole plant and carvacrol) were needed to prevent biofilm formation in *S. pyogenes* ATCC 49399 and the clinical isolate. In contrast, biofilm formed by the clinical isolate was revealed to be susceptible to ethanol extract from top-cut leaves and apigenin with MBIC=MIC. The positive control, penicillin G showed the lowest MBIC value of 0.016-0.064 μg/mL.

5.4.3 Effect of selected summer savory phytochemicals on *S. pyogenes* established biofilm

Selected summer savory phytochemicals and penicillin G were tested for their metabolic anti-biofilm activity on one-day old, established biofilm using the MTT reduction assay to assess the viability and metabolic activity in biofilm cells (Table 6.4). In contrast to the previous anti-biofilm formation assay, apigenin showed the least eradication activity except in clinical isolate, where MBEC was 64 μg/mL. Ethanol extract from top-cut leaves was the most effective agent in removing metabolic activity in established biofilms made by *S. pyogenes* ATCC 19615 and *S. pyogenes* ATCC 49399 giving the lowest MBEC values of 71 μg/mL for both strains. Carvacrol displayed the most biofilm inhibition among the two isoprenoid phytochemicals treatments against the three strains of *S. pyogenes* with MBEC of 469 μg/mL. Essential oil extracted from fresh whole

plant showed a better efficiency in disrupting metabolic activity in established biofilm of *S. pyogenes* ATCC 49399 than carvacrol. In comparison, penicillin G was revealed to be less efficient in abolishing metabolic activity in the established biofilm with MBEC values of 400 μg/mL.

5.5 DISCUSSION

Of the fifteen summer savory phytochemicals components (polyphenols, isoprenoids and extracts), essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, and apigenin showed a strong anti-*S. pyogenes* activity. However, all of these phytochemicals exhibited at least 1,000-fold weaker inhibitory activity than penicillin G, which exhibited an MIC value of 0.004 μg/mL consistently with a previous study (*269*). The abundance in carvacrol may illustrate the markedly inhibitory effect of essential oil extracted from fresh whole plant on the growth of *S. pyogenes* as compared to other essential oils tested. Previous studies also support this result. In a study conducted in Turkey, the essential oil extracted from aerial parts of summer savory plants was used as antimicrobial agent against different microorganisms including *S. pyogenes*. The significant inhibitory ability (MIC=250 μg/mL) was explained owing to the high concentration of carvacrol (26.1%) and thymol (28.9%) (*177*). Similarly, as indicated in Mihajilov-Krstev *et al.* (2009), summer savory essential oil showed high anti-*S. pyogenes* activity (MIC=MBC=12.5 μg/mL) resulting from a sufficient amount of carvacrol (67%). In addition to the anti-bacterial activity elicited by itself, carvacrol has been demonstrated to exhibit synergic capacity when combined with erythromycin against erythromycin-resistant *S. pyogenes* strains. Notably, in the same study, the MICs were in the range of 64 to 256 μg/mL (*161*), corresponding with MIC values in our study, which ranged from 117 to 234 μg/mL for all three *S. pyogenes* strains. Further, application of essential oil extracted

from fresh whole plant revealed a pronounced effect on decreasing the growth of planktonic cells, however, the anti-*S. pyogenes* ability was weaker than pure carvacrol. This is probably due to the positive correlation between anti-*S. pyoegenes* activity and carvacrol: the higher the content of carvacrol, the stronger the inhibitory activity against planktonic *S. pyogenes*. This is consistent with a previous study where the relation between the abundance of single phytochemical and anti-microbial activity was indicated (*271*). Considering the effects of polyphenol constituents in comparison to the crude polyphenol-rich extracts (water and ethanol extracts), synergic impact among bioactive constituents were demonstrated. Apigenin, the main polyphenol component in ethanol extracts (47.0-140.6 mg/100g of dried extract) was capable of effectively inhibiting the *S. pyogenes* strains with MICs of 7.8-15.6 μg/mL. Rosmarinic acid, which is the major constituent of water extract and ethanol extract from top-cut leaves, showed a marginal anti-*S. pyogenes* effect on all three strains. Previous studies have also observed that rosmarinic acid exhibited an anti-bacterial ability (*272, 273*). In the study of Slobodnikova *et al*. (2013), the anti-bacterial effect of rosmarinic acid on *Staphylococcus aureus* was detected in the range of 156 to 1,250 μg/mL. Similarly, MICs of rosmarinic acid against eight pathogenic bacteria including *Staphylococcus epidermidids*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis*, *Staphylococcus lugdunensis*, *Pseudomonas aeruginosa*, *Corynebacterium*, *Mycobacterium smegmatis*, and *Staphylococcus warneri* were found between 300-1,200 μg/mL in Abedini *et al*. (2013) (*272*). The MIC of 600 μg/mL obtained in the present study is in aggrement with the those findings. In comparison to the meaningful inhibitory activity of major polyphenols, crude extracts except for the ethanol extract from top-cut leaves did not exert remarkable effect on combating *S. pyogenes*. The probable explanation might be differences in the concentrations of apigenin and rosmarinic

acid in the various water extract and ethanol extracts. Contrarily, the ethanol extract from top-cut leaves, sufficient in both phytochemicals was illustrated to be the most effective polyphenol-rich extract, in which, the MIC value was 36-71 μg/mL and MBC value was 285 μg/mL. It is noteworthy that this MIC value was between that of apigenin (MIC=8-16 μg/mL) and rosmarinic acid (MIC=600 μg/mL). The enhancing inhibitory effect of the extract might result from phytochemicals acting in a synergic manner (*274-277*). Though individual phytochemicals play a significant role in bacteria growth reduction, combinations of these components may exhibit a synergic effect in contributing to a higher anti-microbial activity than the sum of the individual phytochemicals would warrant.

Investigation of kinetic kill curves quantitatively confirmed the inhibitory effect of selected summer savory phytochemicals against *S. pyogenes*. Both polyphenols and isoprenoids were revealed to work in a dose- and time- dependent manner. Interestingly, essential oils (carvacrol and essential oil extracted from fresh whole plant) demonstrated their bactericidal activity within a short incubation period at high concentrations (4 × MIC and 8 × MIC) while polyphenols showed a complete bacterial reduction effect during a longer exposure time at the same corresponding concentrations. Similar observations were reported in previous studies where essential oils exhibited their lethal ability within a few hours while prolonged exposures of 24 h was required for polyphenols to express their bactericidal effect (*278-280*).

Agar dilution is a promising method to mimic the process of incorporating phytochemicals into honey lozenges due to the high temperature exposure of 50ºC. Generally, MIC results obtained with agar dilution agree with micro-broth dilution results (*281, 282*). However, in this study, different results were observed in the assessment of anti-*S. pyogenes* by using micro-broth dilution and agar dilution, where mostly higher

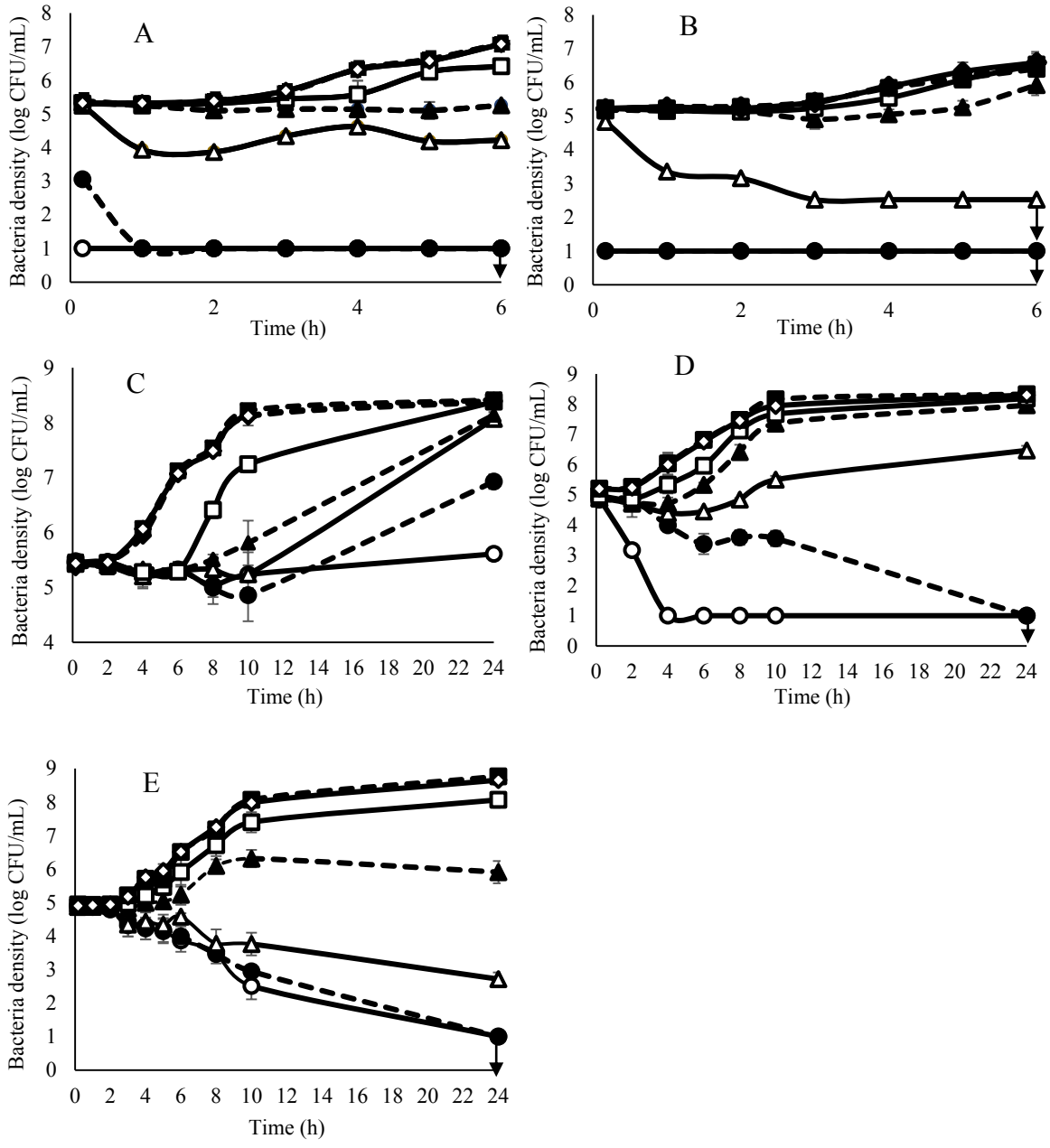

Fig. 5.1 Effect of selected summer savory phytochemicals and extracts on the growth of *Streptococcus pyogenes* 19615 during incubation for 24 h at 37°C. The arrows represent the detection limits (A: 10 CFU/mL; B: 10 CFU/mL and 33 CFU/mL; D: 10 CFU/mL; E: 10 CFU/mL). A: Carvacrol; B: Essential oil extracted from fresh whole plant; C: Apigenin; D: Ethanol extract from top-cut leaves; E: Penicillin G (positive control). ○ = 8 × MIC; ● = 4 × MIC; △ = 2 × MIC; ▲ = MIC; □ = 1/2 × MIC; ■ = bacteria control; ◇ = highest concentration of solvent control (0.9% for carvacrol, 1% for fresh essential oil, 0.7% for apigenin and 1% for ethanol extract from top-cut leaves, 0.003% water for penicillin G).

Table 5.3 Minimum biofilm inhibitory concentration (MBIC) of selected summer savory phytochemicals against *Streptococcus pyogenes* strains

Test samples	MBIC (μg/mL)		
	ATCC 19615	ATCC 49399	Clinical
Essential oil extracted from fresh whole plant	1,250 (4 × MIC)	1,250 (8 × MIC)	1,250 (8 × MIC)
Carvacrol	938 (4 × MIC)	938 (8 × MIC)	938 (8 × MIC)
Ethanol extract from top-cut leaves	142 (2 × MIC)	71 (2 × MIC)	36 (MIC)
Apigenin	63 (4 × MIC)	31 (4 × MIC)	31 (MIC)
Penicillin G	0.016 (4 × MIC)	0.064 (16 × MIC)	0.064 (16 × MIC)

*** Clinical: the clinical isolate of *S. pyogenes*.**

Table 5.4 Minimum biofilm eradication concentration (MBEC) of selected summer savory phytochemicals against *Streptococcus pyogenes* strains

Test samples	MBEC (μg/mL)		
	ATCC 19615	ATCC 49399	Clinical
Essential oil extracted from fresh whole plant	313 (1/4 × MBC)	156 (1/8 × MBC)	625 (1/2 × MBC)
Carvacrol	469 (1/2 × MBC)	469 (1/2 × MBC)	469 (1/2 × MBC)
Ethanol extract from top-cut leaves	71 (1/4 × MBC)	71 (1/4 × MBC)	71 (1/4 × MBC)
Apigenin	> 125 (> 8 × MIC)	> 125 (> 8 × MIC)	63 (4 × MIC)
Penicillin G	400 (25,000 × MBC)	400 (6,250 × MBC)	400 (25,000 × MBC)

Clinical: the clinical isolate of *S. pyogenes

MICs were reported in agar dilution assay. This difference might be due to the subjective interpretation of different methods used (*283*). Micro-broth dilution method showed inherently higher subjective interpretation as compared with the viable cell growth in agar dilution method (*283*). Besides, limited solubility of test compounds in solidified agar substrate may also illustrate the higher MICs using agar dilution method.

Numerous studies have addressed the anti-bacterial capacity of polyphenols and isoprenoids (*159, 284*), but the mode of action of those phytochemicals has not been well documented. Polyphenols are proposed to destroy the integrity of bacteria membrane, inhibit nucleic acid synthesis and disrupt the energy metabolism through the inhibition of ATP synthase (*28, 280, 285*). In general, essential oils are reported to penetrate the bacterial membrane due to their lipophilic activity causing the increase in membrane permeability and membrane damage, which ultimately leads to cell death (*286-288*). Active groups like hydroxyl groups are found to be responsible for interfering with the proton motive force and running up the energy deficit to eventually cause cell death. Furthermore, the toxicity of essential oils upon bacteria is also influenced by the position of the hydroxyl groups presented in the phytochemicals (*289*). Further studies such as outer membrane permeability test, intracellular K+ efflux, and nucleotide leakage studies are required to investigate the mechanism of action of summer savory phytochemicals in impairing the growth of planktonic *S. pyogenes*.

In addition to significant anti-*S. pyogenes* activity, selected summer savory phytochemicals (essential oil extracted from fresh whole plant, carvacrol, ethanol extract from top-cut leaves, and apigenin) also showed anti-biofilm activity although penicillin possessed the most suppressive activity with the MBIC of 0.016-0.064 μg/mL. MTT reduction assay was employed as an indicator of the metabolic activity of biofilm biomass.

The results suggested that selected summer savory phytochemicals exerted their biofilm inhibitory effect at low concentrations between 31-1,250 μg/mL. Lower concentrations of phytochemicals have been reported to be effective in a study of three Thai plant species (*Boesenbergia pandurate* (Roxb.) Schltr., *Eleutherine americana* Merr. and *Rhodomyrtus tomentosa* (Aiton) Hassk.). The inhibitory effect on biofilm formation was detected at significantly low concentrations of 0.24-125 μg/mL for 24 h and 48 h using safranin staining method (*170*). The difference may be due to differences in the phytochemicals, biofilm incubation time and MBIC assay method, which assessed antimicrobial concentration required to reduce the amount of biofilm biomass (safranin) versus the metabolic activity in the biofilm (MTT). Previous investigations also indicated remarkable anti-biofilm activity of essential oils against *S. pyogenes*. A biofilm inhibition of 35-40% was demonstrated following the treatment with essential oils from Patchouli (*Patchouli cablin*) at the lowest test concentration of 5% for 24 h (*14*). In this study, biofilm inhibitory effects of 25.3%-66.3% and 38.9%-43.1% on the three *S. pyogenes* strains were detected after the treatment of carvacrol and summer savory essential oil extracted from fresh whole plant for 72 h at the 1/2 × MBIC concentration, respectively (data not shown). It is obviously notable in the current study that equal or higher concentrations of phytochemicals (MIC to 8 × MIC) was needed to inhibit the biofilm formation than to impair the growth of its planktonic cells. This observation is supported by the exploration in anti-bacterial and anti-biofilm capacity of *Mutellina purpurea* essential oil and α-pinene against *Staphylococcus epidermidis*, where the MBICs of those essential oils were of 1-2 times higher than MICs (*290*). The disruptive effect of phytochemicals on biofilm formation might result from the inhibition of planktonic cell growth (*12*). Although, recent studies have revealed that the impairment of the biofilm pathway is unlikely to rely on

interference with bacterial growth since growth and biofilm inhibition can occur simultaneously (*164, 291*). In a study of crude extracts against *St. aureus* biofilm formation, different fractions showed various biofilm inhibitory activities with/without influencing the growth of planktonic cells (*165*). Besides, the failure of biofilm establishment has been found to result from negative impact of phytochemicals on bacteria motility and attachment to the surface (*166*).

Polyphenol-based phytochemicals were superior to isoprenoids in the anti-biofilm assays. Apigenin was the most active phytochemical in suppressing the biofilm formation of *S. pyogenes* indicating that the inhibitory effect of bacterial growth might be involved in the mode of mechanism. However, limited or no inhibitory ability of this phytochemical against established biofilm was detected. Notably, other selected phytochemicals including carvacrol, essential oil from fresh whole plant and ethanol extract from top-cut leaves all showed relatively greater inhibitory effect on formed biofilm than penicillin G. The high concentration in removing established biofilm of this antibiotic was found in this study (MBEC=400 μg/mL). The failure in removing formed biofilm of *S. pyogenes* at high concentration of penicillin (> 400 mg/L) has been indicated (*16, 292*). Numerous studies have confirmed the difficulty in removing the established biofilm of pathogenic bacteria using phytochemicals. Lemongrass essential oil was reported to disrupt biofilm formation but unable to eradicate formed biofilm of *S. aureus* (*143*). Proprietary cranberry extracts (Cran A, Cran B, Cran C) also showed inability to inhibit established biofilm even at the highest concentration (10 mg/mL) against though their inhibitory effects on biofilm made by *St. aureus* and *St. epidermidis* were demonstrated (*168*). As expected, carvacrol exerted potent anti-biofilm activity, which has been presented in previous studies (*169, 293*). Possible explanations for biofilm eradication by phytochemicals may include the

disruption of extracellular polymeric substance (EPS) synthesis, extracellular matrix damage, and interference of bacterial cells attachment (*145, 293, 294*). Further microscopic analysis would be necessary to visualize the biofilm formation and eradication following the treatment with phytochemicals.

The biofilm of the three analyzed *S. pyogenes* strains showed varying biofilm susceptibilities upon exposure to selected summer savory phytochemicals. This might be because of different serotypes based on the M protein among those strains. The M protein, encoded by *emm* gene, is an important virulence factor in virulence and biofilm formation in *S. pyogenenes* (*13, 76*). In this study, the serotype of all *S. pyogenes* strains remains unknown except *S. pyogenes* ATCC 19615, which has been reported to belong to serotypes 5 and 49 (*295*). Therefore, the identification of the remaining strains would provide a better understanding in the comparison of biofilm formation ability.

Furthermore, concerning the potential pharmaceutical application of summer savory phytochemicals, the biomass and metabolism of biofilm are equally important targets. Different methods such as crystal violet staining, MTT reduction method or CFU counting method has been used in numerous anti-biofilm research studies (*143, 270*). The over estimation of using MTT reduction method has been found in previous study since most of lethally collapsed cells might still possess metabolism (*145*). Hence, more effective investigating methods should be established in the future to fully reveal the anti-biofilm activity of summer savory phytochemicals.

5.6 CONCLUSION

This is the first report of revealing the anti-bacterial and anti-biofilm activities of summer savory bioactive phytochemicals against *S. pyogenes*. Summer savory phytochemicals such as essential oil extracted from fresh whole plant, carvacrol, ethanol

extract from top-cut leaves showed pronounced inhibitory effect on planktonic bacteria and sessile cells at low concentrations of 8-313 μg/mL and 31-1,250 μg/mL, respectively. These bioactive phytochemicals could be considered as potential anti-*S. pyogenes* agents. Further research is required to purify the active fractions and components of essential oil and ethanol extract of summer savory. Additional studies of cytotoxicity of those phytochemicals would also be important before any recommendation is made for industrial application.

CHAPTER 6 DISCUSSION

S. pyogenes is an essential human pathogen responsible for a wide spectrum of diseases ranging from pharyngitis to severe invasive diseases. Penicillin and erythromycin are often prescribed for the treatment of pharyngitis. Increasing number of reports have addressed the therapeutic failure in using these antibiotics. Non-steroidal drugs including nimesulide and diclofenac are commonly used as anti-inflammatory therapy. However, side effects such as cardiovascular and gastrointestinal injury have been demonstrated. Therefore, recently, several research studies have focused on investigating alternative medicinal agents. Plant-based natural products including polyphenols and isoprenoids provide a potential choice due to their inherent pharmacological properties. Summer savory (*Satureja hortensis*) is a traditionally culinary herb which has been used as a folk medicinal treatment for various ailments. Crude extracts and essential oils extracted from this plant and their bioactive constituents have been extensively studied to possess anti-inflammatory and anti-bacterial activities. Thus, this study aimed to determine the inhibitory effects of bioactive phytochemicals derived from summer savory such as polyphenols, isoprenoids, and their major components in two different cell model systems of inflammation (THP-1 differentiated macrophages and human tonsil epithelial cells) and *S. pyogenes*.

The abundance in rosmarinic acid and apigenin, which are mostly reported as the major polyphenol phytochemicals in summer savory crude extracts, was confirmed using UPLC-MS. However, in order to fully investigate the contribution of polyphenols, more compounds such as luteolin-7-glucoside, apigenin-7-rutinoside, and apigenin-7-glucoside need to be quantified as there have been shown to be additional phenolic components of

summer savory extracts in some studies.

The study in PMA-differentiated LPS-induced THP-1 macrophages showed the anti-inflammatory activity of summer savory phytochemicals by lowering the protein secretion of pro-inflammatory cytokines including IL-6, TNF-α, COX-2, and PGE_2 in non-cytotoxic concentrations. COX-2 and PGE_2 are significantly associated with the biochemical pathway of NF-κB. Thus, the evaluation of this pathway would provide a better understanding of cellular mode of action related to the inhibitory effect of summer savory phytochemicals. Similarly, the production of pro-inflammatory mediators such as IL-8, ENA-78, GCP-2, and hBD-2 was markedly suppressed by selected summer savory phytochemicals including polyphenols (ethanol extract from top-cut leaves, apigenin, and rosmarinic acid) and isoprenoids (commercial essential oil, essential oil extracted from leaves, carvacrol, p-cymene and β-myrcene) in LTA- or PGN- stimulated human tonsil epithelial cells. An exception was that LTA-activated IL-8 expression, where none of phytochemicals showed the impairment activity. NF-κB and MAPK signaling pathways have been demonstrated to be responsible for *S. pyogenes* infection. Hence, further studies would be necessary to fully investigate the role of those phytochemicals in preventing pharyngitis *in vitro*.

Isoprenoids, such as carvacrol and essential oil extracted from fresh whole plant and phenolic compounds (apigenin and ethanol extract from top-cut leaves), were screened according to their relatively low anti-*S. pyogenes* and anti-biofilm concentrations. Isoprenoids showed their lethal activity in planktonic cells within a short incubation time, while phenolic compounds indicated no bactericidal ability or a longer killing period. Different anti-bacterial mechanisms might explain the variation. Polyphenols play their role by destroying the integrity of the bacterial membrane, inhibiting nucleic acid synthesis

and energy metabolism, while essential oils usually affect the membrane permeability leading to cell death. Synergic effect of bioactive constituents and predominant amount of carvacrol were suggested in the inhibitory activity of polyphenols and isoprenoids, respectively. Therefore, this study promotes the need for further studies in determination of the mode of action. The impairing activity of selected summer savory phytochemicals in sessile cells was also observed in both biofilm formation and the eradication of established biofilm. Higher doses of phytochemicals were needed to eradicate established biofilm than to prevent biofilm formation. This might be due to the protection of established EPS from hostile environment. Further microscopic analysis should be implemented to visualize the anti-biofilm ability of those phytochemicals. It was apparent in this study that different strains of *S. pyogenes* possessed different anti-biofilm ability. The serotype characterized by M protein has been reported to be associated with the diversity of biofilm formation. Thus, the identification of *S. pyogenes* serotypes remains as a future improvement. MTT was used as a biofilm metabolic indicator in the biofilm studies. However, it has been reported MTT could give overestimation of cell viability (*145*). Other methods (crystal violet and colony counting) would be beneficial to confirm the anti-biofilm activity of summer savory phytochemicals.

In conclusion, *in vitro* studies showed significant anti-inflammatory, anti-*S. pyogenes* and anti-biofilm activities of summer savory phytochemicals. Carvacrol, apigenin and ethanol extract of top-cut leaves were found to be the most effective phytochemicals based anti-inflammatory, anti-*S. pyogenes* and anti-biofilm assays. Among those selected phytochemicals, carvacrol could be a candidate for incorporation into honey lozenges designed for obtaining relief from sore throat due to its relatively low price and high efficiency. However, further investigations on *in vivo* effects, cytotoxicity and sensory

tests are necessary to ensure efficacy, safety, and consumer acceptance of summer savory phytochemicals before industrial application.

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