CARVACROL POSSESSES ANTI-BACTERIAL ACTIVITY AGAINST STREPTOCOCCUS PYOGENES AND INFLAMMATION

by

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DEDICATION

Dedicated
To my inspiring parents, Ranjani and Kamal Wijesundara,
and to my ever-loving husband, Buddika,
and
my siblings
for being the
pillows, role models, catapults,
cheerleading squad and sounding boards
I have needed

and

To all my teachers with Love...!

"When I am down, and, oh, my soul, so weary
When troubles come, and my heart burdened be
Then, I am still and wait here in the silence
Until you come and sit awhile with me
You raise me up, so I can stand on mountains
You raise me up to walk on stormy seas
I am strong when I am on your shoulders
You raise me up to more than I can be……"
-Rolf Løvland-

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ABSTRACT

Streptococcal pharyngitis, caused by *Streptococcus pyogenes,* is a significant health issue worldwide. Beta-lactam antibiotics and non-steroidal anti-inflammatory drugs are recommended for treatment and pain management. This research stems from the global interest in identifying phytochemicals as novel antimicrobials, anti-inflammatory, and analgesic agents for overcoming therapeutic challenges. In this study, carvacrol (5-isopropyl-2-methyl phenol), a monoterpenoid phenolic compound, was investigated for anti-streptococcal, anti-biofilm, and anti-inflammatory activities. The mechanisms of action of these activities were investigated using *in vitro* models of bacterial cells, human tonsil epithelial cells, and isolated cell membranes. Carvacrol showed significant inhibition of growth and biofilm formation by *S. pyogenes*. It also could eradicate preformed *S. pyogenes* biofilms in a concentration- and time-dependent manner. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) of carvacrol against *S. pyogenes* were 125 µg/mL, 250 µg/mL, and 125 µg/mL, respectively. In addition, Carvacrol exhibited instantaneous bactericidal activity against *S. pyogenes.* Carvacrol appeared to target the streptococcal cell membrane, and carvacrol treatment altered the membrane fluidity, polarization, permeability, and phospholipid composition. As a result, carvacrol treatment led to cytoplasmic content leakages, such as lactate dehydrogenase and nucleic acids. The hydrophobic carvacrol primarily interacted with acyl chains of bacterial membrane phosphatidylglycerol, phosphatidylethanolamine, and cardiolipins. Carvacrol also inhibited cell surface hydrophobicity, acidity, and downregulated genes (*speB*, *srtB*, *luxS*, *covS*, *dltA*, *ciaH*, and *hasA*) involved in biofilm formation. In addition, carvacrol possessed anti-inflammatory activities in cultured human tonsil epithelial cells by suppressing the production of pro-inflammatory cytokines such as interleukin-8, interleukin-6, granulocyte chemotactic protein-2, and epithelial-derived neutrophil-activating protein, and other inflammatory biomarkers (human β defensin-2, cyclooxygenase-2, and prostaglandin E2). Therefore, the above results collectively suggest that carvacrol has the potential to be used as a safe and efficacious natural agent in the management of streptococcal pharyngitis.

LIST OF ABBREVIATIONS USED

AEA	N-Acylethanolamine
AI-2	Autoinducer 2
ANOVA	Analysis of variance
AMP	Antimicrobial peptides
AP	Activation protein
ATCC	American Type Culture Collection
BHI	Brain heart infusion
CFU	Colony-forming unit
Cer	Ceramides
Ch	Cholesterol
CGM	Complete growth medium
CL	Cardiolipin
COX-2	Cyclooxygenase-2
CV	Crystal violet
D5DG	Deuterated diglyceride
DG	Diglyceride
DHFA	Dihydrofolic acid
DMSO	Dimethyl sulfoxide
DLCL	Dilysocardiolipin
DPH	1,6 diphenyl 1,3,5 hexatriene
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune-sorbent assay

ENA-78	Epithelial cell-derived neutrophil-activating protein 78
EPS	Extracellular polymeric substances
FBS	Fetal bovine serum
GAS	Group A *Streptococcus*
GC-MS	Gas chromatography-Mass spectrometry
GCP-2	Granulocyte chemotactic protein
HBD-2	Human β-defensin-2
HDMS	Hexamethyldisilazane
Hr	Hours
HRP	Horseradish peroxidase
HTonEpiC	Human tonsil epithelial cells
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kDa	Kilo Dalton
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LPG	Lysophosphatidylglycerol
MAPK	Mitogen-activated protein kinase
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBIC	Minimum biofilm inhibitory concentration
MIC	Minimum inhibitory concentration
Min	Minutes
mRNA	Messenger ribonucleic acid
MTS	3- (4,5-dimethylthiazol-2-yl) -5- (3 – carboxymethoxyphenyl)

	-2- (4-sulfophenyl) - 2H – tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa B
NHP	Natural health product
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
OAHFA	OAcyl-(gamma-hydroxy) fatty acid
PA	Phosphatidic acid
PABA	P-aminobenzoic acid
PAMPs	Pathogen-associated patterns
PBS	Dulbecco's Phosphate buffered saline
PC	L-α-Phosphatidylcholine
PE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
PG	L-α-Phosphatidyl-DL-glycerol sodium salt
PGE_2	Prostaglandin E2
PGN	Peptidoglycan
PLL	Poly-L-lysine
PMS	Phenazine methosulfate
P/S solution	Penicillin/streptomycin solution
QS	Quorum sensing
s	Seconds
SEM	Scanning electron microscopy
SPH	Sphingosine

TEpiCGS	Tonsil epithelial cell growth supplement
TEpiCM	Tonsil epithelial cell medium
TG	Triglyceride
THFA	Tetrahydrofolic acid
TLR	Toll-like receptor
TMB	3, 3', 5, 5'- Tetramethylbenzidine
TNF-α	Tumor necrosis factor-alpha
TNS	Trypsin neutralization solution
v/v	Volume by volume
WHO	World Health Organization

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CHAPTER 1. INTRODUCTION

1.1. STREPTOCOCCUS PYOGENES INFECTIONS

Streptococcus pyogenes, also commonly known as Group A *Streptococcus* (GAS), is a significant human pathogen and is responsible for a broad spectrum of diseases, from relatively mild, superficial infections, such as Streptococcal pharyngitis, impetigo, and scarlet fever; to more invasive infections including cellulitis and necrotizing fasciitis. In addition, it is associated with life-threatening intoxications such as Toxic Shock Syndrome. Albeit rare in North America, *S. pyogenes* infections can occasionally be associated with post-infectious complications, such as acute rheumatic fever (ARF), rheumatic heart disease, and post-streptococcal glomerulonephritis (PSGN) (Bryant and Stevens, 2020; Stevens and Bryant, 2016; Wong and Stevens, 2013).

Like many infectious diseases, developing countries shoulder the burden of *S. pyogenes* infections and post-infectious complications. (Sims Sanyahumbi et al., 2016). Furthermore, poverty-associated living conditions in developing countries encourage faster disease transmission, and the lack of efficient prevention and treatment programs perpetuates the problem in these countries. (Efstratiou and Lamagni, 2016).

Rapid antibiotic therapy, surgical debridement of necrotic tissue, appropriate supportive care, and intravenous immunoglobulin are the treatments used to manage invasive Group A Streptococcus infection. Given the enormous economic and health burdens associated with *S. pyogenes* infections, several investigators have discussed the need for improved treatment strategies and potential vaccine candidates to reduce further the burden of *S. pyogenes* (Andrejko et al., 2021; Miller et al., 2022).

S.pyogenes was thought to be the fifth most lethal pathogen in the world (Lozano et al., 2012). However, this estimation is based on incomplete data. Many countries do not have appropriate population-based laboratory surveillance systems and chronically underreport the true incidence of many infectious diseases. According to available statistics, it was estimated that 18.1 million people suffered from invasive *S. pyogenes* diseases worldwide, with 1.78 million new cases and 500,000 deaths from infections and sequelae each year (Carapetis et al., 2005; WHO, 2004).

1.2. STREPTOCOCCAL PHARYNGITIS

Pharyngitis is defined as the inflammation of the mucous membrane and underlying structures of the pharynx, including the nasopharynx, uvula, soft palate, and tonsil (Langlois and Andreae, 2011). It is among the most common clinical manifestations for which people seek medical attention and accounts for 2% of outpatient primary healthcare visits for adults and 6% for children in the United States (Wessels, 2011).

Non-infectious factors include airborne pollutants, pollen allergies, smoking, secondhand smoke exposure, excessive alcohol consumption, seasonal allergies, and chemical irritations caused by occupational or hazard-related irritants, which can damage the epithelial lining of the throat and increase the risk of bacterial infection (Renner et al., 2012).

Viral infections are the most common cause of acute pharyngitis (90% of cases in adults, >70% cases in children), while bacterial infections account for 15% – 35% of all cases in children and 5% – 10% of cases in adults (Choby, 2009; Cooper et al., 2001; Renner et al., 2012). Although co-infections may occur, most cases are strictly viral, with

Rhinoviruses, Adenoviruses and Coronaviruses being the most common. In addition, Epstein-Barr and Influenza A and B are occasionally implicated (Shulman, 1989).

S. pyogenes is the leading cause of bacterial pharyngitis; however, Groups C and G streptococci, *Neisseria gonorrhoeae*, *Corynebacterium diphtheriae*, *Chlamydia pneumoniae*, *Yersinia enterocolitica*, *Arcanobacterium haemolyticum*, and *Mycoplasma pneumoniae* are less common causes (Renner et al., 2012; Shulman, 1989).

Although reported across all age groups, streptococcal pharyngitis is significantly more common among those under 16 years of age (Chiappini et al., 2011). In temperate climates, the peak incidence occurs in late autumn, winter, and early spring (Chiappini et al., 2011; Cirilli, 2013).

Streptococcal pharyngitis is typically self-limiting and usually resolves in 3-7 days. It has an initial incubation period of approximately 2 to 5 days and is highly communicable during the acute phase of the illness (Chiappini et al., 2011). Some reports have suggested that the transmission rate from untreated patients to be high as 35% in close contacts, such as family members or schools (Shaikh et al., 2010a). The risk of transmission decreases significantly after appropriate antibiotics have been initiated (Choby, 2009).

Those colonized in the posterior pharynx but otherwise display no clinical symptoms are thought to be *S. pyogenes* carriers (DeWyer et al., 2020; Shaikh et al., 2010a). They can occasionally (family units) be a source of spread but generally are less likely to be a significant source due to the lower bacterial density in their pharynx (Gerber et al., 2009). In addition, they have little or no risk of developing suppurative or non-suppurative complications.

1.3. PREVALENCE AND ECONOMIC BURDEN OF STREPTOCOCCAL PHARYNGITIS

S. pyogenes is the most common bacterial cause of acute pharyngitis and accounts for 15% - 37% of cases of pharyngitis in children and 5% -10% in adults (Shaikh et al., 2010b; Shulman et al., 2012); however, it is rare in children younger than three years of age (Chiappini et al., 2017).

Although little economic data exist for Streptococcal pharyngitis (Miller et al., 2022), specifically, the estimated cost of invasive *S. pyogenes* disease and acute upper respiratory infections was $6.08 billion annually (Andrejko et al., 2021). The medicinal and non-medicinal societal cost per case of Streptococcal pharyngitis is estimated to be around $205 US (medical: $118; non-medical: $87), with almost one-half being attributable to non-medical expenses, such as child care, transportation, non-prescription drugs, and health products (Pfoh et al., 2008). When all the associated costs of Streptococcal pharyngitis are tallied, $224 - 539 million is spent annually in the United States (Pfoh et al., 2008).

1.4. POST-INFECTIOUS COMPLICATIONS OF STREPTOCOCCAL PHARYNGITIS

Streptococcal pharyngitis is associated with both suppurative and non-suppurative complications. Suppurative consequences of Streptococcal pharyngitis include otitis media (Segal et al., 2005) and peritonsillar and retropharyngeal abscesses (Herzon and Martin, 2006). Non-suppurative complications, such as acute rheumatic fever (ARF) (Oliver et al., 2020), post-streptococcal glomerulonephritis (PSGN) (Almroth et al.,

2005), and pediatric autoimmune neuropsychiatric disorder associated with *S. pyogenes* (Maini et al., 2012), may occur several weeks after infection.

Non-suppurative complications are assumed to be the outcome of abnormal immune reactivity in tissues after immunologically severe *S. pyogenes* infection. For example, PSGN is caused by the deposition of antigen-antibody-complement complexes on the basement membrane of kidney glomeruli and leads to kidney inflammation (Eison et al., 2011). Rheumatic heart disease, the most severe manifestation of ARF, can result in irreversible damage to heart valves. Specific M proteins on *S. pyogenes* strains have been highly associated with this (Kirvan et al., 2014). Although the prevalence of ARF has disappeared in developed countries, it continues to be a significant cause of morbidity and mortality in developing countries (Carapetis et al., 2016). Antibiotic treatment of Streptococcal pharyngitis within ten days of onset is known to minimize suppurative complications and ARF. However, post-streptococcal glomerulonephritis can occur irrespective of appropriate treatment.

1.5. ETIOLOGY: STREPTOCOCCUS PYOGENES

S. pyogenes is a beta-hemolytic Gram-positive coccus, fermentative, facultative anaerobe, and non-motile bacteria that grow in pairs or as short to moderate-length chains 0.6-1.0 μm in diameter (Bryant and Stevens, 2020). The cell envelope of *S. pyogenes* contains two primary functional layers: the cytoplasmic membrane and the cell wall (Figure 1.1). The cell wall is thick and aids in maintaining the bacterium's cell shape and integrity (Beeby et al., 2013; Hirt et al., 2018). In addition, the cell wall serves as a support to anchor other cell envelope components and protects bacteria from external influences such as mechanical stresses, external environmental hydrolases, membrane

toxic compounds, and high cytoplasmic osmotic pressure (Schlegel and Zaborosch, 2003).

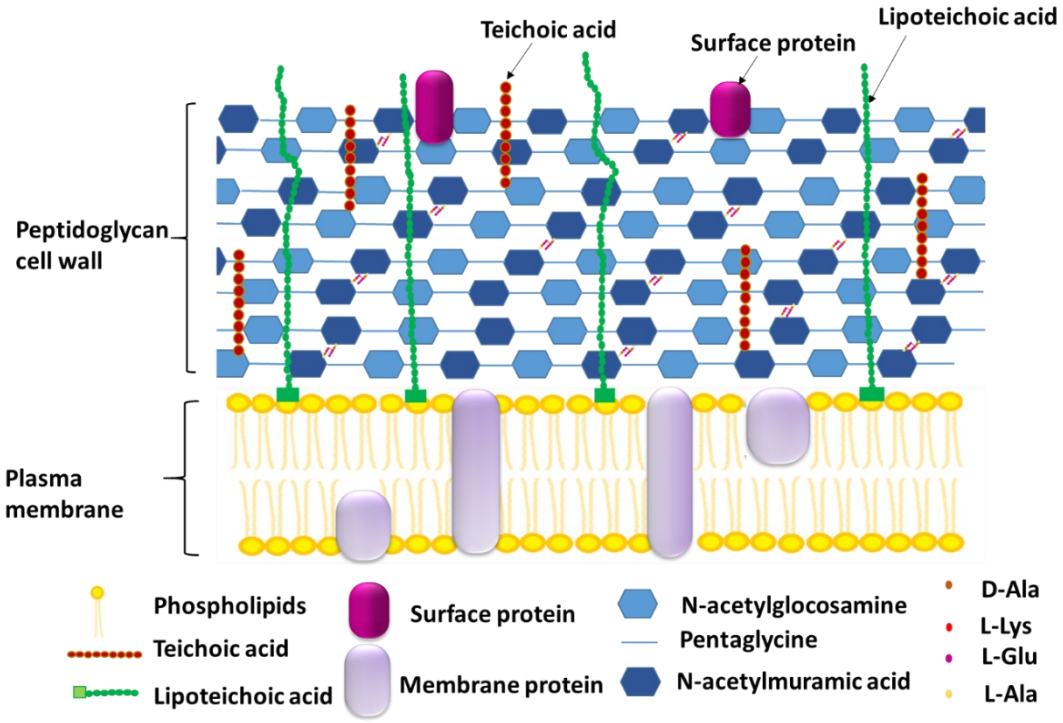

Figure 1.1 Schematic representation of the main components of *Streptococcus pyogenes* cell envelope.

The cell wall of *S. pyogenes* is primarily made of polysaccharides and peptidoglycan (PGN) (Beeby et al., 2013). The PGNs are polymers of alternative arranged *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) linked by a β-glycoside bond and cross-linked by a peptide that contains L- and D-amino acids [L-alanine, D-glutamic acid, L-lysine, D-alanine (L-Ala-D-Glu-L-lys-D-Ala)](Beeby et al., 2013; Meroueh et al., 2006; Vollmer et al., 2008). Rebecca Lancefield devised a serological classification scheme of beta-hemolytic Streptococci based on their carbohydrate composition. *S. pyogenes* was assigned to Group A due to the

peptidoglycan-anchored surface rhamnose polysaccharide in the bacterial wall (Lancefield antigen, Group A carbohydrate) (Ralph and Carapetis, 2013b).

The cytoplasmic membrane of *S. pyogenes* performs essential roles for their survival and cellular functions, including acting as a physical barrier, maintaining proton gradients, energy storage, lipid domain formation and participation in protein recruitment or dispersion, acting as primary and secondary messengers, and forming exosome vesicles (micelles) (Bryant and Stevens, 2020; Epand et al., 2016; Malanovic and Lohner, 2016b). In addition, the cytoplasmic membrane plays a central role as a permeability barrier for regulating the ions and molecules (including nutrients, enzymes, electrons, toxins, antibiotics, and drugs) diffusion across the cells. The electric potential difference across the membrane in bacterial cells is called "membrane potential" (Salton and Freer, 1965).

The membrane of *S. pyogenes* comprises several phospholipids organized in a symmetrical bilayer and many proteins that are localized to specific locations (Epand and Epand, 2009). The phospholipids found in the *S. pyogenes* cell membrane are phosphatidylglycerol (P.G.), lysyl-phosphatidylglycerol (LPG), cardiolipin (CL), phosphatidylethanolamine (P.E.), phosphatidylcholine (P.C.), and phosphatidylinositol (P.I.) (Parsons and Rock, 2013; Sohlenkamp and Geiger, 2016).

S. pyogenes cell wall consists of PGN- or cell membrane-anchored lipo-glycans and glycol-polymers such as teichoic acids (TA), lipoteichoic acid (LTA) (Metzgar and Zampolli, 2011; Schäffer and Messner, 2005; Weidenmaier and Peschel, 2008) as well as surface proteins (e.g. M protein, fibronectin-binding proteins, collagen adhesin, and protein A) (Fischetti, 2016; Metzgar and Zampolli, 2011). These molecules play

important roles in host-cell adhesion, inflammation, and immune modulation (Percy and Gründling, 2014).

The *S. pyogenes* M protein, a cell surface molecule encoded by the *emm* gene, can be divided into several *emm* types or M serotypes. Modern molecular typing utilizes whole genome sequencing and subdivides these into *emm* types (Metzgar and Zampolli, 2011; Reglinski and Sriskandan, 2015). More classical schemes use M-protein precipitin reactions (Lancefield) or T-protein agglutination reactions (Griffith) (Bryant and Stevens, 2020). M protein is composed of two identical polypeptide chains that form an alpha-helical coiled-coil arrangement, anchored in the cell membrane and spanning the cell wall of *S. pyogenes*. More than 200 M protein types have been discovered to date, and their antiphagocytic actions are important for the virulence of *S. pyogenes* (Bryant and Stevens, 2020). Furthermore, *S. pyogenes* contains other virulence factors contributing to *S. pyogene's* pathogenicity. These include streptokinase, streptodornase, hyaluronidase, streptolysin O, streptolysin S, cysteine protease-SpeB, superantigen proteins, and several exotoxins (Ashurst and Edgerley-Gibb, 2020; Castro and Dorfmueller, 2021).

The cytoplasm is vital for cell functions such as cell growth, metabolism, and replication (Wang and Levin, 2009). About 80% of the cytoplasm of *S. pyogenes* consists of water, with the remaining 20% consisting of cytoplasmic components such as gases, nutrients, enzymes, and biological waste. Ribosomes, nucleoids (circular DNA, RNA), and occasionally inclusion granules are also tightly packed (Schlegel and Zaborosch, 2003).

1.6. PATHOPHYSIOLOGY OF STREPTOCOCCAL PHARYNGITIS

1.6.1 *S. pyogenes* adhesion to the host surface

S. pyogenes is an obligate human pathogen. Streptococcal pharyngitis begins with the attachment of *S. pyogenes* to the upper respiratory mucosa of the pharynx (Terao, 2012). Adhesion of *S. pyogenes* is a two-step process that begins with weak interactions and progresses to more specialized, high-affinity binding (Brouwer et al., 2016). Host-derived protective adaptations include mucous and salivary fluid flow mechanisms (Frenkel and Ribbeck, 2015; Murakami et al., 2012) and exfoliation of the epithelium designed to dislodge bacteria (Bisno et al., 2003b; Cunningham, 2000). In response, *S. pyogenes* expresses various several adherence factors such as hyaluronic acid (HA), fimbriae or pili, LTA, M protein, and fibrinogen-binding protein to counteract these forces (Castro and Dorfmueller, 2021; Nobbs et al., 2009).

LTA, a surface carbohydrate, facilitates reversible initial adhesion to host surfaces through weak hydrophobic interactions (Brouwer et al., 2016). The second stage of adhesion, a functionally irreversible adhesion, is initiated by surface pili, followed by multiple, higher affinity events through lectin-carbohydrate and protein-protein interactions (Brouwer et al., 2016; Courtney et al., 2002).

The cell surface protein adhesins facilitate interactions with different host components, allowing *S. pyogenes* to colonize various tissue sites in the human body (Walker et al., 2014). Sortase enzymes attach these adhesive proteins covalently to the peptidoglycan (PGN) in the cell wall, covalently to modified lipoproteins in the cell membrane, and non-covalently to components of the cell surface (Walker et al., 2014). In addition, several extracellular matrix proteins of the host cell surface, including

fibronectin, fibrinogen, collagen, vitronectin, and laminin, have also been targets of *S. pyogenes* adhesins (Cunningham, 2000; Singh et al., 2012).

1.6.2. *S. pyogenes* biofilm formation

The *S. pyogenes* cell surface proteins are responsible for colonization and phagocytosis resistance (Tart et al., 2007). M proteins bind with various host proteins to prevent activation of the alternate complement pathway and evade phagocytosis by macrophages and destruction by leucocytes (Cusick et al., 2012; Sanderson-Smith et al., 2007). Furthermore, molecular mimicry by hyaluronic acid, a compound of the capsule of *S. pyogenes,* which is chemically similar to those of human connective tissues (cardiac, skeletal, and smooth muscles and neuronal tissues), permits the *S. pyogenes* to evade phagocytosis by macrophages and destruction by leucocytes (Bisno et al., 2003a). *S. pyogenes* may grow extracellularly to form colonies and construct biofilms that shield them from host defenses.

Bacteria form a biofilm complex, an important part of pharyngeal colonization and persistence (Fiedler et al., 2015). Biofilms are multicellular communities embedded in a matrix of bacterial extracellular polymeric substances (EPS), adhering to the surface (Flemming and Wingender, 2010). The EPS matrix contains polysaccharides, nucleic acids, and proteins, which allow *S. pyogenes* to survive and proliferate in a hostile environment (Flemming and Wingender, 2010; Lewis, 2001). The biofilm expands continually after the initial attachment of planktonic cells, and *S. pyogenes* proliferates in the pharynx and detaches as individual cells from the bulk after maturation (Hall-Stoodley et al., 2004; Post et al., 2004). The bacteria then disengaged from the biofilm matrices and regained their planktonic state, invading another surface or forming a new biofilm (Fiedler

et al., 2015; Hall-Stoodley et al., 2004). A graphical representation of the development stages of the biofilm of *S. pyogenes* is presented in Figure 1.2.

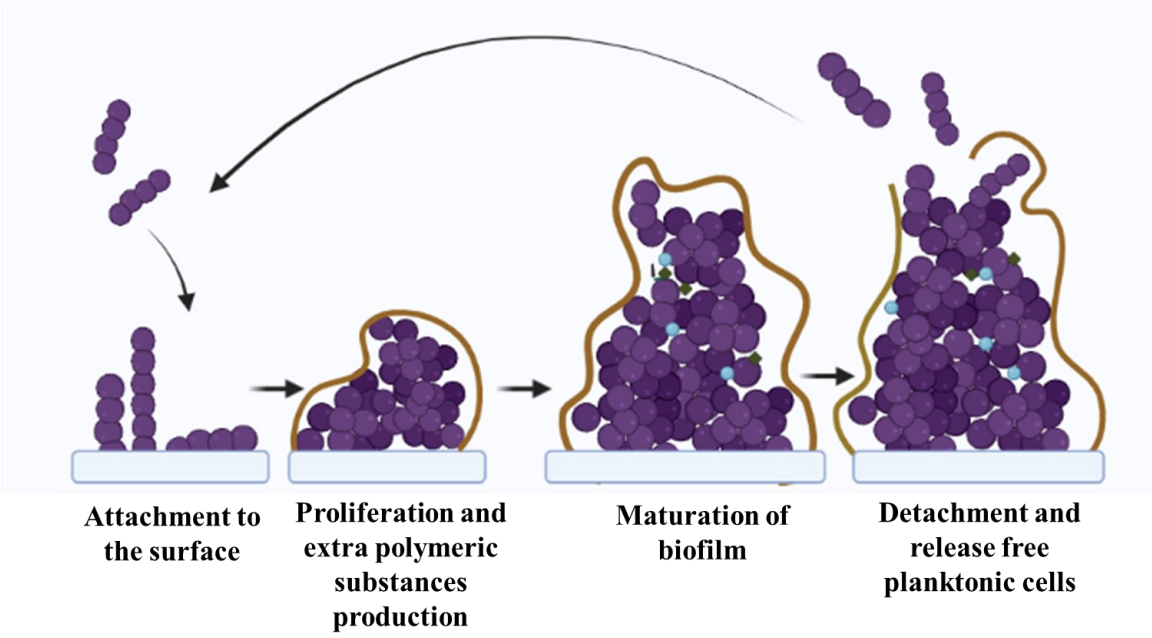

Figure 1.2 Development of *Streptococcus pyogenes* biofilm on the human cell surface. The figure was created using BioRender.com.

1.6.3. Release of chemical signals and expression of virulence genes

When the host environment is favorable, *S. pyogenes* begins to spread and form a community (Tart et al., 2007). Cell-cell communication occurs by exchanging extracellular signaling molecules known as auto-inducers (Waters and Bassler, 2005). Auto-inducers are peptides that are secreted after being processed from bigger pro-peptides, such as autoinducer-2 (AI-2) (Marouni and Sela, 2003). A signal transduction cascade is started when these auto-inducers reach a critical concentration as a function of cell density, which regulates gene expression (Bassler, 1999). Controlling gene expression in response to cell density is also known as quorum sensing (Q.S.), which communicates not only inside

bacterial societies but also involves interactions between surrounding bacterial species, altering specific behaviors of the entire community (Bassler, 1999; Tart et al., 2007; Waters and Bassler, 2005).

1.6.4. Intracellular invasion

S. pyogenes can enter human epithelia, and proteins, including M and F proteins, play a role in streptococcal epithelial cell invasion (Cywes and Wessels, 2001). The M1 serotypes of *S. pyogenes* were found to have a significant frequency of intracellular invasion of tonsil epithelial cells (Dombek et al., 1999). After the intracellular invasion, *S. pyogenes* spread rapidly to various organs through the bloodstream (Terao, 2012). Extracellular products such as hyaluronidase, DNases, streptokinase, streptolysins, and streptococcal pyrogenic exotoxin B distribute components that increase pus liquefaction and assist bacteria in invading tissue planes (Bisno et al., 2003b). Furthermore, hyaluronidase degrades hyaluronic acid, which is found in connective tissue, and DNases are responsible for DNA degradation (Bisno et al., 2003b). As a result, streptococci invade the pharyngeal tissue and cause a localized inflammatory reaction in the throat and tonsils.

S. pyogenes is transmitted from human to human via direct contact; salivary and respiratory droplets by coughing and sneezing (from people with pharyngeal colonization) and nasal discharge or contaminated fomites (Milne et al., 2011). However, chronic asymptomatic pharyngeal and nasopharyngeal colonized carriers may not be risk transmitters due to their relatively small reservoir of organisms (Tanz and Shulman, 2007).

1.7. STREPTOCOCCAL PHARYNGITIS-ASSOCIATED INFLAMMATION

1.7.1. Inflammation

Inflammation is a biological response of the immune system that various harmful stimuli can trigger, including pathogens, damaged cells, toxic compounds, or irritants (Chen et al., 2018b). Streptococcal pharyngitis is the inflammation of the mucous membranes of the oropharynx due to an *S. pyogenes* infection. Typical tissue-level signs of Streptococcal pharyngitis inflammation are redness, swelling, pain, heat, and loss of tissue function (Ricciotti and FitzGerald, 2011b).

1.7.2. Chemical mediators of inflammation

The local vascular system, the immune system, and the injured cells produce several chemical mediators during inflammation (White, 1999b). For example, pro-inflammatory and pre-inflammatory cytokines, prostaglandin E2 (PGE_2), vasoactive amines (histamine and serotonin), and kinins (bradykinin) are mediators of inflammation (Ricciotti and FitzGerald, 2011b). Prostaglandins (PGs), a class of chemical mediators, are produced from arachidonic acid by the enzymes cyclooxygenase (COX) and lipoxygenase (LOX) (Hla and Neilson, 1992; Ricciotti and FitzGerald, 2011b; White, 1999b). They increase vascular permeability and the efficacy of chemicals that aggregate platelets during blood clotting and are connected with inflammation-related discomfort and fever (Ramani et al., 2015).

S. pyogenes antigens, such as LTA and PGN, are recognized by Toll-like receptors (TLR) with myeloid differentiation factor 88 (MyD88) signaling adaptor and transcription factor nuclear factor-kappa B (NFκB) (Loof et al., 2010). These signaling processes cause the expression of pro-inflammatory cytokines, chemokines, and other

inflammatory mediators (Takeda et al., 2003). Pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα), interleukins (IL), enzymes [(cyclooxygenase (COX-2)] and pro-inflammatory chemokines such as interleukins (IL-12, IL-23) and granulocyte chemotactic protein-2 (GCP-2), have vasoactive and chemotactic properties (Schett, 2011), which can promote inflammation in response to tissue injury (Ramani et al., 2015). Anti-inflammatory cytokines such as IL-4, IL-10, IL-11, and IL-13 down-regulate the inflammatory response by giving negative feedback that suppresses the activity of pro-inflammatory cytokines (Schett, 2011).

1.7.3. Signs and symptoms of *S. pyogenes* inflammation

The signs and symptoms of Streptococcal pharyngitis extensively overlap with viral pharyngitis (Choby, 2009). Therefore, diagnosis of Streptococcal pharyngitis based solely on clinical findings, such as the patient's history or physical examination, is challenging (Choby, 2009). However, clinical symptoms suggestive of Streptococcal pharyngitis include sore throat (typically of sudden onset), pain in swallowing, fever, headache, and rarely abdominal pain, nausea, and vomiting (Choby, 2009; Gerber et al., 2009). In addition, other clinical features include tonsillar-pharyngeal erythema with or without exudates, uvular inflammation, anterior cervical lymphadenitis, soft palate petechiae, and swollen red uvula (Gerber et al., 2009).

1.7.4. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed to manage inflammatory conditions (Cremonesi and Cavalieri, 2015). Common NSAIDs for managing inflammation associated with Streptococcal pharyngitis are summarized in Table 1.1. The primary mechanism of action of NSAIDs is the inhibition of cyclooxygenase (COX), the

enzyme catalyzing the synthesis of prostaglandins (PGs) (Cremonesi and Cavalieri, 2015; Ricciotti and FitzGerald, 2011a; Tulunay, 2000). COX-2, a membrane-anchored COX enzyme, is an inducible enzyme activated by *S. pyogenes* wall antigens, such as PGN and LTA, as well as by cytokines (Goldmann et al., 2010; Simmons et al., 2004). NSAIDs block COX enzymes and limit PGs synthesis (Ricciotti and FitzGerald, 2011b), reducing inflammation, discomfort, and fever.

Table 1.1 Common NSAIDs for the management of inflammation associated with Streptococcal pharyngitis.

NSAIDs name	Treatment	% Relative effect compared to the control	Reference
Aspirin	800 mg, single dose	reducing throat pain by 55% at 1 hr	(Schachtel et al., 1991)
Ibuprofen	400 mg, single dose	reducing throat pain by 32% to 80% after 2-4 hr, and 70% at 6 hr (adult), reducing throat pain by 25% in children after 2 hr	(Schachtel et al., 1988)
Nimesulide	200 mg/twice a day, daily	has shown superior antipyretic potency to indomethacin, ibuprofen, aspirin, and paracetamol in rats with yeast-induced fever	(Davis and Brogden, 1994)
Niflumic acid	1,000 mg/day for 4-5 days	reducing throat pain by 17% after two days, 33% after four days	(Sauvage et al., 1990)
Morniflumate suppositories	400 mg/twice a day for four days with antibiotics	34% reduction in pharyngeal pain after four days	(Manach and Ditisheim, 1990)
Tiaprofenic acid	for five days with antibiotics	reducing throat pain by 14% after two days and 93% after five days in children	(Benarrosh and Ulmann, 1989)

NSAIDs have been linked to nausea, vomiting, diarrhea, constipation, decreased appetite, rash, dizziness, headache, and tiredness. Furthermore, some individuals are allergic to NSAIDs and may experience shortness of breath upon taking one (Davis and Brogden, 1994; Kim et al., 2013).

1.8. CURRENT ANTIBIOTIC TREATMENTS AND PREVENTION METHODS FOR STREPTOCOCCAL PHARYNGITIS

Streptococcal pharyngitis is usually a self-limiting infection that resolves within a few days, even without treatment. However, it is recommended to start appropriate antibiotic treatments immediately. Treatment goals for individuals with Streptococcal pharyngitis include eradicating *S. pyogenes* from the throat, lowering the risk of bacterial transmission, and preventing suppurative and non-suppurative consequences (Ashurst and Edgerley-Gibb, 2020; Hayes and Williamson, 2001; Rafei and Lichenstein, 2006). Moreover, patients are usually no longer contagious after 24-48 hrs of the treatment (Chiappini et al., 2017).

Only patients with positive *S. pyogenes* throat culture or positive results from a fast antigen detection test should be treated with antibiotics. The world health organization (WHO), the Infectious Diseases Society of America, and the Canadian Pediatric Society recommend a 10-day course of oral penicillin (250 mg, 2−3 times/day/children and 3−4 times/day/adults) (Gerber et al., 2009; Shulman et al., 2012). Patient allergies, bacteriological and clinical efficacy, potential side effects, compliance, length of therapy, dosage frequency, and cost must be considered when choosing an antibiotic (Choby, 2009). Antibiotics with approved indications of Streptococcal pharyngitis are summarized in Table 1.2.

Table 1.2 Approved antibiotic regimes for Streptococcal pharyngitis.

Class of antibiotics	Antibiotic	Dose and route	Frequency	Duration of therapy	Reference
Penicillins	Penicillin VK	≤ 27 kg: 250 mg, orally > 27 kg: 500 mg, orally	2-3 times	10 days	*FDA (Gerber et al., 2009)
	Benzathine penicillin G	≤ 27 kg: a single dose of intramuscular injection 600,000 units of intramuscular injection > 27 kg: a single dose of 1.2 million units of intramuscular	-	One dose	*FDA (Gerber et al., 2009)
		≤ 27 kg: 250 mg > 27 kg: 500 mg	2-3 times	10 days	(Bisno et al., 2002)
Cephalosporin	Cephalexin	Child: 25 to 50 mg/kg/day Adult: 500 mg	2-4 divided doses 2 times daily	10 days	(Casey, 2007)
	Cefadroxil	Child: 30 mg/kg per day	2 divided doses	10 days	(Gerber et al., 1986)
		Adult: 1 g	Once daily		
	Cefaclor	30 mg/kg/day	4 divided doses	10 days	(Gerber et al., 1986)
	Cefuroxime axetil	15 mg/kg	2 divided doses	10 days	(Scholz, 2004)
	Cefixime	8 mg/kg	Single dose	10 days	(Block et al., 1992)
	Cefdinir	14 mg/kg	Single dose	5 days	(Nemeth et al., 1999)

Class of antibiotics	Antibiotic	Dose and route	Frequency	Duration of therapy	Reference
Macrolides	Azithromycin	Child: 12 mg/ kg	Once daily	5 days	(Gerber et al., 2009)
		Adult: 500 mg on day 1; 250 mg on day 2	Once daily		
	Erythromycin ethylsuccinate	Child: 30-50 mg/kg/day > 27 kg: 400 mg/kg	2-4 divided doses 4 times daily	10 days	(Casey, 2007)
	Erythromycin esotlate	Child: 20 to 40 mg/kg/day.	2-4 divided doses	10 days	(Adam and Scholz, 1996)
		Adult: not recommended **	-	-	
Lincosamides	Clindamycin	7 mg/kg/day (Max. = 300 mg/dose)	3 times daily	10 days	(Tanz et al., 1991)

Dosages are given based on the patient weight in Kg.

*The United States Food and Drug Administration (FDA) recommended.

**Adults receiving erythromycin isolate may develop cholestatic hepatitis; the incidence is higher in pregnant women, in whom the drug is contraindicated.

Although several antibiotics are approved for use in Streptococcal pharyngitis, oral penicillin or amoxicillin remain the drugs of choice. Some reports have suggested that first-generation oral cephalosporin, such as cephalexin, may have reported higher bacteriological eradication rates than penicillins (Brook, 2017; Casey, 2007). Alternatives for penicillin allergy or hypersensitive patients include oral cephalosporins, clindamycin, or macrolides (Gerber et al., 2009).

1.9. CHALLENGES IN CURRENT TREATMENTS AND PREVENTION METHODS

Penicillin allergies are reported by approximately 10-15% of the US population, with elderly and hospitalized patients reporting allergies at higher rates (van Driel et al., 2021). Although many patients report being allergic to penicillin, true allergies, such as clinically significant IgE-mediated penicillin hypersensitivity reactions, are rare (1 in 10,000 to 1 in 40,000) (Shenoy et al., 2019; van Driel et al., 2021).

Many studies have suggested several potential explanations for antibiotic treatment challenges for *S. pyogenes*, such as co-pathogenicity of β-lactamase-producing normal pharyngeal flora, tolerance to penicillin, *S. pyogenes* co-aggregation, bacterial interference, intracellular localization, poor patient compliance (inappropriate dose, duration of therapy, or choice of antibiotic), recurrent exposure to *S. pyogenes*, non-infectious carrier stage, and reacquisition from close contact or fomite (Brook, 2001; Pichichero and Casey, 2007; Pichichero et al., 2000). However, there is no evidence that *S. pyogenes* has become resistant to penicillin.

When huge bacterial loads exacerbate severe infections, penicillin's potency may be reduced in the stationary phase of bacterial development because it targets cell wall

synthesis (Brook, 2001). However, previous studies proved that the contribution of penicillin tolerance to treatment failure plays a minimum role. For example, it accounts for less than 0.1% of *S. pyogenes* isolates collected from pharyngitis patients after ten days of penicillin therapy (Orrling et al., 1996; Vega et al., 2013).

Although current guidelines also recommend alternative antibiotics for patients with a penicillin allergy or hypersensitivity, there are a few challenges associated with their usage in the long term (Choby, 2009; DeWyer et al., 2020). Though some studies have shown the higher bacteriologic eradication rate of macrolides in Streptococcal pharyngitis treatment compared to penicillins, given the high rates of macrolide resistance, they are not considered a better treatment option. Moreover, some studies suggest that the reported higher bacteriologic success rate may also be due to the inclusion of patients who were *S. pyogenes* carriers rather than acutely infected (Brook, 2017; Chiappini et al., 2011; D et al., 2013). On the other hand, resistance to macrolides, such as erythromycin, clarithromycin, and azithromycin, has been reported among *S. pyogenes* isolates in some regions of the United States, Canada and Europe (Katz et al., 2003; Richter et al., 2005). Furthermore, cephalosporins and macrolides have a broader spectrum of activity and generally have higher side effects than penicillin.

Compared to planktonic cultures, the *S. pyogenes* biofilm matrix offers protection against antibiotics and contains environmental promoters that aid in establishing drug resistance (up to a 1,000-fold decrease in sensitivity) (Bueno, 2014; Post et al., 2004). Although the matrix may not be effective at inhibiting antibiotic penetration, it may retard the rate of penetration, which induces the expression of genes within the biofilm that arbitrate tolerance.

Additionally, the slow-growing bacterial cells in the biofilm matrix may reduce susceptibility to antibiotics. Because of deeply located in the biofilm structure, these slow-growing bacterial cells experience nutritional stress (limitation). Those surviving bacterial cells can go from dormant to later becoming active and producing biofilms (Taraszkiewicz et al., 2013). As a result, scientists and physicians are eager to develop better alternatives for preventing/reducing *S. pyogenes* biofilm formation or boosting the efficacy of recommended antibiotics' against *S. pyogenes* biofilm growth inhibition, disruption, or eradication.

Another difficulty with more prolonged antibiotic therapy is patient noncompliance. Patients who experience symptomatic relief without finishing the specified medication time may discontinue the antibiotic course. Furthermore, recurrent infection with the same serotype, following initial treatment, may be associated with milder symptoms (career stage); these individuals are contagious to others in their environment and are susceptible to acute rheumatic fever (Pichichero and Casey, 2007; Pichichero et al., 2000). Chronic carriers are at low risk of disease transmission or acquiring invasive *S. pyogenes* infections, and carriers are often not treated (Choby, 2009). Some studies show that demographic and clinical factors, such as the patient's age, are also associated with treatment failure. Younger children aged 1-8 years are more likely to have *S. pyogenes* treatment failures and significantly more common recurrences than older children aged 13 to 19 (Pichichero et al., 1998). Furthermore, higher rates of microbiological failure are shown in carrier-stage patients under 15 years old.

1.10. EXPLORATION OF NATURAL ANTI-BACTERIAL AGENTS

1.10.1. The need for novel anti-bacterial therapy discovery

The emergence of pathogenic Gram-positive bacteria resistance to many effective current antibiotics and the lack of new alternative developments are significant challenges (Karaman et al., 2020). The public view of antibiotics as trustworthy medication has been changing due to the emergence of drug-resistant bacteria, which continue to evolve. *S. pyogenes* is resistant to tetracyclines (Pires et al., 2005), macrolides (Pires et al., 2005; Silva-Costa et al., 2015b), and lincosamides (Pires et al., 2005), with sporadic resistance to fluoroquinolones (Richter et al., 2003).

With the diminishing pharmaceutical investments in novel drug development, there is significant pressure for novel antibiotic molecules to treat infections. However, new anti-bacterial drug development is time-consuming and rarely economically feasible for most companies. Therefore, we require complementary and alternative drug therapy/therapeutic agents to prevent and manage issues associated with those highly infectious bacterial infections

1.10.2 Mechanisms of antibiotic actions of anti-bacterial agents

Anti-bacterial agents have a unique mechanism of action owing to their molecular structure and degree of affinity to specific target sites within bacterial cells. Antibiotics are either bactericidal or bacteriostatic (Patil and Patel, 2021). However, a lack of significant bacterial kill may not be adequate therapy for some severe infectious diseases caused by Gram-positive bacteria.

The mechanisms of action of various antibiotic drugs vary from inhibition of growth to causing bacterial death (Table 1.3). Antibiotic mechanisms of anti-bacterial

drugs interfere with cell wall synthesis, nucleic acid synthesis, and ribosome function (Calvo and Martínez-Martínez, 2009; Kapoor et al., 2017).

Table 1.3 Various mechanisms of action of anti-bacterial drugs

Mode of Action	Target	Drug Class	References
Inhibit cell wall biosynthesis	Penicillin-binding proteins	β-Lactams: penicillins, cephalosporins, monobactams, carbapenems	(Martin et al., 2022)
	Peptidoglycan subunits	Glycopeptides	(Mesleh et al., 2016)
	Peptidoglycan subunit transport	Bacitracin	(Siewert and Strominger, 1967)
Inhibit biosynthesis of proteins	30S ribosomal subunit	Aminoglycosides, tetracyclines	(C.P et al., 2019)
	50S ribosomal subunit	Macrolides, lincosamides, chloramphenicol, oxazolidinones	(Anandabaskar, 2021)
Disrupt membranes	Lipopolysaccharide, inner and outer membranes	Polymyxin B, colistin, daptomycin	(Huang, 2020; Ledger et al., 2022; Sabnis et al., 2021)
Inhibit the nucleic acid synthesis	RNA	Rifampin	(Mosaei and Zenkin, 2020)
	DNA	Fluoroquinolones	(Hooper, 2001)
Antimetabolites	Folic acid synthesis enzyme	Sulfonamides, trimethoprim	(Capasso and Supuran, 2014)
	Mycolic acid synthesis enzyme	Isonicotinic acid hydrazide	(Bernardes-Genisson et al., 2013)

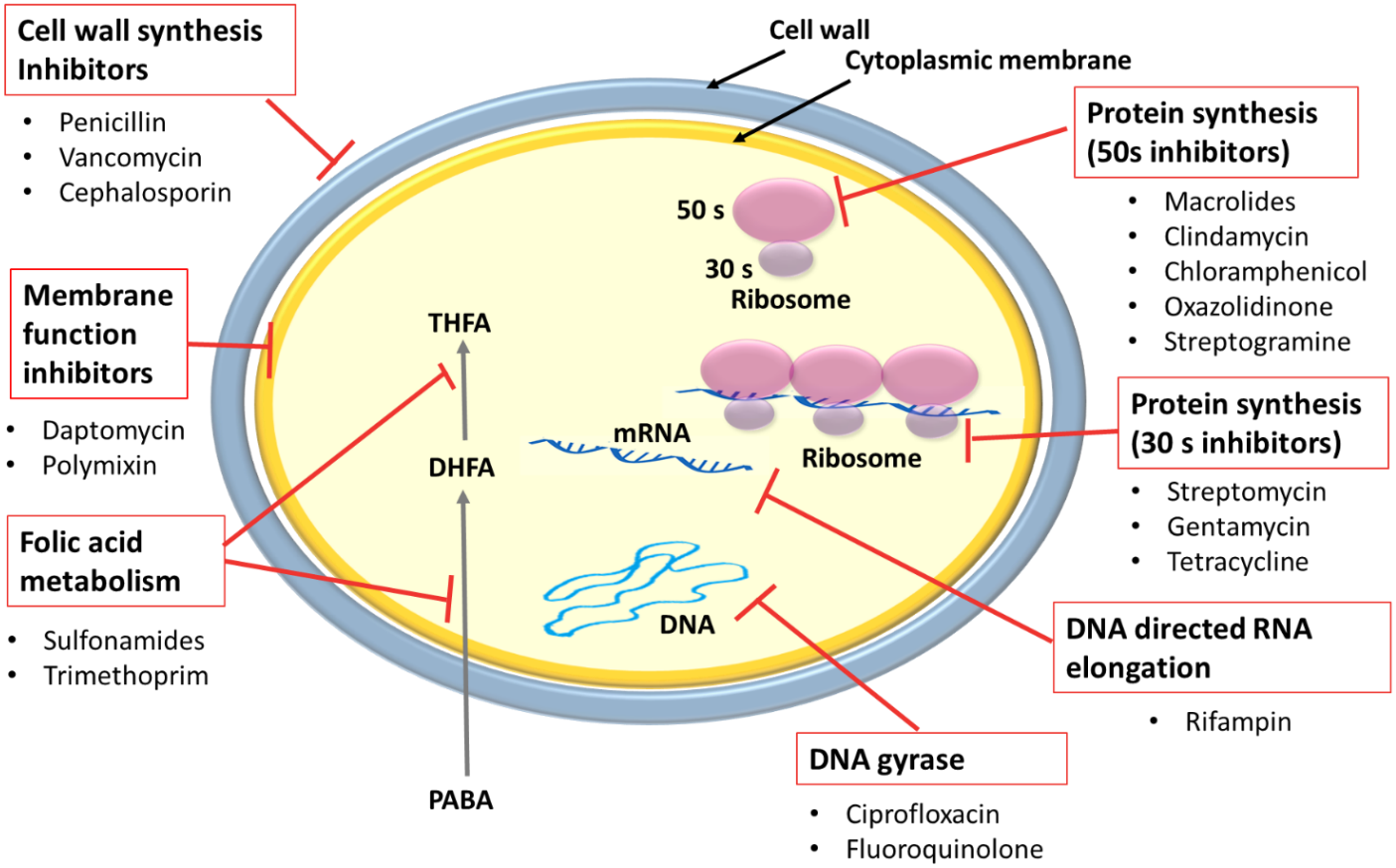

Figure 1.3 Types of the mechanisms of action of conventional antibiotics against Gram-positive bacteria.

PABA: P-aminobenzoic acid, DHFA: dihydrofolic acid; THFA: Tetrahydrofolic acid.

1.10.3 Novel antibiotic targets

In addition to the conventional antibiotics and targets summarized in Table 1.3, new approaches are being explored for the next generation of safe and effective antibiotic compounds. For example, researchers are focusing on new drugs that target virulence compounds responsible for quorum sensing, biofilm formation, bacterial secretion systems, enzymes for tissue penetration (Rasko and Sperandio, 2010; Sintim et al., 2010), and efflux pumps (Du et al., 2021).

1.10.4 Novel sources of anti-bacterial agents

Plants, animals, microbes from extreme habitats, and marine-derived bioactive chemicals have been assessed for anti-bacterial agents (Casciaro et al., 2020; Lini and Zyju, 2017). In recent years, significant progress has been made in discovering potential new antimicrobials from these natural sources (Mantravadi et al., 2019; Miethke et al., 2021).

Plant extracts and their bioactive compounds (Altemimi et al., 2017; Asif, 2015; Wijesundara and Rupasinghe, 2019a) are excellent sources of anti-bacterial compounds. Phenolics, terpenes, alkaloids, and antimicrobial peptides (AMPs) are plant-derived compounds. In addition, some animals are potential sources of anti-bacterial chemicals. The tested animal-derived antimicrobials have been extracted from various bodily sections, secretions, and venoms from honeybees, spiders, scorpions, and snakes. Extreme ecosystems such as hot springs (Mahajan and Balachandran, 2017), thermal sulphurous springs (Mahajan and Balachandran, 2017), abandoned mine waste disposal sites (Stierle and Stierle, 2005; Trenozhnikova and Azizan, 2018), are being investigated for bioactive antimicrobial substances from novel species. Bioactive chemicals derived from marine sources (sponges, coelenterates, echinoderms, marine microbes, and algae)

have been used in natural and synthesized forms (Stincone and Brandelli, 2020). Variabillin, asperidol, eunicin, cycloeudesmol, aplasmomycin, himalomycins, pelagiomycins, and prepacifenol are examples of novel antibiotic agents from marine sources (Doshi et al., 2011).

1.10.5 Advanced and novel technologies in anti-bacterial agent discovery

Technological advancements have aided in the discovery of novel antimicrobials from a variety of sources. However, understanding the chemical structure, mechanism of drug-host interactions, and prospects for changes that improve the efficacy of novel agents is just as important as identifying a new compound. Conventional techniques such as bioinformatics (Ndagi et al., 2020), nanotechnology (Pelgrift and Friedman, 2013), metagenomics (Dos Santos et al., 2017), metabolomics (Panter et al., 2021), synthetic biology (Trosset and Carbonell, 2015), and novel microbial culture methods, next-generation DNA sequencing (Dunne et al., 2012), mass spectrometry (Haddad and Kümmerer, 2014), and nuclear magnetic resonance spectroscopy (Maurer et al., 1998) have played critical roles in drug discovery. Techniques that target the enzymes or proteins responsible for antimicrobial resistance, new drug delivery systems like nanoparticles and combination drug therapy are currently being used or proposed as potential alternatives to standard antibiotics (Ali et al., 2020; Lini and Zyju, 2017). In addition, CRISPR and other phage-based technologies and innovations may open up new avenues for infection control (Wan et al., 2021).

1.10.6 Challenges in the discovery of new anti-bacterial agents

Despite considerable efforts in developing novel anti-bacterial agents with therapeutic promise in laboratory studies (*in vitro*), the number of anti-bacterial drugs in the pipeline

and those influential in clinical trials remains low. The main challenges to the successful clinical development of new anti-bacterial drugs are the financial and regulatory hurdles (Bettiol et al., 2015). Pharmaceutical companies are less motivated to invest in developing novel anti-bacterial classes due to their lengthy development times of returns/results, complex clinical trials, and uncertain success rates (Harbarth et al., 2015; Schuhmacher et al., 2016). Despite the growing demand for novel antibiotics, pharmaceutical and biotechnology companies are reluctant to develop novel antibiotics because of possible market failures. Moreover, those who started R&D investigations may tend to cease the procedure and reduce productivity due to higher R&D costs or low return on investment (Harbarth et al., 2015; Pammolli et al., 2011). It has been reported that the number of novel antibiotics developed and approved has gradually declined over the recent past.

1.10.7 Phytochemicals as natural anti-bacterial agents against *S. pyogenes*

Phytochemicals are secondary metabolites (Mera et al., 2020) found in various plant parts (seeds, roots, leaves, stems, flowers, and fruits) and serve as the defense system for plants to protect them from all the biotic (pathogens, insects, and herbivores) and abiotic stresses (environmental stresses) (Asif, 2015).

Plant extracts rich in phytochemicals have been utilized in traditional medicines to treat diseases and illnesses since ancient times. In addition, phytochemicals have been identified and isolated for drug discovery and development, food applications, or cosmeceutical purposes (Altemimi et al., 2017; Kumar et al., 2022). To research the therapeutic use of plants, a complete understanding of phytochemistry and comprehensive knowledge of the phytochemical makeup of plant extracts, which can

then be used to generate various medications, are required. Furthermore, phytochemicals or their derivatives have been assessed for their pharmacological activities, such as antimicrobial, anti-inflammation, anti-cancer, and anti-allergic activities (Macé et al., 2017a; Mera et al., 2020; Suresh and Abraham, 2020; Tewtrakul et al., 2008; Wijesundara et al., 2017b).

During the past few decades, pharmaceutical industries and scientific communities have explored phytochemicals as potential anti-bacterial agents against Gram-positive pathogens, including *S. pyogenes* (Hu et al., 2018; Macé et al., 2017a; Savoia, 2012). In addition, the aqueous or organic extracts from plant parts that showed anti-bacterial activities were found to have a distinct phytochemical profile such as alkaloids, terpenes (monoterpenes and sesquiterpenes), quinones, and polyphenols such as phenolic acids, flavonoids, and saponins (Saleem et al., 2010).

Phytochemicals exert their anti-bacterial activity through different mechanisms of action. Disruption of the cell wall structure, degradation of the cytoplasmic membrane, leakage of membrane constituents, alteration of membrane permeability, inhibition of microbial enzymes, and inhibition of the primary biochemical pathway are commonly accepted mechanisms of action for *S. pyogenes* (Arunachalam et al., 2016; Javed et al., 2020; Oliveira et al., 2015; Rehman et al., 2021). On the other hand, phytochemicals can be excellent alternatives for conventional antibiotics, especially against drug-resistant *S. pyogenes* strains, because of their multiple mechanisms against bacterial resistance development, such as inhibition of efflux pump, quorum sensing, biofilm formation, and suppression of virulence factors (Banerjee et al., 2017; Joshi et al., 2016; Kifer et al.,

2016; Man et al., 2018; Rehman et al., 2021; Saleem et al., 2010; Wijesundara and Rupasinghe, 2018b).

Besides being natural compounds, most phytochemicals are harmless/less toxic to host cells and less susceptible to bacterial resistance strategies (Joshi et al., 2016; Man et al., 2018; Rehman et al., 2021). Therefore, phytochemicals offer a strategy to combat bacterial resistance, widen the effective spectrum, and have the potential for synergistic combinations with conventional antibiotics.

1.11. CARVACROL

Carvacrol (5-isopropyl-2-methyl phenol, Figure 1.4) is a phenolic monoterpenoid. It is the primary natural constituent in the essential oil fraction of aromatic plants belonging to the family Lamiaceae, such as oregano (*Origanum vulgare*), pepperwort (*Lepidium flavum*), and thyme (*Thymus vulgaris*). Carvacrol was detected in aromatic essential oils, aqueous extracts or ethanol-like solvent extracts of leaves and flowering shoots of various plants (Coccimiglio et al., 2016; Dash et al., 2021; Wijesundara and Rupasinghe, 2018b; Wijesundara and Rupasinghe, 2019a, b). The percentage composition of carvacrol in those extracts and essential oils from different sources ranges from trace amounts to higher levels, approximately between 2.5% - 90% (El Khoury et al., 2019; Lukas et al., 2009; Panda et al., 2021; Wijesundara and Rupasinghe, 2018b; Wijesundara and Rupasinghe, 2019a).

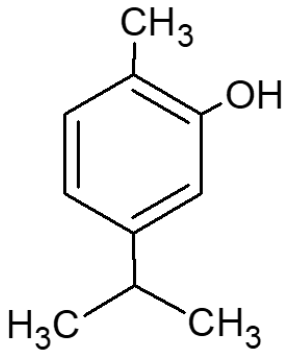

Figure 1. 4 The chemical structure of carvacrol

Carvacrol is soluble in ethanol, carbon tetrachloride, and diethyl ether but not in water. It has a density of 0.9772 g/cm^3 at 20 °C, a boiling point of 237–238 °C, and a melting point of 1°C. Carvacrol- containing plants/herbs is commonly used as a spice in culinary and as a therapy/prevention agent for infections in traditional medicine. Furthermore, the American Food and Drug Administration has classified it as "A Generally Recognized-As-Safe" (GRAS) and a compound used commercially as a food-flavoring agent. Therefore, Carvacrol alone or associated with one or more synergistic products could be incorporated into formulations for applications in biomedicine and food packaging (Ruiz-Rico et al., 2022).

Carvacrol has shown several biological activities *in vivo* and *in vitro*, including anti-oxidant (Rúa et al., 2019), antimicrobial (Mauriello et al., 2021; Rúa et al., 2019), anti-cancer (Elgamal et al., 2021; Trindade et al., 2019), anti-inflammatory (Ezz-Eldin et al., 2020a), immunomodulatory (Ezz-Eldin et al., 2020a), anti-aging (Elgamal et al., 2021), anti-hypertensive (Jamhiri et al., 2019), and anti-diabetes (Morocho et al., 2018).

1.12. THE ANTI-BACTERIAL EFFECT OF CARVACROL AND ITS MECHANISM(S) OF ACTION

Carvacrol is known to be active against a diverse range of bacteria. For example, it is active against *Staphylococcus aureus* (Hasanvand et al., 2021), *Pseudomonas aeruginosa* (Hasanvand et al., 2021), *Listeria monocytogenes* (Karatzas et al., 2001), *Escherichia coli* (Patil et al., 2013), *Campylobacter jejuni* (Johny et al., 2010), and *Salmonella typhimurium* (Giovagnoni et al., 2020).

More interestingly, carvacrol has been reported to have no inhibitory effect or may even promote the growth of beneficial intestinal bacteria such as lactobacillus, bifidobacteria, and firmicutes (De Blasio et al., 2021; Pancu et al., 2021; Ruiz-Rico et al., 2022). Therefore, it supports its use in food, feed additives, and medical applications.

Although the anti-bacterial mechanism of carvacrol is not thoroughly studied, evidence suggests that it might involve one or several targets of the bacterial cell. The anti-bacterial activity of carvacrol has been attributed to its hydrophobic nature, free hydroxyl group, and its ability to delocalize electron system (Friedman, 2014). In addition, carvacrol may disrupt the cell membrane making the membrane permeable to protons and ions and causing loss of integrity. Furthermore, cytoplasmic membrane disruption affects the membrane-associated energy-transducing system (Ben Arfa et al., 2006). Carvacrol is reported to interfere with biofilm formation and maturation. In addition, the role of carvacrol in the interference of bacterial quorum sensing during the biofilm formation process has been explored (Burt et al., 2014). However, there is a gap in understanding the mechanism of carvacrol against *S. pyogenes*. As far as I am aware,

only one preliminary study (Magi et al., 2015b) on anti-*Streptococcus pyogenes* activity is available.

1.13. PROBLEM STATEMENT

Treating Gram-positive infections, including *S. pyogenes* causing infections in hospitals and the community, is a significant problem. *S. pyogenes*, one of the most adaptable human pathogens, is the leading cause of bacterial pharyngitis worldwide. Most Streptococcal pharyngitis cases are treated with an oral penicillin course with no issues. However, searching for alternative treatments is preferable due to the previously mentioned challenges (1.9) associated with antibiotic treatments and as a precaution for future resistance developments.

As it takes a substantially extended period to develop a new drug, there has been increased interest in novel functional foods and natural health products (NHP) for treating Streptococcal pharyngitis. Moreover, consumer demand is increasing for products derived from natural sources or substances to improve food quality and human health.

Typically, a drug or an NHP development begins with basic research to understand and identify the potential target/s of the selected new molecule/drug candidate and its cellular or molecular level interaction with the targets. Then, it is necessary to confirm that these molecules are effective and safe for humans. Finally, pre-clinical testing using cultured cells and experimental animal models is used to conduct safety and efficacy tests before any molecules are tested on humans.

Essential oils and other plant products have traditionally been used in medicine to treat respiratory tract infections as natural treatments. Phytochemical-rich essential oils from aromatic herbs such as oregano, thyme, summer savory, mint, and lavender are

mixtures of those phytochemicals reported being effective against *S. pyogenes* (Savoia, 2012). Previously, I screened more than 60 extracts (ethanol extracts, aqueous extracts, and essential oils) from herbal plants for anti-*S. pyogenes* activity (Wijesundara and Rupasinghe, 2018b; Wijesundara and Rupasinghe, 2019a, b; Wijesundara et al., 2017b). Carvacrol was found to be the most abundant compound in phytochemical profiles of the most effective extracts against *S. pyogenes*, and it was chosen for further study of its activity and mechanisms. However, no systematic studies are available that assessed carvacrol's mode of action, the target of *S. pyogenes*, safety, and the possibility of its usage in NHP development.

Sore throat and difficulty in swallowing are inflammatory symptoms of Streptococcal pharyngitis. Most NSAIDs (Table 1.1) for *S. pyogenes* pharyngeal inflammation are orally administered drugs. However, this oral route is a disadvantage for pediatric and elderly patients with trouble swallowing. Therefore, throat lozenges or vapors would be alternative approaches to delivering anti-inflammatory actives because of their patient compliance, simplicity, faster ingestion, and quicker assimilation and clinical impact. Therefore, there is a chance of developing a carvacrol-incorporated novel type of NHP form for the oral route, one that dissolves quickly in saliva without the need to swallow the drug. Thus, it would be much more efficient to create NHP with both anti-bacterial and anti-inflammatory properties. Therefore, one of our long-term research goals is to identify natural products with anti-inflammatory activities and then develop carvacrol-incorporated NHPs for sore throats associated with Streptococcal pharyngitis. Hence, this thesis research drives the first fundamental steps of NHP development against *S. pyogenes,* causing pharyngitis.

1.14 RESEARCH HYPOTHESIS

I hypothesize that carvacrol inhibits the planktonic growth of *S. pyogenes* by interfering with membrane integrity and functions, inhibits biofilm formation by suppressing biofilm-associated gene expression and reduces inflammation by suppressing the production of pro-inflammatory biomarkers.

1.15. RESEARCH OBJECTIVES

1.16.1. Overall objective

Therefore, the overall objectives of this thesis research were to determine the anti-bacterial and anti-biofilm activities of carvacrol on *S. pyogenes* and subsequently investigate the underlying cellular mechanisms of these activities. Furthermore, the anti-inflammatory activity of carvacrol against *S. pyogenes*-induced tonsil cell inflammation was also studied.

1.16.2. Specific objectives

Specific research objectives were as follows:

Chapter 2:

1. To assess carvacrol's in vitro anti-bacterial activity and time-to-kill against planktonic *S. pyogenes*.
2. To observe the morphological damages to the bacterial cells by carvacrol.
3. To determine the effect of carvacrol on cytotoxicity using a human tonsil epithelial cell model system *in vitro.*
4. To study the interactions of combinations of carvacrol and other antibiotics prescribed for Streptococcal pharyngitis.

Chapter 3:

1. To assess the bactericidal activity of carvacrol against *S. pyogenes* and carvacrol-induced physiological and functional changes of the bacterial membrane.
2. To confirm the membrane target of carvacrol using the protoplast model *in vitro.*
3. To evaluate the influence of carvacrol on the membrane phospholipid composition of *S. pyogenes* and its potential mechanism of action.

Chapter 4:

1. To understand the effect of bacteriostatic concentration of carvacrol on lipid profile changes of *S. pyogenes*.
2. To study the effects of carvacrol on the morphological integrity of the membrane and its fluidity function using an *S. pyogenes* cell membrane isolate system *in vitro.*

Chapter 5:

1. To assess the effects of carvacrol against *S. pyogenes* biofilm formation at the cellular and gene expression levels *in vitro*.
2. To assess the biofilm eradication activities of carvacrol using preformed biofilms *in vitro.*
3. To understand the interaction of carvacrol with LuxS protein using *in silico* molecular modeling and docking study.

Chapter 6:

1. To investigate the anti-inflammatory properties of carvacrol using the human tonsil epithelial cell (HTonEpiC) model system-induced with bacterial antigens *in vitro*.

CHAPTER 2. CARVACROL EXHIBITS RAPID BACTERICIDAL ACTIVITY AGAINST STREPTOCOCCUS PYOGENES THROUGH CELL MEMBRANE DAMAGE

The data presented in this section have been published in a peer-reviewed article.

Niluni M. Wijesundara, Song F. Lee, Zhenyu Cheng, Ross Davidson and H.P. Vasantha Rupasinghe (2021). Carvacrol exhibits rapid bactericidal activity against *Streptococcus pyogenes* through cell membrane damage. Scientific Research, 11(1): 1487 (2021). https://pubmed.ncbi.nlm.nih.gov/33452275/

Author Contributions: Niluni M. Wijesundara performed all the experiments, analyzed the data, and drafted the manuscript. H.P. Vasantha Rupasinghe, the principal investigator, acquired the funds and resources, supervised the project, and reviewed the manuscript. All the co-authors (Niluni M. Wijesundara, Song F. Lee, Zhenyu Cheng, Ross Davidson, and H.P. Vasantha Rupasinghe) have made intellectual contributions to the manuscript and read and approved the final manuscript.

2.1. ABSTRACT

Streptococcus pyogenes is an important human pathogen worldwide. The identification of natural antibacterial phytochemicals has renewed interest due to the current scarcity of antibiotic development. Carvacrol is a monoterpenoid found in herbs. We evaluated carvacrol alone and combined it with antibiotics against four strains of *S. pyogenes in vitro.* The inhibitory effect of bacterial growth of carvacrol was assessed alone and combined with selected antibiotics. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol against *S. pyogenes* were 125 µg/mL (0.83 mM) and 250 µg/mL (1.66 mM), respectively. Kill curve results showed that carvacrol exhibits instantaneous bactericidal activity against *S. pyogenes*. We also demonstrated carvacrol's potential mechanism of action by compromising the cell membrane integrity. Carvacrol-induced membrane integrity changes lead to leakages of cytoplasmic content, such as lactate dehydrogenase enzymes and nucleic acids. Furthermore, we confirmed dose-dependent rupturing of cells and cell deaths using transmission electron microscopy. The chequerboard assay results showed that carvacrol possesses an additive-synergistic effect with clindamycin or penicillin. Therefore, Carvacrol alone, combined with clindamycin or penicillin, can be used as a safe and efficacious natural product for managing streptococcal pharyngitis.

Keywords: Streptococcal pharyngitis, antibacterial activity, strep throat, antibiotics, phytochemicals

2.2. INTRODUCTION

Streptococcus pyogenes, also known as group A Streptococcus (GAS), a Gram-positive, aerotolerant anaerobic coccus, is a human pathogen responsible for significant morbidity and mortality. *S. pyogenes* is responsible for a myriad of diseases, ranging from the mild, non-invasive throat and skin infections (Ibrahim et al., 2016; Williamson et al., 2016) to invasive, life-threatening diseases, including streptococcal toxic shock syndrome (Rodríguez-Nuñez et al., 2011; Schmitz et al., 2018) and necrotizing fasciitis (Chua et al., 2017). If untreated, *S. pyogenes* infections can develop into severe suppurating infections or non-suppurative complications such as rheumatic heart disease (Greenberg and Kaskel, 2017; Martin and Green, 2006; Martin et al., 2015). *S. pyogenes* has also become one of a single organism's top ten infectious causes of mortality (Ralph and Carapetis, 2013a). The global prevalence of severe *S. pyogenes* infection cases is estimated at over 18 million, with approximately 1.78 million new cases yearly (Soderholm et al., 2018).

Streptococcal pharyngitis, commonly known as "strep throat," is responsible for high medical and social costs (Pfoh et al., 2008). Although observed in patients of any age, the prevalence is highest in 5- to 15-year-old children (Cirilli, 2013), presumably because of multiple exposures and low immunity. *S. pyogenes* is responsible for 37% of sore throats in patients <16 years of age, whereas it is implicated in only 5-15% of adults and 24% of infants less than five years of age (Shaikh et al., 2010a; Shulman et al., 2012). Therefore, antibiotic therapy is imperative to eradicate *S. pyogenes* from the throat to decrease the risk of transmission (Shulman et al., 2012) and prevent suppurative and non-suppurative complications (van Driel et al., 2016b).

Appropriate antibiotic selection requires consideration of bacteriological and clinical efficacy, administration frequency, therapy duration, potential side effects, patients' allergies, compliance, and cost (Choby, 2009). Despite the genetic diversity of *S. pyogenes* and the massive exposure over several decades, the organism remains sensitive to penicillin and other commonly used beta-lactam antibiotics. Therefore, the Infectious Diseases Society of America, Canadian Pediatric Society, and world health organization (WHO) recommend a 10-day course of oral penicillin V potassium (250 mg, 2-3 times/day for children, and 250 mg four times/day or 500 mg twice/day for adults). Beta-lactam antibiotics exhibit bactericidal effects by inhibiting the synthesis of bacterial cell walls (Wessels, 2011). Specifically, they prevent cross-linking between peptidoglycan chains through the DD-transpeptidase enzyme, a penicillin-binding protein (Sauvage and Terrak, 2016).

Although penicillin remains the first choice of drug for streptococcal pharyngitis, other antibiotics are shown to be effective in eradicating *S. pyogenes*. First-generation oral cephalosporins and macrolides are recommended for patients with penicillin allergies as alternative treatment options (Wessels, 2011). Macrolides bind to 30 S ribosomal RNA target sites of *S. pyogenes*, inhibiting protein synthesis. Unfortunately, macrolide resistance has been well described either due to active efflux (*mef* genes) or target modification (*erm* genes) (Dinos, 2017; Silva-Costa et al., 2015b).

Carvacrol, also known as cymophenol (2methyl-5-propan-2-yl phenol, Figure 1A), is a monoterpene phenolic compound of the *Thymus* and *Oregano* family of herbal plants (Wijesundara and Rupasinghe, 2018b). Carvacrol alone or carvacrol-rich essential oils have been studied for biological activities such as anti-oxidant (Yildiz et al., 2021),

anti-inflammatory (de Carvalho et al., 2020b), anti-cancer (de Carvalho et al., 2020b), antipyretic (Singh et al., 2016), and analgesic properties (Singh et al., 2016). In addition, Carvacrol exhibits antimicrobial activities against fungi (Vinciguerra et al., 2018), Gram-positive (Chang et al., 2017), and primarily Gram-negative bacteria (Allaoua et al., 2018; Lee et al., 2017). In Gram-negative bacteria, carvacrol depolarizes the cytoplasmic membranes (Siroli et al., 2018; Xu et al., 2008). Furthermore, carvacrol appears to affect ATP synthesis and subsequently reduce the other energy-dependent cellular processes, such as the synthesis of enzymes and toxins (Nostro and Papalia, 2012). However, the antibacterial mechanisms of carvacrol against human pathogenic Gram-positive bacteria have not been reported sufficiently.

European Union food improvement agents (207-889-6) and the joint FAO/WHO expert committee on food additives (JECFA number 710) have classified carvacrol as a flavoring agent. The study's specific objectives were to investigate the efficacy of carvacrol against *S. pyogenes*, understand the mechanism of action on cell integrity, and determine cytotoxicity to human cells. Furthermore, this study evaluated the synergistic effects of carvacrol with antibiotics.

2.3. MATERIAL AND METHODS

2.3.1. Media, chemicals, and bacterial strains

Carvacrol, penicillin G sodium salt, DMSO (≥ 99.8%), penicillin V potassium (VK), LTA, ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide, >95%), poly-L-lysine (PLL), Dulbecco's phosphate-buffered saline (PBS), [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) and phenazine methosulfate (PMS) were obtained from Sigma-Aldrich Ltd., Oakville, ON, Canada. Four *S. pyogenes* strains, ATCC 19615 and ATCC 49399, a clinical isolate (originated from a pharyngitis patient) and an erythromycin-resistant strain (*Spy* 1558, erm), were used in the study and were grown in brain heart infusion (BHI), Oxoid Ltd., Nepean, ON, Canada. Cultures cryopreserving, sub-culturing, and inoculum preparation (1×10^6 CFU/mL) were performed as described previously (Wijesundara and Rupasinghe, 2018b).

2.3.2. Growth inhibition assays

Macro-dilution method

The MIC was determined according to the Clinical and Laboratory Standards Institute (CLSI) method. Two-fold serial dilutions of carvacrol (0.125 to 2000 μg/mL) were made from stock in DMSO and combined with *S. pyogenes* suspension, and bacteria growing in 1% DMSO served as diluent control. The MIC was the lowest carvacrol concentration with no visible growth in tubes after 24 hrs incubation at 37 °C.

Micro-dilution method

The micro-broth dilution assay was conducted similarly to the above method but in 96-well plates as per the guidelines of CLSI. Carvacrol (0.125 to 2000 µg/mL) was incubated with bacterial suspension (200 µL). Absorbance was measured at optical density (OD) = 600 nm after 24 hrs incubation at 37 °C.

2.3.3. Bactericidal activity

The MBC was determined by pipetting 30 µL from wells that showed no visual growth in MIC experiments onto BHI agar, and the plates were incubated at 37 °C for 24 hr. The lowest carvacrol concentration with no visible bacterial colonies (assumed to eliminate ≥ 99.9% of the initial inoculum) was considered the MBC.

2.3.4. Synergistic effect of carvacrol

The checkerboard method assessed the synergistic effects of carvacrol with antibiotics (Rand et al., 1993). Briefly, 50 µL of each antibiotic and carvacrol (from 2-fold serial dilutions) were added into 100 µL of bacterial suspension in a 96-well plate. OD_{600} was measured after 24 hr incubation. Fractional Inhibitory Concentration Index (FICI) was calculated according to the formula: $FICI_A$ = [(MIC A Combination)/ MIC A alone]

$FICI_A = MIC_{A+B}/MIC_A$, $FIC_B = MIC_{B+A}/MIC_B$,

FICI = Σ [(MIC_A Combination)/ MIC A alone] + (MIC_B Combination)/ MIC B alone

$FICI = FICI_A+FICI_B$

Where MIC_A value is the MIC of compound A alone, the MIC_B value is the MIC of compound B alone, and MIC_{A+B} value is the MIC of compound A in the presence of compound B, and *vice versa* MIC_{B+A}. FICI values were interpreted accordingly as

synergy (FICI ≤ 0.5), additive synergy (>0.5 FICI ≥1.0), Indifference /No interaction (>1.0 FICI ≥4.0), and antagonism (FICI >4.0).

2.3.5. Time-kill Analysis

Spectrophotometric method

The bactericidal effect of carvacrol on the planktonic growth of *S. pyogenes* (ATCC 19615, clinical isolate, and *Spy* 1558) was determined by time-kill curve analyses as previously described (Wijesundara and Rupasinghe, 2018b). Bacterial suspensions of *S. pyogenes* were grown with (1/4 × MIC, 1/2 × MIC, MIC, and 2 × MIC) or without carvacrol in 100 μL of BHI in 96-well plates and plates were incubated at 37 °C. Growth dynamics were measured spectrophotometrically at OD = 600 nm every hour for 24 hr.

The viable cell counts method

The cell suspensions of *S. pyogenes* strains (ATCC 19615, clinical isolate, and *Spy* 1558) were incubated in the presence of carvacrol or penicillin G in 96-well plates. Bacteria grown in 1% of DMSO and BHI served as controls. Then the plates were incubated at 37 °C in a humid 5% CO_2 with shaking. Bacterial growth was monitored over a 6 hr period and the at the 24^{th} hour. Then, the viable bacterial count was measured at selected time points using a previously described method (Chen et al., 2003) with some modifications. Bacterial suspensions were diluted using sterile saline water (1:9) and 20 μL drop-spotted onto BHI agar. Colonies were counted after 24 hr incubation. The time taken to kill initial bacterial loads was assessed by plotting the *log* CFU/mL versus incubation time.

2.3.6. Transmission electron microscopy (TEM)

The morphological changes of *S. pyogenes* were observed following incubation with carvacrol (1/4 × MIC, 1/2 × MIC, and MIC). After 16 hr, samples were centrifuged (5,000 × *g*, 10 min, 4 °C), washed with PBS (0.1 M, pH 7.4) and fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate trihydrate buffer for 2 hr at 4 °C. Then, cells were fixed with 1% (w/v) osmium tetroxide (in 0.1 M cacodylate buffer) for 4 hr at room temperature. Rewashed cells were dehydrated with acetone (50%, 70%, 95%, and 100%), followed by ethanol (30%, 50%, 70%, and 90%). Then 100% Epon Araldite resin was added and hardened for 48 hr in a 60 °C oven. Thin sections (approximately 100 nm thickness) were cut using a microtome (Reichert-Jung Ultracut E Ultramicrotome, EquipNet Inc., Canton, MA, USA) with a diamond knife and were placed on 300 mesh copper grids, which were then stained as follows: 2% aqueous uranyl acetate for 10 min, distilled water rinse for 2 × 5 min, lead citrate for 4 min, a quick rinse with distilled water and air dry. Samples were observed using a transmission electron microscope (JEM 1230, JEOL Inc., Peabody, MA, USA) at 80 kV, and images were captured using a digital camera (ORCA-HR, Hamamatsu Photonics, Japan). An average of 200 cells from two independent experiments was analyzed for each treatment. Morphological changes were quantified as a percentage of ruptured and dead cells.

2.3.7. Cytoplasmic content leakage

2.3.7.1. Release of 260-280-nm absorbing materials

The integrity of the cell membrane of carvacrol-treated cells was evaluated by measuring the release of cell constituents at 260 nm and 280 nm. Briefly, 10 mL of cell cultures were incubated at 37 °C under agitation for 24 hr in the presence of carvacrol (1/8 × MIC

to 4 × MIC) and without carvacrol as control. Then, samples were centrifuged (5,000 × *g*, 10 min), and the absorbance of the supernatants was measured at 260 nm and 280 nm using NanoQuant Plate (Tecan Infinite^{TM} M200 PRO, Morrisville, NC, USA) and were analyzed by agarose gel electrophoresis.

2.3.7.2. Release of cytoplasmic nucleic acids

Bacteria from the logarithmic growth phase were collected and centrifuged (10,000 × g, 10 min) and washed once with 10 mM. PBS (pH 7.2) and resuspended to an OD_{600} = 0.6. One mL suspension was treated with carvacrol (1/8 × MIC, 1/4 × MIC, 1/2 × MIC, MIC, 2 × MIC, and 4 × MIC) at 37 °C for 2 hr and centrifuged (10,000 × *g*, 5 min). The supernatants were analyzed by agarose gel electrophoresis. Ethanol precipitations were performed to isolate released nucleic acids and precipitated pellets were dissolved with 10 µL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 7.5). The 10 µL of each sample was then mixed with 2 µL of gel-loading dye (B7024S, New England Biolabs, Ipswich, MA, USA). Carvacrol-triggered nucleic acid leakage was visualized using a Bio-Rad ChemiDoc XRS + system following electrophoresis in 0.8% agarose gel containing ethidium bromide, with 1 × TAE buffer [40 mM Tris base, 0.5 mM EDTA (pH 8.0) and 20 mM glacial acetic acid] at 120 - 140 V for 40 - 45 min. A 100 bp DNA ladder (UBPBio, Lucerna-Chem AG, CH, Luzern, Switzerland) was used as a molecular-weight size marker.

2.3.8. Cell cytotoxicity assay

Cell cytotoxicity was determined using tonsil epithelial cells (HTonEpiCs) (ScienCell Research Laboratory, San Diego, CA, USA) (Wijesundara et al., 2017b). Briefly, 1×10^4/100 µL of TonEpiC cells were seeded in poly-L-lysine-coated 96-well plates, and

media was discarded after 24 hrs of incubation (5% CO_2 at 37 °C) without disturbing the adhered cell layer. A 100 µL of fresh media containing carvacrol was added. A mixture of LTA and PGN (5 mg/mL from each) was used as bacterial antigen controls. After 24 hr-incubation, 10 µL of MTS: PMS (20:1) was added. The absorbance was measured at 490 nm after a 2.5 hr incubation, and results were expressed as percent cell viability compared with untreated cells.

2.3.9. Release of cytoplasmic LDH for bacteria and tonsil cells

The LDH activity in the cell supernatant was measured using the CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's protocol. The 24-hr seeded cells (6000 cells/100 µL density) were washed and replaced with 100 µL fresh growth medium. Both cells and bacterial suspensions were treated with DMSO or carvacrol (1/2 × MIC, MIC, 2 × MIC) at 37 °C. After 4 hr incubation, cells were centrifuged, and 100 µL of supernatant was mixed with 100 µL of CytoTox96 reagent. After 30 min at room temperature, acetic acid (1 M) was added to stop the reaction, and absorbance was measured at 490 nm. Released LDH from HTonEpiC cells and *S. pyogenes* cells by carvacrol were calculated by comparing their respective LDH release treated supernatant with lysis buffer (9% Triton X-100).

2.3.10. Statistical analysis

Statistical analyses were performed using MINITAB statistical software (Version 17.0; Inc., Chicago, IL, USA) and GraphPad Software 5.0 (La Jolla, San Diego, CA, USA). All the experiments were performed in three independent studies with triplicates. Results were presented as means ± standard errors. The statistical analysis was conducted using a

student’s t-test and a one-way analysis of variance. Mean separations were done using Tukey's test. Differences were considered statistically significant at $P < 0.05$.

2.4. RESULTS

2.4.1. Carvacrol inhibits the growth of *S. pyogenes*

The antibacterial activities of carvacrol and four antibiotics against *S. pyogenes* are summarized in Table 2.1. Carvacrol showed growth inhibitory effects against all four tested strains of *S. pyogenes* with a minimum inhibitory concentration (MIC) of 125 μg/mL. The percentage of growth inhibition relative to dimethyl sulfoxide (DMSO, vehicle) control is shown in Figure 2.1 B. Both micro-and macro-broth dilution methods revealed similar findings.

2.4.2. The activity of carvacrol with conventional antibiotics and among antibiotic combinations

The checkerboard assay analyzed the antibacterial activity of carvacrol in combination with antibiotics (Figure 2.6.S1). Table 2.2 is summarized the fractional inhibitory concentration index (FICI) values for different combinations of carvacrol, penicillin G, penicillin VK, clindamycin, and erythromycin. Carvacrol displayed additive synergism with clindamycin, whereas the combinations with other antibiotics showed no significant synergistic effects.

2.4.3. Carvacrol instantaneously kills *S. pyogenes*

The bactericidal effect of carvacrol on the planktonic growth of *S. pyogenes* (ATCC 19615, clinical isolate, and *Spy* 1558) was investigated, and the minimum bactericidal concentration (MBC) values are shown in Table 2.1 and Figure 2.1 C. In addition, the effect of carvacrol against *S. pyogenes* was investigated by studying time-kill kinetics (Figure 2.2), and carvacrol showed concentration- and time-dependent bacterial killing ability. The complete killing of *S. pyogenes* was observed immediately after exposure to

the carvacrol at the concentration of 250 μg/mL, and such killing was observed for all three strains. A significant bacterial count reduction was observed with 125 μg/mL of carvacrol; however, the complete bacterial killing was not observed at the 24th hr. Furthermore, a significant inhibition activity was observed in bacterial growth at 1/2 × MIC of carvacrol. However, reducing the bacterial count at the end of the 24-hr incubation period did not go beyond 3 to 4 log units for three strains. Both methods [viable cell counts method (Figure 2.2 A) and spectrophotometric method (Figure 2.2 B)] gave similar results.

2.4.4. Carvacrol induces morphological changes in *S. pyogenes*

Transmission electron microscopy (TEM) was employed to observe the morphological and ultrastructural alterations induced in three *S. pyogenes* strains upon exposure to 1/8 × MIC, ¼ × MIC, and ½ × MIC of carvacrol treatment compared to the control. Both untreated control (Figure 2.3 Aa, a′, a′′) and vehicle control (Figure 2.3 Ab, b′, b′′) cells remained intact with a complete cell wall, a visible cell membrane, and a homogeneous cytoplasm. In contrast, significant cell deaths (indicated in white arrows) such as ruptured or completely broken cell wall, detached cytoplasmic membrane from the cell wall, dispersion of the intracellular contents, and noticeable cytoplasmic clear zones were observed in ½ × MIC-carvacrol treated ATCC 19615, clinical isolate and *Spy* 1558 (Figure 2.3 Ac, c′, c′′) cells. The percentage of ruptured and dead cells relative to the total cells was calculated (Figure 2.3 B). Cells with distorted shapes compared to the smooth spherical shape of intact cells were also observed. Cellular debris and broken cell wall parts were also abundant.

Morphological changes of disintegrated cells, including membrane fusing, clumping, rupturing, the disintegration of the cell wall and/or membrane, and cytoplasmic disruptions of cells following exposure to ¼ × MIC carvacrol were noticeable in *S. pyogenes* strains (Figure 2.3 A d, d′, d′′). The round shape of most of the intact cells turned into abnormally elongated shapes with ruptured or broken cell walls. The ruptured and dead cell morphologies were observed in all three bacterial strains; however, the percentage of ruptured and dead cells was significantly higher in *Spy* 1558 than in other strains (Figure 2.3 B). Carvacrol at or greater than MIC was highly bactericidal; therefore, it did not generate enough bacterial cell pellets for TEM analysis. TEM images also confirmed that cell density was significantly reduced in all treated samples compared to vehicle control in a concentration-dependent manner.

2.4.5. Carvacrol induces cytoplasmic content leakage

The membrane leakage assays were carried out to assess the integrity of the cell membranes due to cytoplasmic membrane damage of *S. pyogenes*. The release of RNA, double and single-strand DNA was detected only in the 125 (g/mL (MIC) supernatants of carvacrol treatment. The concentrations of RNA, dsDNA, and ssDNA released by ATCC 19615 were estimated to be 87±0.01 ng/µL, 156±19.3 ng/µL, and 82±3.7 ng/µL, respectively, while that by the clinical isolate were 85±2.4 ng/µL, 71±8.2 ng/µL, and 69±2.5 ng/µL, respectively.

Since some 260-280 nm absorbing proteins in cell supernatants can interrupt the absorbance measurement of nucleic acids, to confirm the carvacrol-induced cytoplasmic nucleic acid release, ethanol precipitation was carried out and then visualized, followed by agarose gel electrophoresis. When ATCC 19615 and the clinical isolate were grown in

the presence of 62.5 μg/mL and 125 μg/mL concentrations (1/2 × MIC and MIC, respectively) of carvacrol, cells released visualizable nucleic acids after 24 hr (Figure 2.4 Aa). In addition, when high-density bacterial pellets were treated with carvacrol for 1 hr, the concentration-dependent release of nucleic acid was observed (Figure 2.4 Ab).

The leakage of a common cytosolic enzyme, L-lactate dehydrogenase (LDH), into the cell medium was measured. The LDH release by *S. pyogenes* treated with 125 μg/mL of carvacrol was estimated to be 47 - 71% relative to the total cellular LDH, which was significantly higher than the vehicle control (Figure 2.4 B). Furthermore, the amount of LDH released by carvacrol concentration of 250 μg/mL or more was not significantly different from that of the LDH released by Triton X-100. These results suggest the concentration-dependent disturbance to membrane integrity compromise by carvacrol. In contrast, carvacrol did not cause the release of LDH from cultured human tonsil epithelial cells (Figure 2.4 C).

2.4.6. *In vitro* cell cytotoxicity of carvacrol

The results showed that carvacrol was not cytotoxic for normal human tonsil epithelium (HTonEpiCs) cells at concentrations below or equal to 250 μg/mL (Figure 2.5 A). The viability of HTonEpiCs cells was determined to be 89%. The rest of the carvacrol concentrations tested exhibited safety, as > 95% of the cells were viable following carvacrol treatments (Figure 2.5). The cell morphology agreed with the above observations (Figures 2.5 B and 2.5 C). Results demonstrated that carvacrol within the tested concentration range is not toxic to mammalian cells (Figure 3.4 C); however, selectively toxic to bacterial cells.

2.5. DISCUSSION

Natural compounds have been used to treat infectious diseases worldwide (Lima et al., 2019; Mahady, 2005). Carvacrol is a monoterpenoid phenolic derivative primarily found in essential oils of herbal plants, including thyme and oregano. Extensive studies have been conducted investigating the biological properties of carvacrol for its potential use in clinical applications (Lee et al., 2017); (Baranauskaite et al., 2017; Barnwal et al., 2017). In this study, we investigated the antimicrobial activity of carvacrol against *S. pyogenes* and attempted to understand the potential mechanisms of action. The MIC and MBC values (125 and 250 μg/mL, respectively) for four strains coincide with the results of a previous study (Magi et al., 2015a) that reported MICs of carvacrol ranged from 64 to 256 μg/mL against clinical isolates of *S. pyogenes* isolated from children with pharyngotonsillitis in Italy. In addition, our previous study with oregano essential oils that consist of more than 90% of carvacrol exhibited a similar bactericidal activity (Wijesundara and Rupasinghe, 2018b). Similar to many other essential oil compounds, carvacrol's antimicrobial activity is related to its phenol structure and the presence of delocalized electrons (Ultee et al., 2002b).

The synergistic antibacterial activity of various plant extracts and phytochemicals in the presence of conventional antibiotics has been reported. The mechanism of synergistic actions of plant extracts and phytochemicals is postulated as a modification of active sites on bacterial cells, inhibition of enzymes that catalyze modification or degradation of antibiotics, inhibition of efflux pumps, or increase of membrane permeability (Mgbeahuruike et al., 2019; Nandu et al., 2018; Sanhueza et al., 2017). In addition, essential oils containing carvacrol, cinnamaldehyde, cinnamic acid, eugenol,

and thymol can exert synergistic effects combined with several antibiotics, including macrolides, has also been reported (Langeveld et al., 2014). However, in contrast to the evidence of synergism between several different erythromycin combinations and carvacrol reported (Magi et al., 2015a), we have observed no synergistic effect between the tested erythromycin and carvacrol combinations over all the strains. However, only additive synergy was seen in some concentrations of carvacrol, penicillin VK, carvacrol, and clindamycin, among all the combinations. Therefore, the effective carvacrol-antibiotics combinations of the current study can be further investigated using killing time, cell targets, and mechanisms of action, considering them as a potential therapeutic strategy for common drug-resistant bacteria.

The time-kill assay is a robust and appropriate tool for collecting information about the dynamic interaction between the antibacterial agent and the bacterial strain. Therefore, bacterial killing kinetics of various carvacrol concentrations at different times were assessed. Carvacrol-treated *S. pyogenes* was killed within 5 min exposure at concentrations over 250 μg/mL (1.05 mM), whereas low concentrations (≤ 125 μg/mL) of carvacrol inhibit the growth gradually via a sublethal phase. A previous study reported the detection of dead cells as early as 1 hr after incubation with carvacrol at the MIC using a live/dead assay (Magi et al., 2015a). We had previously investigated the time taken to accomplish complete *S. pyogenes* killing by penicillin G. When the MIC concentration of penicillin G (MIC = MBC = 0.016 μg/mL) was used in the kill curve against *S. pyogenes*, 24 hr were taken for the complete bacterial killing (Wijesundara and Rupasinghe, 2018b). The observed instantaneous bactericidal action of carvacrol in this study suggests that carvacrol may have affected the bacterial membrane integrity. On the

other hand, the observed time-dependent killing and growth inhibition by carvacrol indicate that additional mechanisms can be involved in cellular processes, such as inhibition of protein, nucleic acids, and lipid synthesis.

Ultrastructural morphological abnormalities induced by carvacrol were observed by electron microscopy. Comparisons of TEM images over their respective untreated controls revealed cell wall and membrane destruction by carvacrol and leakage of bacterial cytoplasmic contents when bacterial cells were exposed to carvacrol. The changes were evident with an increase in the concentration, consistent with the time-kill study results and cell leakage assays. These observations are parallel to the scanning electron microscopy observations of a previous study with carvacrol and thymol, an isomer of carvacrol, which was also able to disturb the *S. mutans* membrane and cause the release of cellular contents (Khan et al., 2017c). Moreover, a similar membrane destructive activity of carvacrol against another Gram-positive bacteria, *Staphylococcus aureus* ATCC 43300, was reported previously (Wang et al., 2016). The scanning electron micrograph observations showed that carvacrol-treated cells became rough and wrinkled, with depression appearing on their surfaces at the concentration of 1.03, 2.06, and 4.12 mM for 4-hr treatment (Wang et al., 2016). However, our TEM observation shows more detailed sectional views of the interior of an intact and damaged cell structure instead of the cell surface changes.

Leakage of nucleic acids and LDH enzymes are indicators that confirm the effect of carvacrol on cytoplasmic leakage of *S. pyogenes* cells. Carvacrol caused complete or severe damage to the membrane at higher concentrations and increased the leaching of cytosolic proteins, enzymes, nutrients, and genetic materials. Therefore, correlations

between leakage-inducing concentrations of carvacrol and MIC value suggest that membrane damage is an essential mechanism of action. Similarly, monoterpenes cooperate with cytoplasmic membranes' lipid bilayer, affecting membrane leakage of cellular contents such as ATP, ions, and nucleic acid (Nowotarska et al., 2017; Trombetta et al., 2005; Wang et al., 2016). Wang *et al.* (Wang et al., 2016) discussed the carvacrol-induced intracellular components leakage using β-galactosidase as an indicator. These observations showed that carvacrol treatment affected the integrity of *S. aureus* cell membranes, which likely resulted in a decrease in cell viability, and findings were in line with the concentration-dependent lactate dehydrogenase leakage in carvacrol-treated *S. pyogenes* cells in the present study. Therefore, we could suggest that carvacrol's cell membrane damage mechanism would not differ between these two species. Besides increasing the permeability of the bacterial cell membrane, it was suggested that carvacrol directly binds to genomic DNA as the second key mechanism of action (Wang et al., 2016). Future studies need to be aimed at understanding DNA damage in *S. pyogenes* cells by carvacrol. The ability of carvacrol to cause leakage of cellular contents suggests that its action may cause pores in the bacterial membranes. It is understood that the antimicrobial activity of many small molecules results in the formation of pores in the bacterial membranes and causes leakage of cellular contents (Yenugu et al., 2006).

Carvacrol has also been reported to be safe and exert minimal toxicity on human cells (Khan et al., 2017a). We confirmed the selective cytotoxicity of carvacrol towards bacterial cells over human tonsil epithelial cells. This bacterial selectivity might be attributed to different physicochemical properties of membrane components, especially phospholipids found in bacteria versus mammalian cells. Cholesterol is the major lipid

component of the eukaryotic membrane but not the bacterial cell membrane and may lead to discrimination between bacterial and host cell membranes (van Meer et al., 2008).

As in all Gram-positive bacteria, the cell wall of *S. pyogenes* is composed of thick peptidoglycan (PGN) covered with proteins, teichoic acid, and lipoteichoic acid (LTA) (Fischetti, 2016; Munoz et al., 1967). Hence, carvacrol must cross the bacterial cell wall before interacting with the cytoplasmic membrane; however, the role of the cell wall in interacting with carvacrol is yet to be revealed. We can postulate that cell wall PGN and teichoic acid may allow penetration of carvacrol to the cytoplasmic membrane. Therefore, future studies can be targeted to understand the effect of carvacrol on the inhibition of PGN biosynthesis and induction of PGN degradation. The LTA is present in most Gram-positive bacterial species, including *Streptococcus* (Brown et al., 2013). Fatty acid chains in the LTA are anchored in the membrane, whereas the remaining part of the LTA (glycerol-phosphate or ribitol-phosphate chain) hangs out through the cell wall. Lipophilic ends of these LTA are found on the cell wall surface and might facilitate the easy penetration of small monoterpene hydrophobic compounds such as carvacrol (Veldhuizen et al., 2006).

Therefore, accumulation of carvacrol in the membrane is expected and thereby causes instant membrane disruption. Bacteria would certainly need either modification of the charge of cytoplasmic membrane lipids or their composition to become resistant to those membrane-active antibacterial agents (Van Bambeke et al., 2008). However, our results indicated that bacteria are susceptible to carvacrol. Carvacrol is suggested to exert its bactericidal activity against *S. pyogenes* cells by disturbing cytoplasmic membrane integrity. Therefore, carvacrol has the potential to develop further as a

novel NHP for managing drug-resistant pathogens such as erythromycin-resistant *S. pyogenes*. However, to validate potential carvacrol applications as a new antibacterial agent and other possible mechanisms, including inhibition of protein biosynthesis, nucleic acid biosynthesis of *S. pyogenes*, biofilm formation, and inhibition of quorum sensing, and preventing adhesion, needs to be further investigated. In addition, the effects of carvacrol on bacterial membrane proteins, nucleic acid, and enzymes involved in membrane lipid biosynthesis and degradation need to be explored. Based on the observations, we suggest carvacrol as a potential antibacterial agent to treat or manage infections caused by *S. pyogenes*. Since carvacrol is a "generally recognized as safe" (GRAS) food additive by the United States Food and Drug Administration (Hallagan and Hall, 1995), a significant potential exists for carvacrol as a safe molecule for broader therapeutic applications.

2.6. CONCLUSIONS

This study investigated the antibacterial effect of carvacrol against *S. pyogenes*, particularly potential growth inhibition and rapid bactericidal mechanisms. Carvacrol at 250 μg/mL (1.66 mM) exhibited instantaneous bactericidal activity against three tested strains of *S. pyogenes*. Our study revealed that carvacrol kills *S. pyogenes* primarily by compromising the cell membrane integrity, leading to cytoplasmic content leakage and, ultimately, bacterial cell death. These findings suggest that carvacrol has the potential to develop as an NHP in the form of throat vapor, lozenge, or mouthwash to manage the discomfort associated with streptococcal pharyngitis. Furthermore, carvacrol can be further explored as a promising antibacterial agent with higher cell selectivity for potential clinical applications against drug-resistant pathogens.

Table 2.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (μg/mL) of carvacrol against four strains of *Streptococcus pyogenes*.

Drug	Strains of *Streptococcus pyogenes*							
	ATCC 19615		ATCC 49399		Clinical isolate		*Spy* 1558	
	MIC[a]	MBC[b]	MIC[a]	MBC[b]	MIC[a]	MBC[b]	MIC[a]	MBC[b]
Carvacrol	125	250	125	250	125	250	125	250
Penicillin G	0.008	0.016	0.008	0.016	0.008	0.016	0.008	0.016
Penicillin VK	0.008	0.016	0.008	0.016	0.008	0.016	0.008	0.016
Clindamycin	0.031	0.063	0.031	0.063	0.031	0.063	0.250	0.500
Erythromycin	0.125	0.250	0.125	0.250	0.125	0.250	15.63	31.25

[a] Minimum inhibitory concentration (MIC) was determined as the lowest concentration of the tested compound that inhibited bacterial growth.
[b] Minimum bactericidal concentration (MBC) was determined as the lowest concentration of the tested compound that killed at least 99.9% of the initial inoculums. Data are representative of three independent experiments.

Table 2.2 The fractional inhibitory concentration (FIC) and FIC index (FICI) for the carvacrol and antibiotic combinations against *Streptococcus pyogenes*.

Combinations		ATCC 19615				Clinical isolate			
A	B	FIC A	FIC B	ΣFICI	Interpretation	FIC A	FIC B	ΣFICI	Interpretation
Carvacrol	Penicillin G	0.5	1	1.5	IND	0.5	1	1.5	IND
Carvacrol	Penicillin Vk	0.5	1	1.5	IND	0.5	0.5	1	ADD
Carvacrol	Clindamycin	0.5	0.5	1	ADD	0	1	1	ADD
Carvacrol	Erythromycin	0.25	1	1.25	IND	-	-	-	-

Fractional inhibitory concentrations (FICs) and fractional inhibitory concentration index (FICIs) were calculated FICI = ΣFIC = FIC_A+FIC_B where MIC_A value is the MIC of compound A alone, MIC_B value is the MIC of compound B alone, and MIC_{A+B} value is the MIC of compound A in the presence of compound B, and *vice versa* for MIC_{B+A}.
FICIs were interpreted as SYN, synergy (FICI ≤ 0.5); ADD, additive synergy (> 0.5 FICI ≤ 1.0); IND, indifference/no interaction (> 1.0 FICI ≥ 4.0); and ANT, antagonism (FICI Index > 4.0).

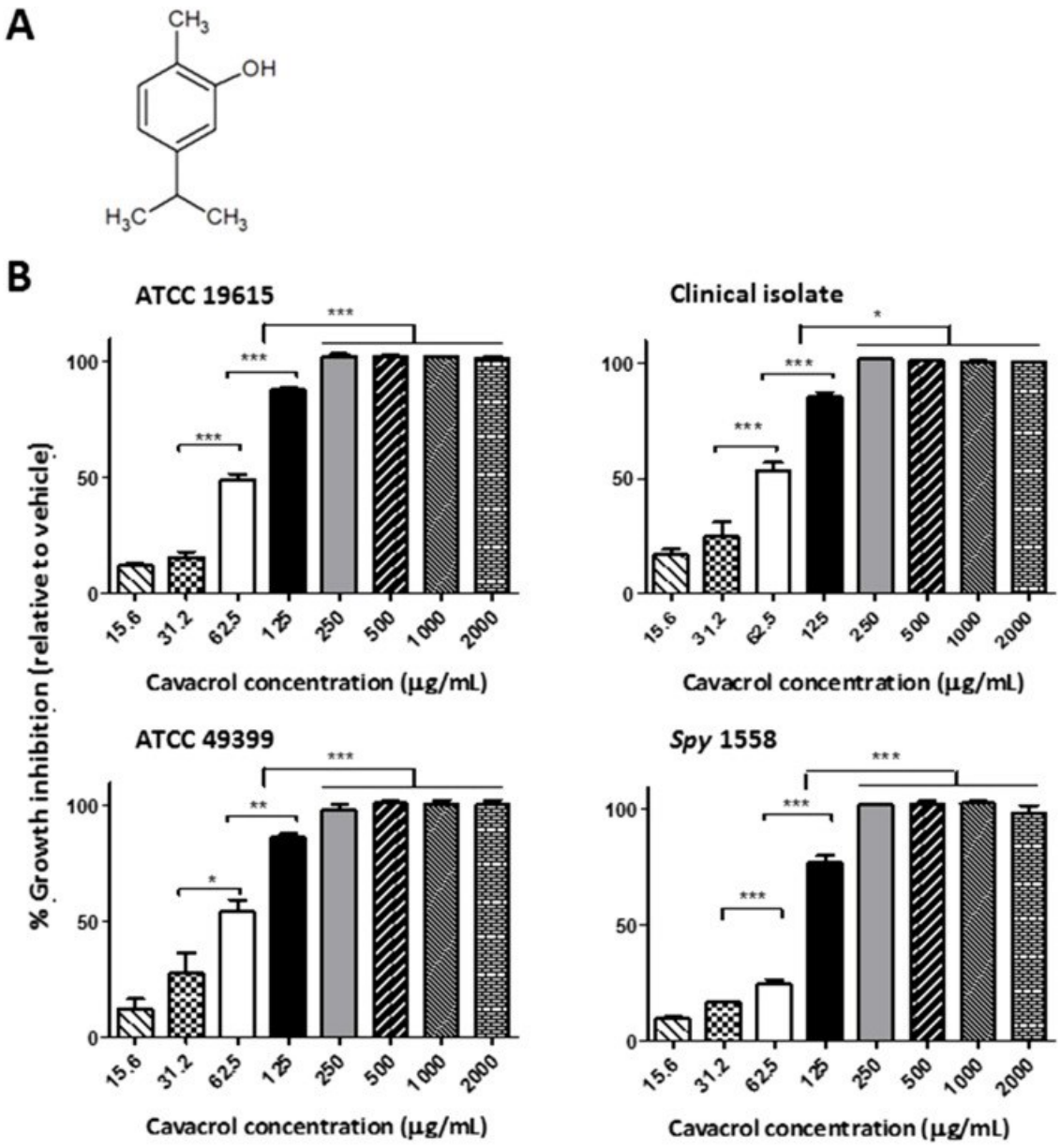

Figure 2.1 Carvacrol inhibits the growth of planktonic *Streptococcus pyogenes* in a concentration-dependent manner.

(A) The chemical structure of carvacrol. **(B)** Inhibition of *S. pyogenes* strains (ATCC 19615, ATCC 49399, a clinical isolate from a pharyngeal patient and an erythromycin-resistant *Spy* 1558) by carvacrol at the given concentrations was measured using micro-broth dilution assay in BHI broth after 24 hr of incubation at 37 °C. Bacterial turbidity

was measured at OD = 600 nm, and percentage growth inhibition was expressed relevant to the vehicle (0.25% DMSO) as the mean ± SE of three independent experiments. Statistical analysis was performed using one-way ANOVA, and the differences among means were compared using Tukey's test; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

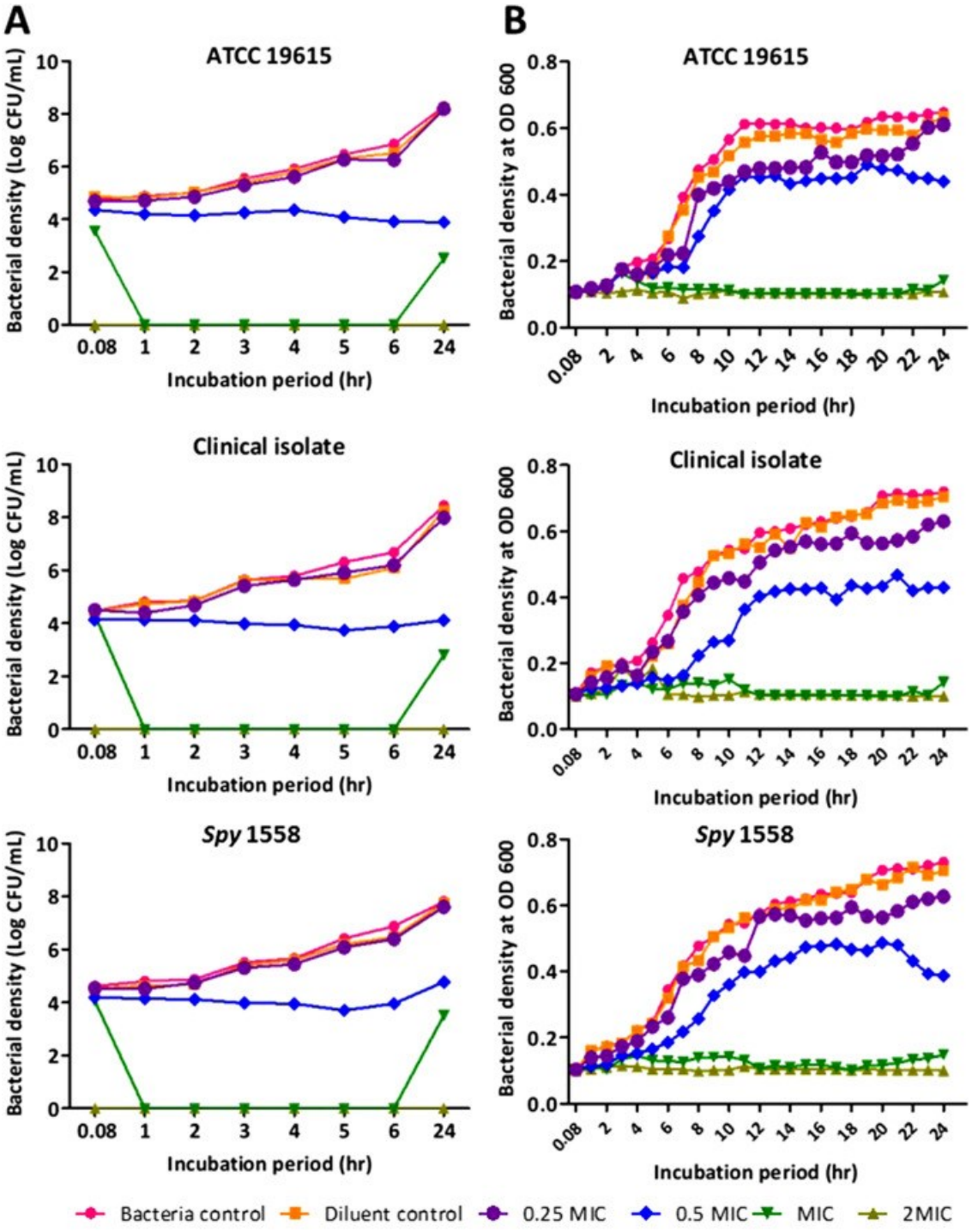
A
B
ATCC 19615
Clinical isolate
Spy 1558
Bacterial density (Log CFU/mL)
Bacterial density at OD 600
Incubation period (hr)
0.08
1
2
3
4
5
6
24
Bacteria control
Diluent control
0.25 MIC
0.5 MIC
MIC
2MIC

Figure 2.2 Carvacrol exerts concentration-dependent growth inhibition on *Streptococcus pyogenes* and an instantaneous bactericidal effect at 2 × MIC.

Time-kill curve plots from **(A)** agar plate count method* and **(B)** spectrophotometric method** on the growth of *S. pyogenes* ATCC 19615, clinical isolate, and erythromycin-resistant *Spy* 1558 in the presence of ¼ × MIC, ½ × MIC, 1 x MIC, and 2 x MIC of carvacrol or 0.25% DMSO (diluent control) and no treatment (bacteria control). The bacteria and carvacrol solutions were prepared in BHI broth and assessed for 24 hr at a 37 °C incubation period. *Cell growth/killing at 0.08, 1, 2, 3, 4, 5, 6, and 24 hr incubation was measured by performing viable cell counts by diluting cultures in saline solution (0.85% NaCl) enumeration on BHI agar plates in duplicate. **The bacterial turbidity at OD = 600 nm was measured using a spectrophotometer in 1 hr intervals over 24 hr incubation period with different carvacrol concentrations or diluent in triplicate. Both methods were performed in three independent experiments. "0" in the scale represents "below the limit of detection."

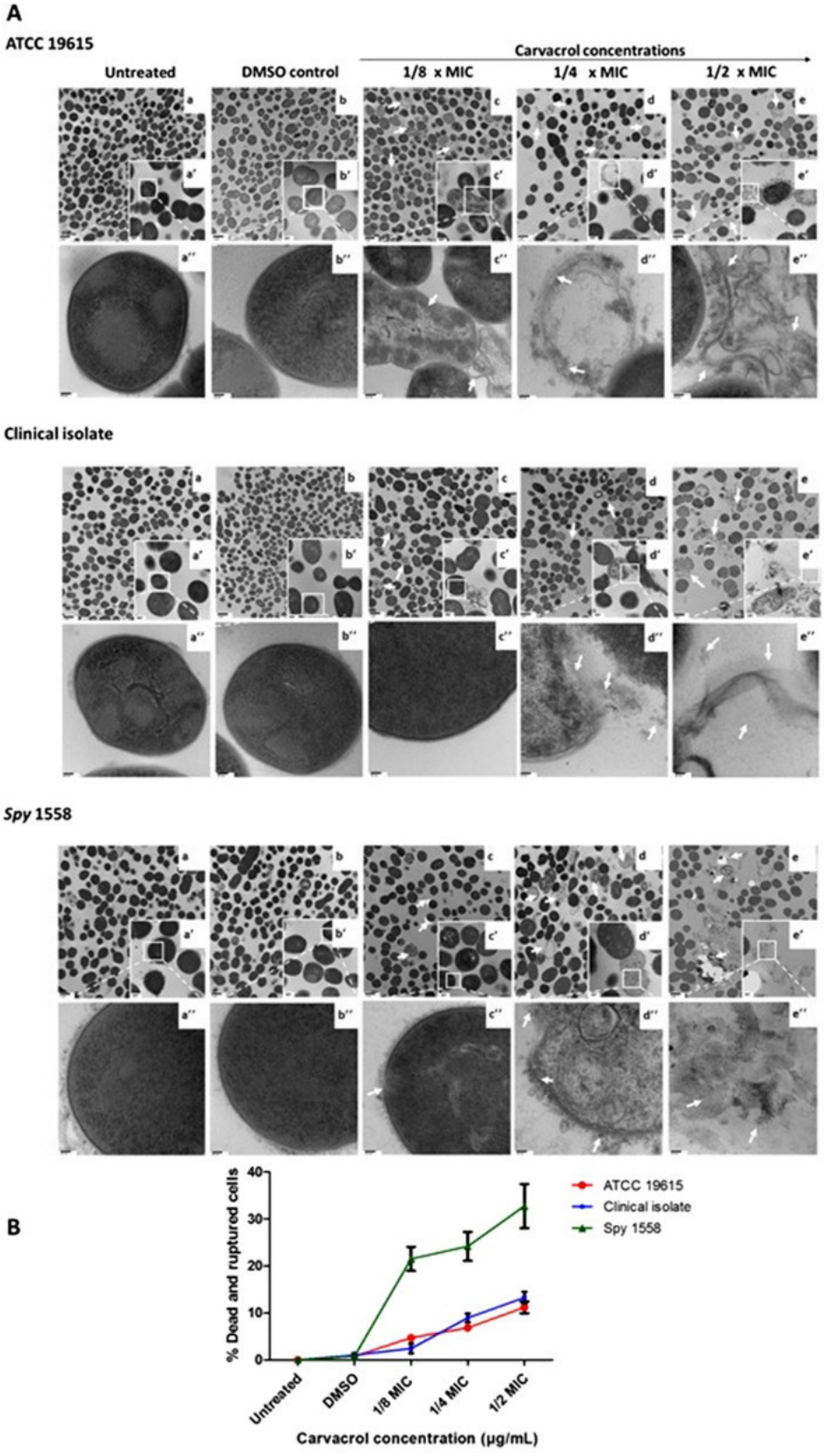
A
ATCC 19615
Carvacrol concentrations
Untreated
DMSO control
1/8 x MIC
1/4 x MIC
1/2 x MIC
Clinical isolate
Spy 1558
B
% Dead and ruptured cells
40
30
20
10
0
Untreated
DMSO
1/8 MIC
1/4 MIC
1/2 MIC
Carvacrol concentration (µg/mL)
ATCC 19615
Clinical isolate
Spy 1558

Figure 2.3 Transmission electron microscopic micrographs of three strains of *Streptococcus pyogenes* treated with or without exposure to carvacrol.

(A) Cell morphology of vehicle control (DMSO) (**b, b′, b′′**), carvacrol at 1/8 × MIC (**c, c′, c′′**), carvacrol at ¼ × MIC (**d, d′**, **d′′**), and carvacrol at ½ × MIC (**e, e′**, **e′′**) were compared with untreated bacterial (**a, a′**, **a′′**) in BHI media. The exponential phase of *S. pyogenes Spy* 1558 cells was treated with different concentrations of carvacrol for 16 h. Images were taken at magnifications of ×10,000, × 50,000 and ×100,000 for a,b,c,d,e , a′,b′,c′,d′,e′ and a′′,b′′,c′′,d′′,e′′, respectively. Arrows indicate dead cell morphologies. MIC, minimum inhibitory concentration; BHI, brain heart infusion. **(B)** Percentage of carvacrol-induced morphological damages determined by TEM. An average of 200 cells from two independent experiments were analyzed per treatment. Morphological changes were quantified as the percentage of ruptured and dead cells to the total cells.

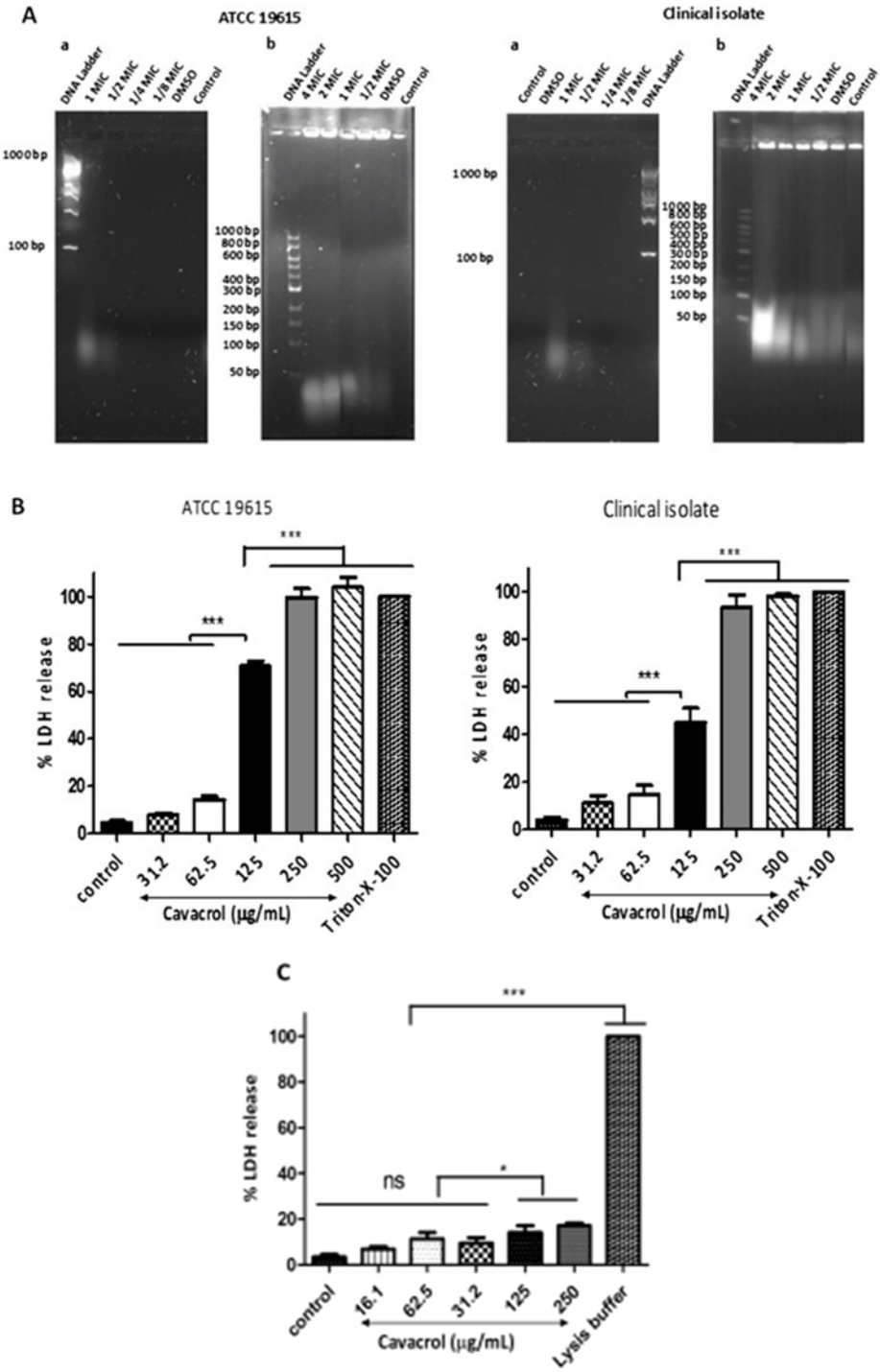
A
ATCC 19615
Clinical isolate
a
b
DNA Ladder
1 MIC
1/2 MIC
1/4 MIC
1/8 MIC
DMSO
Control
4 MIC
2 MIC
1000 bp
100 bp
50 bp
B
ATCC 19615
Clinical isolate
% LDH release
control
31.2
62.5
125
250
500
Cavacrol (µg/mL)
Triton-X-100
***
C
ns
*
16.1
Lysis buffer

Figure 2.4 Carvacrol induces cytoplasmic content leakage.

(A) Carvacrol causes the leakage of cytoplasmic nucleic materials from *Streptococcus pyogenes* in a concentration-dependent manner. Agarose gel (1 %, w/v) electrophoresis and gel red staining of leaked nucleic acid from cell suspensions (1×10^6, OD = 0.02) of ATCC 19615 and clinical isolate strains **(a)** exposed to MIC (125 μg/mL), 1/2 MIC, and 1/4 MIC of carvacrol or vehicle control (0.25% DMSO) over 24 hr. 1 kb ladder as reference. **(b)** Agarose gel (0.8%, w/v) electrophoresis and ethidium bromide staining of genomic DNA recovery by ethanol precipitation from bacteria suspension (OD = 0.6) followed by 2 hr carvacrol treatment. Carvacrol concentration is adjusted to the high bacterial density as MIC= 3750 μg/mL and 1 kb ladder were used as a reference. **(B)** Carvacrol causes lactate dehydrogenase (LDH) leakage from ATCC 19615 and is a clinical isolate of *S. pyogenes.* Overnight incubated cells were treated with different carvacrol or vehicle (0.25% DMSO) for 4 hr. A standard lysis buffer (9% Triton X-100) was included as a positive control and was defined as 100% LDH release. The carvacrol-induced LDH release into culture media was measured using the Promega LDH Cytotoxicity Assay Kit. Data expressed as % LDH release and represented mean ± SE (n = 3), ***P < 0.001, compared among means (ANOVA, Tukey's test). **(C)** Carvacrol does not cause the release of LDH from cultured human tonsil epithelial cells. The seeded human tonsil epithelium cells (TonEpiCs) for 24 hr were treated with different concentrations of carvacrol or DMSO vehicle for 4 hours. A standard lysis buffer was included as a positive control and defined as 100% LDH release. The carvacrol-induced LDH release into cell supernatant was measured using the Promega LDH Cytotoxicity Assay Kit. Data expressed as % LDH release and represented mean ± SEM (n=3), ***P < 0.001, *P < 0.05, compared among means (ANOVA, Tukey's test).

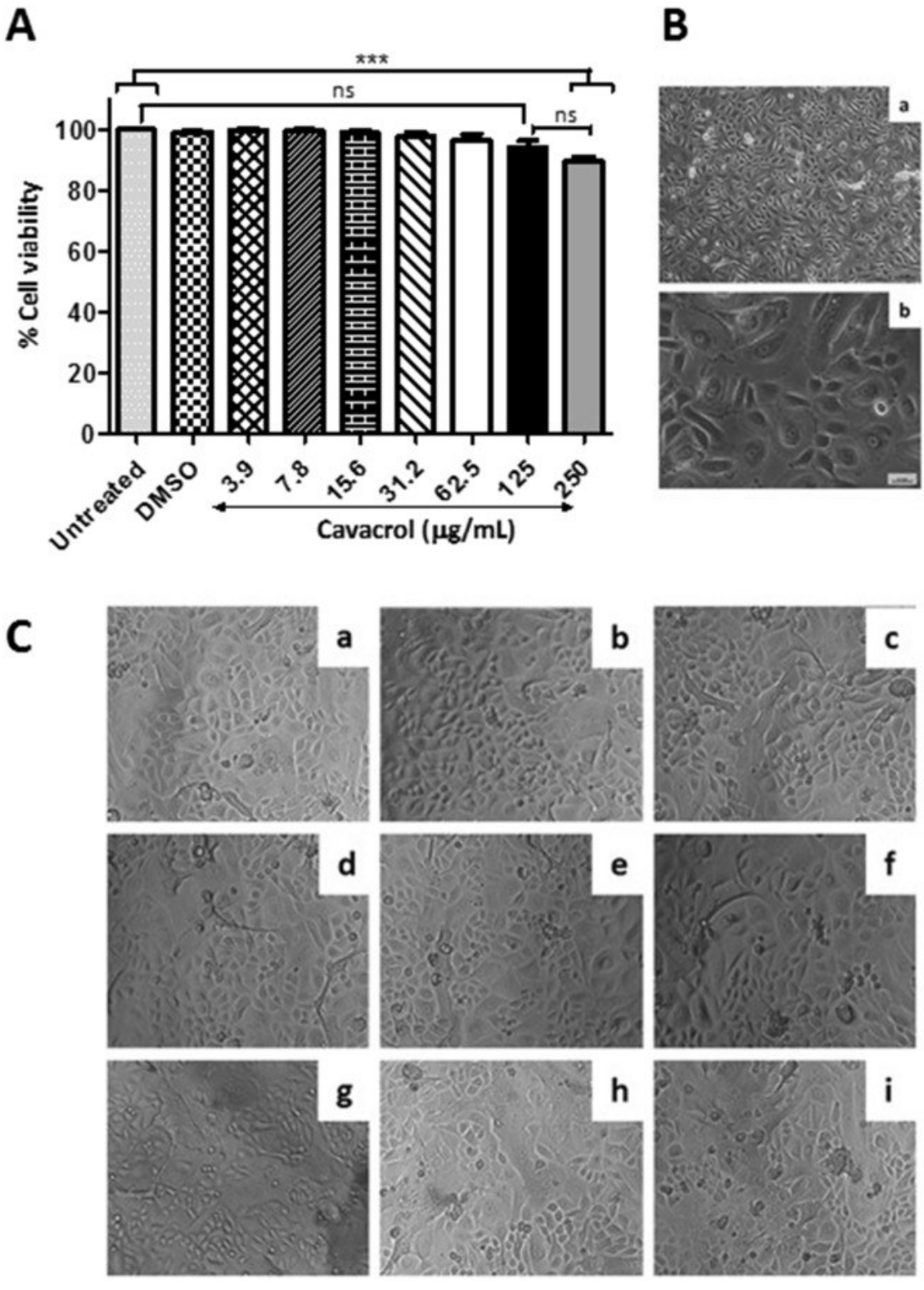
A
***
ns
ns
% Cell viability
100
80
60
40
20
0
Untreated
DMSO
3.9
7.8
15.6
31.2
62.5
125
250
Cavacrol (µg/mL)
B
a
b
C
a
b
c
d
e
f
g
h
i

Figure 2.5 Carvacrol is not cytotoxic to the human tonsil epithelium cells.
(A) Cell viability was measured using the MTS assay after treating human tonsil epithelium cells (TonEpiCs) with 15.6, 31.2, 62.5, 125, and 250 µg/mL carvacrol for 24 hr. Absorbance was measured at 490 nm. Data are shown as mean ± SE from three independent experiments in triplicate. Differences among means were compared with untreated TonEpiCs (ANOVA, Tukey's test). **(B)** Morphology of TonEpiCs grown in a poly-L-Lysine coated flask with tonsil epithelial growth medium at 90% confluence was observed using a phase-contrast microscope. (a) Magnification, ×100; (b) Magnification, ×400. **(C)** TonEpiCs treated with different carvacrol were photographed using a phase-contrast microscope at ×200 magnification. Representative photographs captured 24 hr post-treatment in three independent experiments are shown. (a) untreated; (b) vehicle control (0.25% DMSO) and carvacrol treatments of (c) 3.9 µg/mL, (d) 7.8 µg/mL, (e) 16.1 µg/mL, (f) 31.2 µg/mL, (g) 62.5 µg/mL, (h) 125 µg/mL, and (i) 250 µg/mL.

2.7. SUPPLEMENTARY FIGURES

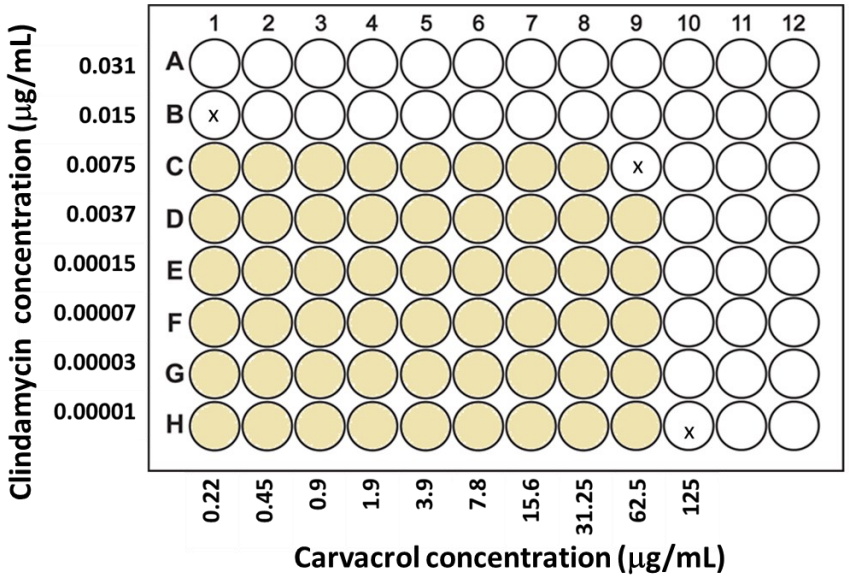

Figure 2.6. S1. Microdilution chequerboard method for fractional inhibitory concentration index (FICI) determination. Shading wells represent the visible growth of *Streptococcus pyogenes*.

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CHAPTER 3. THE BACTERICIDAL ACTIVITY OF CARVACROL AGAINST STREPTOCOCCUS PYOGENES INVOLVES THE ALTERATION OF MEMBRANE FLUIDITY AND INTEGRITY THROUGH INTERACTION WITH MEMBRANE PHOSPHOLIPIDS

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3.1. ABSTRACT

Background: Carvacrol, a mono-terpenoid phenol found in herbs such as oregano and thyme, has excellent antibacterial properties against *Streptococcus pyogenes*. However, its mechanism of bactericidal activity on *S. pyogenes* has not been elucidated. **Objectives:** This study investigated the antibacterial mechanism of carvacrol on cell membrane damage of three *S. pyogenes* strains. **Methods:** Flow cytometry (FCM) experiments were conducted to determine carvacrol's membrane permeabilization and cytoplasmic membrane depolarization activity. *S. pyogenes* protoplasts were used to investigate carvacrol's effects on the membrane, followed by gel electrophoresis. The carvacrol-treated protoplasts were examined by transmission electron microscopy (TEM) to observe ultrastructural morphological changes. The fluidity of the cell membrane was measured by steady-state fluorescence anisotropy. Finally, thin layer chromatographic (TLC) profiling was conducted to identify the affinity of carvacrol for membrane phospholipids. **Results:** Increased membrane permeability and decreased membrane potential from FCM and electron microscopy observations revealed that carvacrol killed microbial cells primarily by disrupting membrane integrity, leading to whole-cell lysis. Ultra-structural morphological changes in the membrane induced by carvacrol over a short period were confirmed using the *S. pyogenes* protoplast and membrane isolate models *in vitro*. In addition, changes in the other biophysical properties of the bacterial membrane, including concentration- and time-dependent increased fluidity, were observed. TLC experiments showed that carvacrol primarily preferentially interacts with membrane phosphatidylglycerol (P.G.), phosphatidylethanolamine (P.E.), and cardiolipins (CL). **Conclusion:** Carvacrol exhibited rapid bactericidal action against *S.*

pyogenes by disrupting the bacterial membrane and increasing permeability, possibly due to affinity with specific membrane phospholipids such as P.E., P.G., and.CL. Therefore, its bactericidal concentration of carvacrol (250 μg/mL) could be used to develop safe and efficacious natural health products for managing streptococcal pharyngitis or therapeutic applications.

KEYWORDS: Carvacrol, streptococcal pharyngitis, *Streptococcus pyogenes*, mechanism of action, membrane phospholipids, permeability

3.2. INTRODUCTION

Carvacrol, 2-methyl-5-(propan-2-yl) phenol, is a monoterpene phenolic compound (Figure 3.4 A) that is reported in various herbal plant extracts belonging to the genera *Thymus* and *Oregano* (Lee et al., 2017; Vinciguerra et al., 2018; Wijesundara and Rupasinghe, 2018b; Wijesundara and Rupasinghe, 2019a). Carvacrol was reported to inhibit the growth of many Gram-positive and Gram-negative pathogenic bacteria, including methicillin-resistant *Staphylococcus aureus, Streptococcus pneumoniae*, and *Haemophilus influenzae* cause various upper respiratory tract infections (Al-Mnaser and Woodward, 2020; Li et al., 2014).

Streptococcus pyogenes is a major upper respiratory tract pathogen that causes diseases with significant morbidity and mortality worldwide (Nelson et al., 2016; Pfoh et al., 2008). One of the common diseases caused by *S. pyogenes* is streptococcal pharyngitis, primarily treated with penicillins, and no significant resistant cases have been reported over the decades (Shulman et al., 2012; van Driel et al., 2016b). However, cases caused by some erythromycin-resistant *S. pyogenes* strains have been identified (Magi et al., 2015a; Silva-Costa et al., 2015b). According to our findings explained in Chapter 2, carvacrol showed effectiveness against an erythromycin-resistant strain of *S. pyogenes,* the *Spy* 1558 strain, at a minimum inhibitory concentration (MIC) of 125 µg/mL. Therefore, we have initiated an understanding of carvacrol's potential mode of action against this bacterium.

Furthermore, cellular content leakage and ultra-structural modification were observed upon carvacrol treatment, suggesting that carvacrol may have affected the cell wall and/or cell membrane. Therefore, carvacrol has the potential to be a promising

alternative to antibiotics to prevent, treat, and manage streptococcal pharyngitis. Carvacrol is also a pain-relieving NHP; hence**,** we need to properly understand how carvacrol works as an antibacterial agent at the cellular level. Several studies have investigated the modes of action of carvacrol on Gram-negative bacteria. For example, carvacrol is shown to disrupt the cell membrane, inhibit the biosynthesis of protein, and interrupt the pathogenic life cycle, including adhesion and biofilm formation (Di Pasqua et al., 2006b; Khan et al., 2017a; Khan et al., 2017b; Lee et al., 2017). However, carvacrol's exact mechanisms of action on *S. pyogenes* have not been reported. In the current study, we investigated the effects of carvacrol on the changes in membrane permeabilization, membrane potential, and membrane fluidity using intact *S. pyogenes* cells and protoplasts.

3.3. MATERIALS AND METHODS

3.3.1. Media and chemicals

Brain heart infusion (BHI) was purchased from Oxoid Ltd (Nepean, ON, Canada) and prepared to the manufacturers' specifications. Carvacrol was purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). It was kept at 4 °C. Sodium chloride (NaCl, ≥ 99.0%), sodium acetate (NaOAc), and magnesium chloride ($MgCl_2$) were purchased from BioShop (BioShop™ Canada Inc., Burlington, ON, Canada), casamino acids (Bacto™, Dickinson and Company, MD, USA), glucose (EM Science, NJ, USA), and phenyl methane sulphonylflurodide (PMSF) (BDH chemicals Inc., ON, Canada) were used for the buffer preparations. Agarose (Thermo Fisher Scientific Inc., MA, USA), vanillin, penicillin G sodium salt, daptomycin, Dulbecco's phosphate-buffered saline (PBS), dimethyl sulphide (DMSO) (≥ 99.8%), ethidium bromide (3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide, >95%), sucrose (1-O-α-D-glucopyranosyl-β-D-fructo-furanoside) and mutanolysin were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada). L-α-phosphatidyl-DL-glycerol sodium salt (P.G.), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (P.E.), L-α-phosphatidylcholine (P.C.), cardiolipin sodium salt (CL), and cholesterol (Ch) were also purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

3.3.2. Bacterial strains and growth conditions

Three *S. pyogenes* strains, ATCC 19615, a clinical isolate (isolated from a pharyngitis patient), and an erythromycin-resistant strain (*Spy* 1558, erm), were used in the study and were all cultured in BHI at 37 °C and 5% CO_2. Stock cultures were stored at − 80 °C in BHI containing 20% glycerol. When required, the subcultures were made on BHI agar

plates and grew in a humid 5% CO_2 incubator (Model 3074, VWR International, West Chester, PA, USA) at 37 °C. Sub-cultures were refreshed every two weeks. The inoculum suspension in the broth was prepared and diluted to 1×10^6 CFU/mL (OD_{600} = 0.02) in the BHI medium, as described previously (Wijesundara and Rupasinghe, 2018b).

3.3.3. Membrane permeability

Cell membrane permeability following carvacrol treatments was assessed by flow cytometry using LIVE/DEAD BacLight™ Bacterial Viability Kit (L7012, Molecular Probes Inc, Eugene, OR, USA). Bacteria were stained with SYTO9, a membrane-permeable green-fluorescent nucleic acid stain or, propidium iodide (PI), a membrane-impermeable red-fluorescent nucleic acid stain.

***Test sample preparation*:** *S. pyogenes* cells from cultures in the mid-exponential growth phase were harvested by centrifugation at 10,000 × *g* for 10 min, washed, and resuspended to an OD_{600} = 0.02. Cell suspensions were then diluted to 10^6 CFU/mL in PBS. Next, the cells were then exposed to carvacrol at 2 × MIC, 1 × MIC, 1/2 × MIC, or DMSO vehicle control for different periods (30 min, 1 hr, 16 hr, and 24 hr) at 37 °C, 5 % CO_2. The cells were stained, as described below**.**

PI staining: PI (3 µL, 20 mM) was incubated with 1 mL of cells for 20 min in the dark. A dead cell control (membrane-compromised cells) was prepared from an overnight culture, and cells were heat-killed at 95 °C for 15 min. The dead cell control (10^6 CFU/mL) was similarly stained. The samples were analyzed by flow cytometry within 1 hr of staining.

SYTO 9 staining: A viable cell sample was prepared from an overnight culture not treated with carvacrol and served as the 100% live-cell control. The test cell sample and viable cell control (10^6 CFU/mL) were incubated with 3 μL of 3.34 mM SYTO 9 in DMSO at room temperature for 15 min. The samples were kept in the dark at room temperature for no more than 1 hr and analyzed by flow cytometry.

Double-staining with PI and SYTO9: Carvacrol- or DMSO-treated cell suspensions were diluted to reach a final density of 10^6 CFU/mL bacteria ($OD_{600} = 0.02$). A mixture of stains was prepared by combining PI (20 mM) and SYTO 9 (3.34 mM) (1:1 ratio), and 3 μL of the combined stain mixture was added to each of the samples (treated samples and DMSO vehicle control sample). The tubes were then incubated at room temperature in the dark for 20 min before measuring the fluorescence of bacterial suspensions with a fluorescence-activated cell sorter (FACS) flow cytometer (CytoFLEX, Beckman Coulter Inc., IN, USA).

Flow cytometry (FCM): To measure the fluorescence intensity, the laser excitation/emission wavelength of 485/542 nm for SYTO 9 and 485/610 nm for PI. Background fluorescence from the medium was determined, and the results were corrected as necessary. Cell suspensions without carvacrol treatment served as the control, and 10,000 events were recorded for each sample. Data acquisition was controlled by CytExpert™ software (Version 2.1, Beckman Coulter Inc., IN, USA) and analyzed using the FACS express software (Version 5, De Novo Software, Glendale, CA, USA).

3.3.4 Fluorescence microscopy of bacterial viability

Microscopic comparison of live/dead cells was performed using LIVE/DEAD BacLight Bacterial Viability Kits (L7007, Molecular Probes Inc, Eugene, OR, USA). Briefly, late exponential phase bacteria (ATCC 19615) resuspended to 1×10^6 cells/mL were treated with carvacrol for 16 hr at 37 °C. Samples treated with DMSO were included as controls. Equal volumes of PI and SYTO 9 were combined, and 3 µL of the dye mixture was added to 1 mL of the bacterial suspension and incubated at room temperature for 15 min in the dark. A 5 µL of the stained bacterial suspension was examined under a fluorescence microscope (Axio Imager 200 M, ZEISS, Gottingen, Germany) equipped with a 63 × magnification oil immersion objective.

3.3.5. Membrane potential

The effects of carvacrol on the membrane potential / cytoplasmic membrane depolarization activity were determined according to the BacLight™ Membrane Potential Kit instructions (B34950, Molecular Probes Inc, Eugene, OR USA).

***Sample preparation*:** Five mL samples of *S. pyogenes* ATCC 19615 (1×10^8 CFU/mL) were treated with carvacrol (2 × MIC, MIC, 1/2 × MIC; MIC = 125 µg/mL) or DMSO vehicle control. Ten microliters of 500 µM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a proton ionophore, were added for 1 mL of each sample. The samples were incubated at 37 °C at 5% CO_2. At 30 min, 1 hr, 16 hr, and 24 hr, the cells were harvested and diluted in BHI to approximately 1×10^6 CFU/mL. The cells (1 mL) were stained with 10 µL of 3 mM of 3, 3′-diethyloxacarbocyanine iodide [DiOC2(3) (Molecular Probes, Sigma, Louis, USA) DiOC2(3), A membrane potential-sensitive fluorescent probe, for 30 min at 37 °C.

FCM: Flow cytometry was performed at excitation and emission wavelengths of 622 nm and 670 nm, respectively. Background fluorescence resulting from the medium was determined. Ten thousand events were recorded for each sample. The data were expressed by mean fluorescence intensity (MFI).

3.3.6. Protoplast experiments

3.3.6.1 Preparation of protoplasts

Protoplasts were cultivated according to the methods described by Linder et al. (1983) and Parks et al. (1980) with modifications. Bacterial culture was harvested at the mid-exponential phase, adjusted to $OD_{600} = 0.6$, and centrifuged for 5 min at 10,000 × g. Cells were washed once in a sterilized protoplast wash buffer (50 mM sodium acetate pH 6.5, 0.2 mM $MgCl_2$) and were re-suspended in 200 µL warmed (37 °C) protoplast buffer (50 mM sodium acetate, 0.2 mM $MgCl_2$, 30% sucrose, 0.1% glucose, 0.05% casamino acids, pH 6.5) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 800 units of mutanolysin. Mutanolysin, an N-acetylmuramidase was used for cell lysis purpose. Samples were incubated at 37 °C for 30 min and were then used to inoculate 2 mL of BHI media containing 30% of sucrose and 0.06 µg/mL of penicillin G. Protoplast cultures were incubated for 20 hr at 37 °C, 5% CO_2. All solutions and media were filtered, sterilized, and stored at room temperature.

3.3.6.2 Confirmation of protoplast formation

Microscopy of protoplast samples

A sample taken immediately from an overnight protoplast culture was examined under a light microscope to confirm that protoplasts had formed. Next, the osmotic support of the

protoplast sample was removed by adding water, and burst-open cells were observed under light microscopy for the presence of burst-open cells. Briefly, the pellet was collected after 1 mL of the overnight culture was centrifuged (5 min, 10,000 g). Next, a sample drop on a glass slide was examined under light microscopy after the pellet had been redissolved in 100 L of water.

Further verification of the proper formation of protoplasts was accomplished by observing the protoplast cultures by transmission electron microscopy (TEM). Briefly, an overnight protoplast culture (1 mL) was centrifuged (5 min, 10,000 × g), and the pellet was washed once in protoplast buffer. Then, as described previously (Wijesundara and Rupasinghe, 2019a), the pellet was fixed, dehydrated, embedded in resin, and then visualized in the thin stained sections.

Gel electrophoresis of protoplast samples

The 1 mL protoplast culture of *S. pyogenes* was centrifuged (10,000 × g, 5 min), and pellets were collected. Then, 0.8% agarose gel electrophoresis was performed for the pellets before and after adding water (100 μL) for leakage of nucleic acids.

3.3.6.3. Confirmation of the effect of carvacrol on bacterial membrane

Effect of carvacrol on protoplast samples

The protoplast of *S. pyogenes* (ATCC 19615) was prepared and cultivated in 10 mL of BHI media containing 30% of sucrose and 0.06 μg/mL of penicillin G (20 hr, 37 °C). Bacteria density was adjusted to OD_{600} = 0.6, and then 2 mL of each bacterial suspension was treated with carvacrol concentrations as 4 × MIC (15 μg/mL) 2 × MIC (7.5 μg/mL), MIC (3.75 μg/mL), 1/2 × MIC (1.88 μg/mL) calculated according to the cell density.

Protoplasts treated with Carvacrol were incubated for 1 hr at 37 °C in the presence of 5% CO_2. Then ethanol precipitated nucleic acid released into the supernatant was collected according to the previously described method in chapter 2.3.7.2 and examined after 0.8% agarose gel electrophoresis.

Transmission electron microscopy of carvacrol-treated protoplast samples.

Samples of each concentration of carvacrol treatment with protoplast were centrifuged (5 min, 10,000 × g), and the pellets were washed in protoplast buffer. Samples were fixed and visualized using TEM according to the method mentioned in 2.6.2. The untreated protoplast was used as a control.

3.3.7. Fluorescence anisotropy

The changes in the anisotropy values were monitored in the membranes of live bacteria using the 1,6-diphenyl 1,3,5-hexatriene (DPH), a hydrophobic fluorescent probe. Briefly, the mid-exponential *S. pyogenes* (*Spy* 1558) cells were adjusted to an OD_{600} of 0.02. Then, cells were treated with or without carvacrol at 37 °C for 1hr and 24 hr. Next, cells were centrifuged, washed twice in PBS, and resuspended in PBS. Then, DPH was added to a final concentration of 2 µM and incubated for 10 min at 37 °C. Steady-state fluorescence anisotropy measurements were performed at 37 °C using a PTI QuantaMaster-4-CW spectrofluorometer (Photon Technology International Inc., Birmingham, England). The fluorescence emission was measured at 37 °C using excitation and emission wavelengths of 356 nm and 423 nm, respectively, and processed using a PTI FeliX32 Analysis module. Fluorescence anisotropy (r) was calculated as:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

Where "*I*" is the fluorescence intensity measured when the excitation and emission polarizers are fixed in the vertical (v) or horizontal (h) position, the instrument determines g to correct for artifacts due to optical components of the fluorimeter.

3.3.9. Thin-layer chromatography (TLC) analysis

Chromatography was performed on 20 × 20 cm silica gel 60 F_{254} glass plates (thickness: 0.5 mm) (Merck Millipore Corporation, Darmstadt, Germany). Phospholipids (P.G., P.E., P.C., CL, Ch), carvacrol, and daptomycin were dissolved in chloroform (25 mg/mL). The individual phospholipid was mixed with carvacrol in chloroform at a ratio of 2:1 (1:1 vol/vol) and incubated for 1 hr at 4 °C. Then, 20 volumes of ice-cold acetone were added to the mixture and incubated for 1 hr at 4 °C. The precipitated materials were collected by centrifugation (21,000 × g, 15 min). The supernatant was also saved and dried under a stream of nitrogen gas under the nitrogen evaporator (N-EVAP™, Organomation Association Inc., Berlin, NJ, USA). The precipitate and the dried supernatant samples were dissolved in 20 µL of chloroform. Approximately 5 µL of each sample, pure phospholipids, carvacrol/daptomycin, were loaded on the TLC plates using a chloroform-methanol-acetic acid solvent system (65:25:10 [vol/vol/vol]). The samples were chromatographed for 1 to 1.5 hr at room temperature (20 ± 1 °C).

After chromatographic separation, the plates were air-dried for 15 min and sprayed with alcoholic vanillin–sulphuric acid solution (1 g of vanillin, 100 mL 95% ethanol, and 10 mL 95% sulphuric acid). The plates were air-dried for 15 min and then heated in a 100 °C oven until the color spots became visible. The spots were captured using photographs and observed under UV using the Bio-Rad™ Gel Doc Imaging System (Bio-Rad Laboratories Inc, MP, CA, USA). The Rf values of the compounds

were determined as the distance moved by the spot front and/distance moved by the solvent front. Spots of phospholipids and carvacrol in the precipitates and supernatants and the unbound carvacrol spots in the supernatants were identified by relating the color, spot size, and Rf values to those of the standard phospholipids and carvacrol. Interactions of phospholipids with daptomycin were considered the positive control.

3.3.10. Statistical analysis

All assays were performed in triplicates, and the results were expressed as the mean ± standard error of three independent experiments. One-way analysis of variance analysis (ANOVA) was performed using Minitab statistical software (Version 17.0, Minitab Inc., State College, PA, USA) and GraphPad Prism software (Version 5.0, LA, CA, USA). Statistical differences were defined as ($p \leq 0.05$), and mean separations among treatments were determined using Tukey's tests.

3.4. RESULTS

3.4.1. Carvacrol increases the permeability of the bacterial cell membrane.

To investigate the effects of carvacrol on cell membrane integrity and cell death, we employed PI and SYTO9 staining followed by flow cytometry. *S. pyogenes* treated with carvacrol showed a concentration- and time-dependent increase in the uptake of PI and SYTO9 stains, suggesting that the bacterial cell membrane has been disrupted (Figure 3.1 and Figure 3.7. S2). However, such an increase was absent in cells not treated with carvacrol. In addition, cells generally showed less red fluorescence in the untreated and DMSO controls, indicating these cells have intact bacterial membranes.

3.4.2. Carvacrol shows a concentration-dependent on increment in dead cells.

The effects of exposure to bacteriostatic concentrations of carvacrol on cell viability and membrane damage were examined using the LIVE/DEAD BacLight® bacterial viability stains. The results showed that cells treated with 1 × MIC of carvacrol displayed an intense and higher red color than cells treated with DMSO control (Figure 3.2). Cells treated with sub-MIC carvacrol levels showed a concentration dependent on color-stained cells. Furthermore, the total number of cells decreased with increasing carvacrol concentrations.

3.4.3. Carvacrol causes depolarization of the cytoplasmic membrane of *S. pyogenes*.

To reveal whether the antibacterial action of carvacrol involves disruption of bacterial membrane potential, red and green fluorescence emitted by DiOC2(3) were measured. The red: green fluorescence ratio indicates the altered membrane potential compared to control cells. The scatter plots of green versus red fluorescence exhibited by the bacterial cells are shown in the right panels of Figure 3.3. Treatment of *S. pyogenes* with the

ionophore CCCP resulted in a decreased red-to-green fluorescence ratio, indicating a reduction in membrane potential; however, DMSO control showed a significant reduction only during the early incubation period (Figures 3.3 and 3.8. S3). Furthermore, the fluorescence intensity ratio of red to green dropped significantly with carvacrol over time. Therefore, carvacrol is more potent in membrane depolarization than CCCP. These results suggested that carvacrol may depolarize bacterial cell membrane concentration time-dependently.

3.4.4. Carvacrol-induced membrane damage in protoplasts

3.4.4.1. confirmation of protoplast formation

Protoplasts were created by incubating ATCC 19615 and clinical isolate cells with mutanolysin in the presence of sucrose as osmotic support. Protoplasts were cultured overnight in the media containing 30% sucrose and penicillin G to prevent the regeneration of the cell wall. Light microscopic examinations of the culture showed that the cells were rounder in shape and more prominent than intact cells. When the osmotic support was removed, these protoplasts lysed. When the protoplasts were cultured in media lacking penicillin G, the cells were stained, Gram-indicating the regeneration of the cell wall.

The gel electrophoresis of protoplast supernatants with the removed osmotic support showed a concentration-dependent nucleic acid leakage (Figure 3.4 A). The results suggest that the nucleic acids were from protoplast lysis. In addition, TEM analysis showed that the protoplasts have an irregular shape and are more translucent than their intact cells (Figure 3.4 B).

3.4.4.2. The effect of carvacrol on protoplast membrane

TEM was used to observe the ultrastructure of *S. pyogenes* protoplasts treated with carvacrol. The untreated protoplasts showed intact membranes and uniformly distributed electron-dense cytoplasm (Figure 3.4 B). In contrast, more detractive morphological features such as cell membrane disruption, cytoplasmic vacuolations, and cell deformation were observed in *S. pyogenes* protoplasts treated with>125 μg/mL of carvacrol. At a lower concentration of carvacrol (62.5 μg/mL), damage to the cell membrane was observed, but to a lesser extent (Figure 3.4 B).

Nucleic acid leakage into the cell medium was assessed to confirm carvacrol treatment leading to cell constituent leakage followed by protoplast membrane disruption. Furthermore, a similar pattern of concentration-dependent leakage of nucleic acids in intact ATCC 19615 and clinical isolate cells (Chapter 2, Figure 2.4) was observed in *S. pyogenes* protoplast of ATCC 19615 and a clinical isolate (Figure 3.4 C). Thus, the results confirmed the disruptive membrane mechanism of action of carvacrol.

3.4.5. Carvacrol causes concentration-dependent membrane fluidity changes.

Fluorescence anisotropy was used for studying the rotational diffusion of DPH within the fatty acyl chains of the cell membrane. The anisotropy values of DPH-integrated intact cells after treatment with carvacrol and controls (triton X -100 and tween-20) are shown in Table 3.1. Different anisotropy values between the untreated, carvacrol treated or positive control cells reflect their membrane fluidity. Our results show a significant decrease in the anisotropy value with increasing carvacrol-treated bacterial cells compared with the control untreated cells, suggesting enhanced fluidity in the cell membrane of carvacrol-treated cells.

3.4.6. Carvacrol preferentially binds to P.G., P.E., and partially to CL in the bacterial membrane.

Preliminary investigations showed that the best solvent system for our experiments is chloroform-methanol-acetic acid (65:25:10), and the ideal detection dye is alcoholic vanillin solution (Figure 3.5 A and B). Carvacrol-treated P.G. and P.E. precipitates showed spots of their respective phospholipids, and their respective supernatants showed no unbound carvacrol spots. Therefore, initially added carvacrol was assumed to be bound entirely with P.G. and P.E. However, both CL-treated carvacrol precipitant and supernatant showed CL spots, whereas its supernatant additionally showed an unbound carvacrol spot (Figure 3.5C and S 4A). Observation of CL samples suggested that carvacrol may be partially bound with CLs.

Two spots of P.C. phospholipids were detected in the supernatant of the P.C. treated sample with a spot similar to the initially added carvacrol. Therefore, we suggest that P.C may break down by carvacrol's effect but may not be bound with broken structures. Observation of no spots in Ch treated precipitate and Ch and carvacrol spots similar to initially added spots suggested carvacrol may not be bound with Ch. Furthermore, their respective precipitants of daptomycin-treated samples showed P.G., P.E., P.C., and CL spots and no trace of unbound daptomycin in the supernatant of those four phospholipids (Figure 3.5D and S 4B). Carvacrol and daptomycin left the supernatants with prominent P.C. fractions (two different spots).

3.5. DISCUSSION

Carvacrol is a phenolic monoterpenoid present primarily in essential oils of herbal plants, including thyme and oregano (Wijesundara and Rupasinghe, 2018b). Several studies have reported the biological properties of carvacrol and suggested its potential clinical and natural health product applications (Allaoua et al., 2018; Chavan and Tupe, 2014; Magi et al., 2015a). We previously reported the rapid bactericidal activity of carvacrol against *S. pyogenes*. In this study, we noted, for the first time, the mode of action of carvacrol, which appears to target the membrane. Carvacrol alters membrane fluidity damaging the membrane integrity, and may interact with membrane phospholipids, P.G., P.E., and CL.

Carvacrol-induced cell membrane integrity losses and changes in permeability, potential and fluidity

We observed that carvacrol could permeabilize the cytoplasmic membrane and disrupt membrane potential. Membrane potential is the difference in electric potential across the membrane in intact bacterial cells (Stratford et al., 2019). The intact bacterial cell has a well-organized cytoplasm and membranes, which play an essential role in bacterial cytoskeletal spatial organization and cell division proteins (Strahl and Hamoen, 2010). Maintaining a constant proton gradient is crucial for bacterial cellular functions (Hurdle et al., 2011; Silhavy et al., 2010). Loss of integrity, depolarization, and ion fluctuations in the membrane simultaneously be affected its cellular functions, particularly ATP synthesis (Dimroth et al., 2000), active transport, cell division, and signal transductions (Strahl and Hamoen, 2010).

We measured the membrane potential by the fluorescence intensity of molecular probes DiOC2(3). This molecule exhibits green fluorescence in bacterial cells, but the fluorescence shifts to red emission as the molecule self-associates at the higher cytosolic concentrations caused by higher membrane potential (Chawla and Singh, 2013). In many Gram-positive bacteria, the red-to-green ratio is proportional to proton gradient intensity (Khater et al., 2020). Interestingly, treatment with carvacrol caused a decrease in the green-to-red fluorescence ratio, suggesting that the mechanism of action involves disruption of membrane potential (depolarization of the membrane) in a concentration and time-dependent manner.

Proton ionophores such as CCCP increase the proton permeability of the bacterial membrane, thereby dissipating membrane potential by eliminating the proton gradient (Perry et al., 2011). Therefore, CCCP was used as a positive control in this study. In addition, *S. pyogenes* cells treated with CCCP showed depolarizing activity as expected in the early phase and recovered at the end of the incubation period. Our results were compatible with a previous study of CCCP on *S. pneumoniae* (Clementi et al., 2014).

The results of the protoplast experiment confirmed that the ultimate target of carvacrol is the bacterial plasma membrane. TEM observations of protoplasts treated with carvacrol, such as uneven shape, intense cytoplasmic vacuolations, and cellular debris, revealed that carvacrol may have interacted with the lipid bilayer of *S. pyogenes* and induced structural changes. Furthermore, the observed nucleic acid leakage from the protoplast of *S. pyogenes* upon carvacrol treatments agreed with the results from the nucleic acid leakage pattern of intact bacterial cells upon similar treatments. Therefore,

the cell membrane was the target of carvacrol against *S. pyogenes*. Similarly, others have suggested that the cytoplasmic membrane is the primary cellular target of essential oils containing carvacrol and thyme (Nowotarska et al., 2017; Trombetta et al., 2005).

Carvacrol is a phenolic compound with a characteristic feature of the hydroxyl group on an aromatic ring. This phenolic hydroxyl group of carvacrol (Figure 1A) is crucial for its bactericidal activity. The importance of the phenolic structure of carvacrol and similar compounds such as thymol, cymene, and menthol in depolarizing the bacterial membrane was described against another Gram-positive pathogen, *Bacillus cereus* (Ultee et al., 2002a). It was further suggested that carvacrol acts as a proton exchanger to destabilize the cytoplasmic membrane, thus decreasing the bacterial membrane's pH gradient. The collapse of the proton motive force and depletion of the ATP pool eventually lead to cell death (Ultee et al., 2002a). Therefore, we suggest that the hydroxyl group of carvacrol may destabilize the cytoplasmic membrane phospholipids of *S. pyogenes*.

External environmental stress, such as temperature, drugs, and internal factors, such as the length and the degree of saturation of fatty acid tails, are a few factors that can influence the bilayer fluidity of bacterial membrane (Casares et al., 2019; Denich et al., 2003). Proper membrane fluidity is vital for maintaining bacteria's fundamental membrane barrier function. Decreasing membrane fluidity affects cell rigidity, leading to the malfunction of bacteria, whereas increasing fluidity causes cellular structural damage. Improper membrane fluidity interferes with essential bacterial cellular processes, such as maintaining membrane potential. Low membrane fluidity affects membrane leakiness in Gram-positive *B. subtilis*, which was reported, which indicates the weakening of

membrane barrier functions and membrane homogeneity (Gohrbandt et al., 2019). In contrast, it has been reported that the corresponding changes in the membrane fluidity had surprisingly little impact on membrane potential in *B. subtilis* (Gohrbandt et al., 2019). Our previous findings in Chapter 2 agree with this pattern, where leakage of cytoplasmic biomolecules (proteins, DNA, and RNA) from the *S. pyogenes* membrane after carvacrol treatment was observed.

Current anisotropy results show a decrease in anisotropy values when cells are incubated with carvacrol which explains an increase in membrane fluidity. The DPH probe was used in the experiment, and it is a highly hydrophobic molecule that inserts into the lipid core of the bacterial membrane (Aricha et al., 2004). DPH is oriented parallel to the axis of the lipid acyl chain, and its mode of motion is assumed to resemble the rotational diffusion of the lipid chains (Aricha et al., 2004). Therefore, membrane micro-viscosity/fluidity is explained as the rotational diffusion of the fatty acyl chains in the bacterial membrane interior (phospholipids) (Aricha et al., 2004). However, bacterial membrane phospholipids have different movements, such as lateral diffusion in the membrane plane and rotation around an axis perpendicular to the membrane plane (Svobodova and Svoboda, 1988). Carvacrol caused an anisotropy decrease, indicating more significant alterations in configurations of the phospholipid bilayers, degree of lipid packing, and membrane thickness. Furthermore, microscopic observations of cell damages also support the above results that carvacrol induced loss of bacterial membrane fluidity, which ultimately changed the cell shape.

Interaction between carvacrol and membrane phospholipids

Bacteria contain several different phospholipids (P.G., P.E., P.C., and CL) in their cytoplasmic membrane but in very different amounts. The lipophilic monoterpenes have been reported to integrate with the membrane phospholipids and cause leakages (Nowotarska et al., 2014; Ultee et al., 2002a). Carvacrol, a lipophilic monoterpene, may also play a similar role in its antibacterial activity. However, the interaction between carvacrol and membrane phospholipids in *S. pyogenes* is not fully established. Therefore, it is interesting to investigate what type of membrane phospholipids of *S. pyogenes* are affected by carvacrol treatment and suggest a potential mechanism of its interaction with the bactericidal activity of carvacrol.

The TLC experiments suggest that carvacrol does interact with specific phospholipids such as P.E., P.G., and CL. A previous study showed that carvacrol, cinnamaldehyde, and geraniol could modify the lipid monolayers consisting of P.E., P.G., and CL by integrating into the phospholipid monolayer, forming aggregates of antimicrobial-lipid complexes, reducing the packing effectiveness of the acyl chains (tails) of phospholipids, increasing the membrane fluidity, and altering the total dipole moment in the monolayer bacterial cell membrane model (Nowotarska et al., 2014).

Phospholipid acyl chains in the membrane lipids are connected through the “van der Waals” interactions (Sohlenkamp and Geiger, 2015). Therefore, external disturbance to these non-covalent bonds causes the fluidization of membrane lipids (Sohlenkamp and Geiger, 2015). Similar fluidization monoterpenes, namely eucalyptol, pulegone, terpineol, and thymol, were reported previously (Gharib et al., 2018). Furthermore, these

compounds were observed to fluidize the liposomal membrane by interacting with the alkyl chains of P.C. liposomes (Gharib et al., 2018).

Based on the present study, we suggest that carvacrol, an isomer to thymol, may also interact with the hydrophobic acyl chains of bacterial membrane phospholipids, creating a fluidizing effect on the lipid membrane (Figure 3.6). The expansion of lipid chains by carvacrol may destabilize the membrane and, consequently, the leakage of cytoplasmic content, as we reported here and previously (Chapter 2, Figure 2.4). Interference of carvacrol with bacterial membranes of *B. cereus* was reported as a mechanism of action of carvacrol previously and shown that it changes the permeability of H+ and K+, obliterates essential functions and ultimate causes cell death (Ultee et al., 2002a).

In general, the accumulation of hydrophobic phenolic compounds in hydrophobic phases, which occupy more than the usual space between fatty acyl chains, subsequently causes conformational changes in the phospholipid bilayer (Fan et al., 2017). According to the observations, we suggested that carvacrol may accumulate in the membrane hydrophobic phases, affecting the intimate arrangement and stability of the phospholipid bilayer. Changes in the membrane phospholipid bilayer expand the membrane, as illustrated in Figure 3.6, which becomes more permeable and decreases membrane potential.

Daptomycin is a cyclic lipopeptide antibiotic with a broad spectrum of activity against Gram-positive bacteria, including *Streptococcus* spp (Ledger et al., 2017). Although mechanisms of actions of daptomycin have not yet been precisely defined, it has been previously investigated and found to be due to the direct influence on the

inhibition of biosynthesis of the cell membrane and/or cell wall component (including PGN, LTA) (Heidary et al., 2017). Daptomycin's mechanisms against Gram-positive bacteria interact with the membrane P.G. (Heidary et al., 2017; Taylor and Palmer, 2016). According to our TLC findings, we also suggest that daptomycin interacts with membrane P.G., P.E., P.C., and CL.

Carvacrol causes changes in cell membranes, such as depolarization, increased permeability, and leakage of the cytoplasmic contents, ultimately leading to bacterial cell death. We also found that carvacrol may preferentially target the membrane phospholipids of P.E., P.G., and partially CL. Therefore, we suggested that carvacrol kills *S. pyogenes* by disrupting the cytoplasmic membrane. To our best understanding, this is the first report that shows the primary target of bactericidal carvacrol is the membrane of *S. pyogenes*. Therefore, being a cell membrane-targeted novel antibacterial agent, with its plant origin, non or less toxic to human cells, and generally regarded as safe status, carvacrol is a powerful natural agent for treating drug-resistant pathogens erythromycin-resistant *S. pyogenes*.

3.6. CONCLUSION

We investigated the mechanism of action of carvacrol against *S. pyogenes* and its rapid bactericidal activities. The *S. pyogenes* membrane provides a target for carvacrol. According to our findings, carvacrol reduces the membrane potential of *S. pyogenes* cells while increasing membrane permeability and fluidity, concentration-dependently. Furthermore, we proposed that carvacrol's bactericidal activity is mediated by preferential binding to negatively charged phosphatidylglycerol, cardiolipin, and zwitterionic phosphatidylethanolamine in bacterial cell lipid bilayers. These findings suggest that carvacrol is a possible candidate for developing novel natural health products such as throat vapors, throat lozenges, or mouthwashes to manage streptococcus pharyngitis.

3.7. FUNDING AND ACKNOWLEDGMENTS

We gratefully acknowledge funding from the Collaborative Research and Development Grant program (CRDPJ 448052) of the Natural Sciences and Engineering Research Council (NSERC) and Canada and Island Abbey Foods Charlottetown, PE, Canada.

Table 3.1 Changes of membrane fluidity in the presence of carvacrol on *Streptococcus pyogenes Spy* 1558 live cells.

Samples	DPH Anisotropy value*	
	1 hr	24 hr
PBS	0.012 ± 0.01	0.022 ± 0.01
DMSO	0.012 ± 0.01	0.023 ± 0.01
Carvacrol (1 × MIC)	0.012 ± 0.01	0.018 ± 0.01
Carvacrol (2 × MIC)	0.005 ± 0.00	0.047 ± 0.00
Triton X - 100	0.009 ± 0.01	0.042 ± 0.00
Tween-20	0.030 ± 0.00	0.034 ± 0.00

*DPH anisotropy values represent the mean ± standard error. DPH; 1,6 diphenyl 1,3,5 hexatriene. MIC, minimum inhibitory concentration, 125 µg/mL); DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

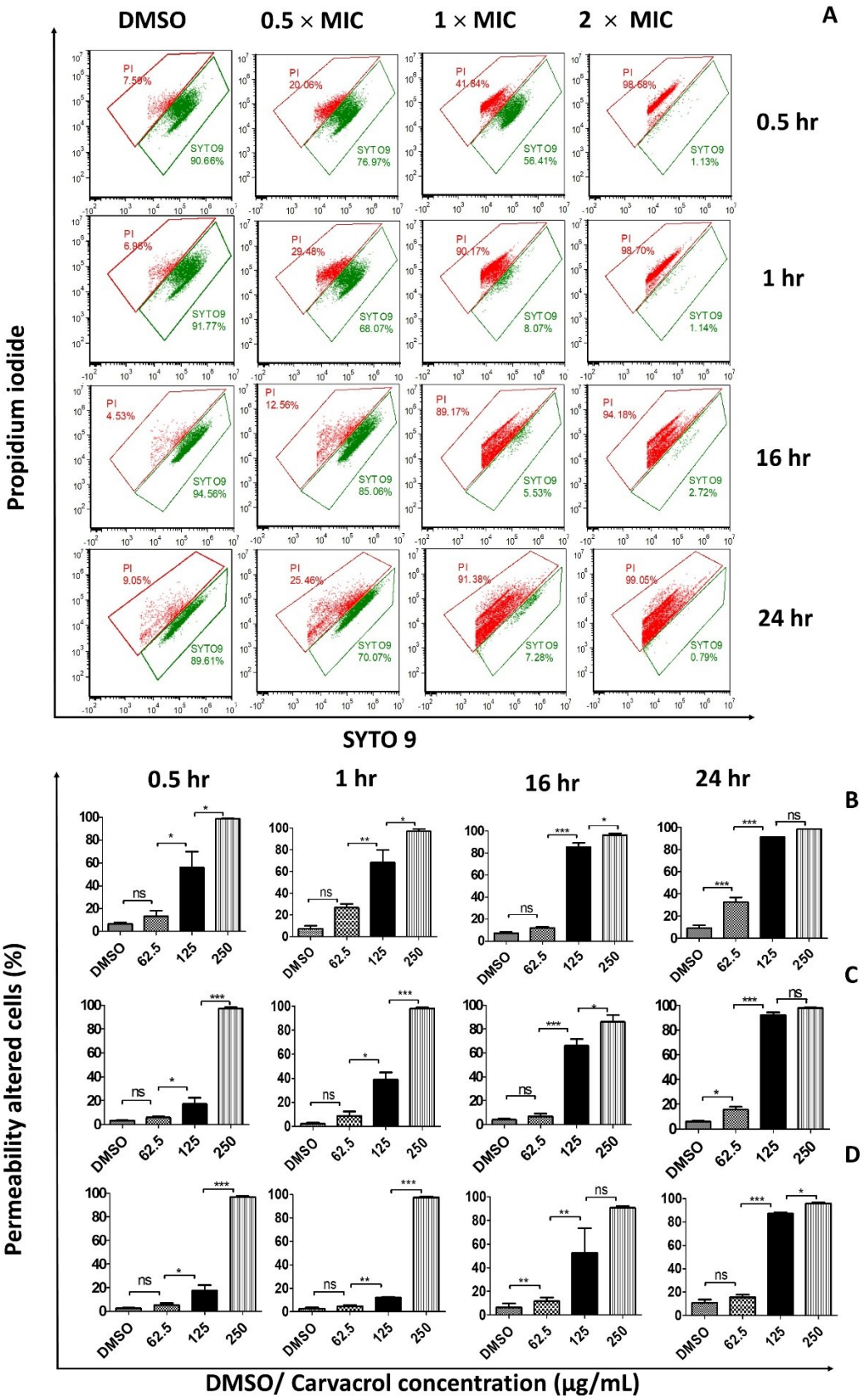
A
DMSO
0.5 × MIC
1 × MIC
2 × MIC
0.5 hr
1 hr
16 hr
24 hr
Propidium iodide
SYTO 9
PI 7.58%
SYTO9 90.66%
PI 20.06%
SYTO9 76.97%
PI 41.84%
SYTO9 56.41%
PI 98.68%
SYTO9 1.13%
PI 6.96%
SYTO9 91.77%
PI 29.48%
SYTO9 68.07%
PI 90.17%
SYTO9 8.07%
PI 98.70%
SYTO9 1.14%
PI 4.53%
SYTO9 94.56%
PI 12.56%
SYTO9 85.06%
PI 89.17%
SYTO9 5.53%
PI 94.18%
SYTO9 2.72%
PI 9.05%
SYTO9 89.61%
PI 25.46%
SYTO9 70.07%
PI 91.38%
SYTO9 7.28%
PI 99.05%
SYTO9 0.79%
B
C
D
Permeability altered cells (%)
DMSO/ Carvacrol concentration (µg/mL)
DMSO
62.5
125
250
ns

Figure 3.1 Carvacrol increases the permeability of the *Streptococcus pyogenes* bacterial cell membrane in a concentration- and time-dependent manner.

Bacteria cells were treated with different carvacrol concentrations or vehicle control for 0.5, 1, 16, and 24 hr, stained with SITO 9/PI and analyzed by flow cytometry. Red and green fluorescence intensity was measured using PC5.5 and FITC channels. Laser excitation/emission wavelengths of 485/542 nm for SYTO 9 and 485/610 nm for PI were used. **(A)** Representative FACS cytograms of vehicle control (0.25 % DMSO) or 0.5 × MIC, 1 × MIC, 2 × MIC of carvacrol treated ATCC 19615 strain over four different incubation periods. The population of SYTO-9-positive and PI-positive cells was shown in the green and red polygonal. The bar graphs show the cell permeability changes of **(B)** ATCC 19615, **(C)** Clinical isolate, and **(D)** *Spy* 1558 strains treated with different carvacrol concentrations over different incubation periods. Data shown are the percentages of membrane-altered cells (percentage of PI-positive cells) and expressed as mean ± SEM from three independent experiments. (Differences among means were compared using Tukey's test; *P < 0.05, **P < 0.01, and ***P < 0.001. MIC: Minimum inhibitory concentration = 125 μg/mL. PI: Propidium iodide.

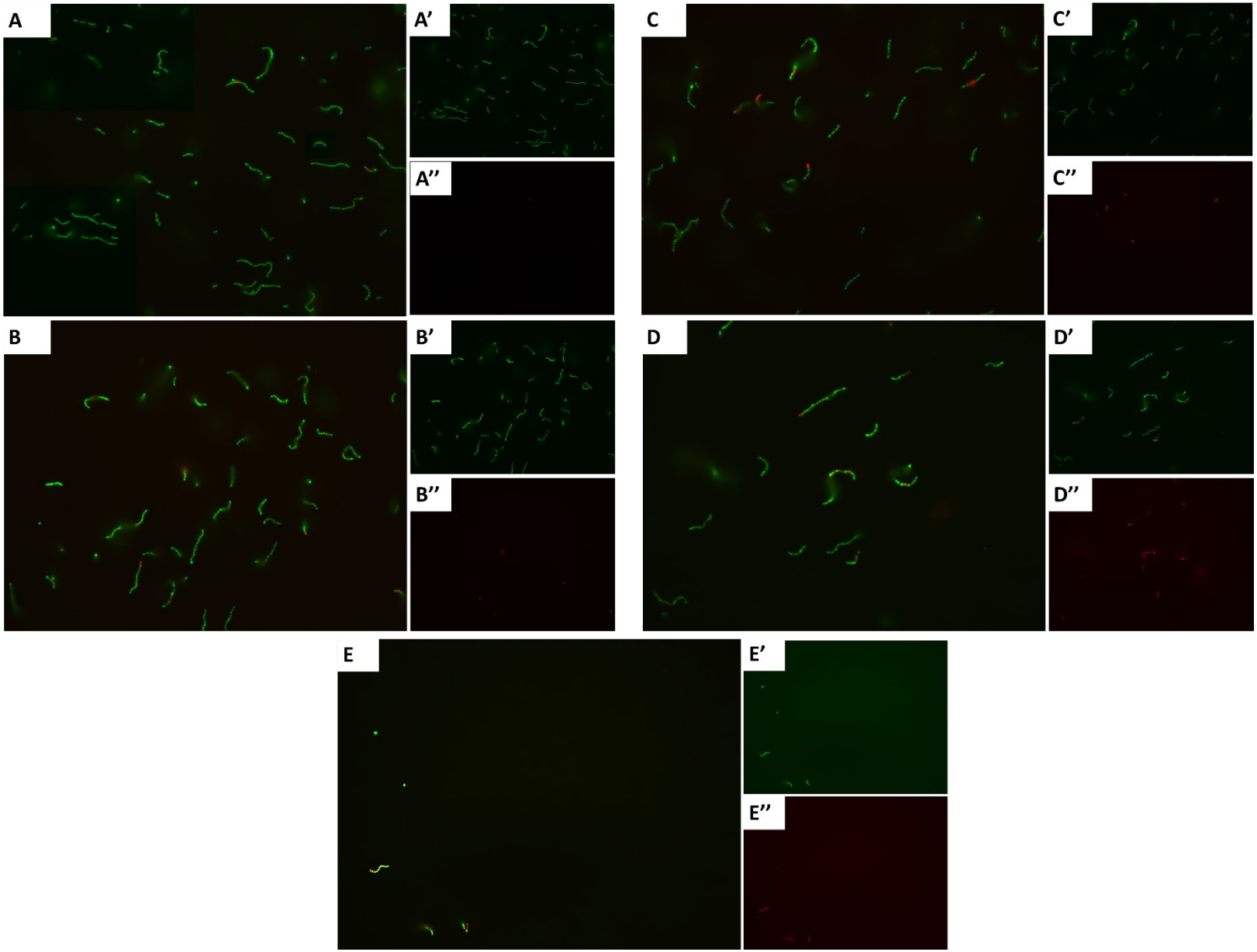
A
A'
A''
C
C'
C''
B
B'
B''
D
D'
D''
E
E'
E''

Figure 3.2 Bacterial viability visualized in the fluorescence microscopy for *Streptococcus pyogenes* cells.

(A) untreated control and treated with carvacrol at (B) 0.125 × MIC (C) 0.25 × MIC (D) 0.5 × MIC and (E) 1 × MIC concentrations. MIC = 125 μg/mL. The cells are labeled with Live/Dead BacLight® bacterial-viability dyes, as STYO9 in green and propodeum iodide (PI) in red. Cells with a damaged membrane/dead show red color fluorescence, whereas cells with a viable and intact membrane show green fluorescence.

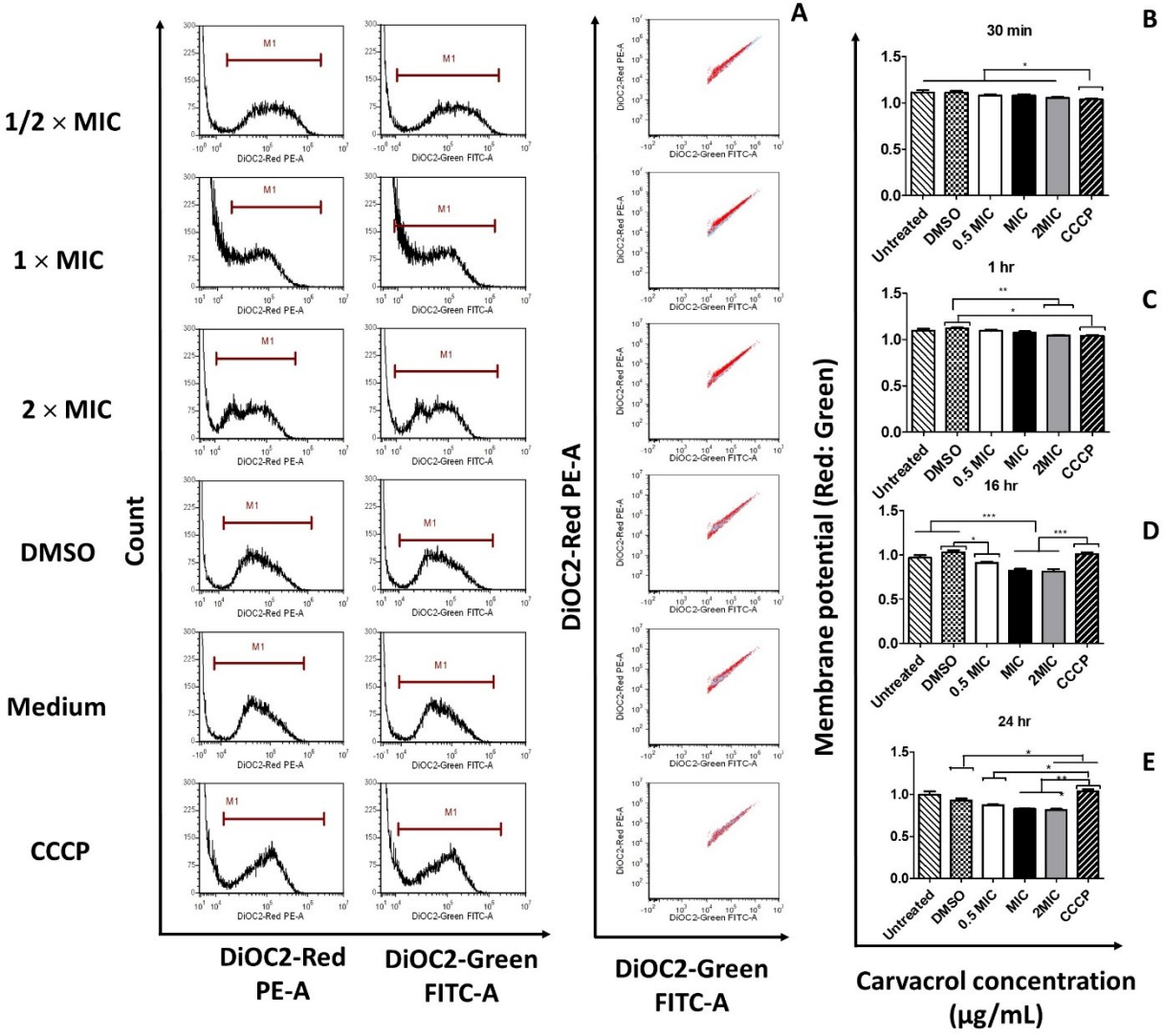
1/2 × MIC
1 × MIC
2 × MIC
DMSO
Medium
CCCP
Count
DiOC2-Red PE-A
DiOC2-Green FITC-A
M1
A
B
C
D
E
30 min
1 hr
16 hr
24 hr
Untreated
DMSO
0.5 MIC
MIC
2MIC
CCCP
Membrane potential (Red: Green)
Carvacrol concentration (µg/mL)

Figure 3.3 Effect of carvacrol treatments on the potential membrane changes of *Streptococcus pyogenes*.

Bacterial suspensions were treated with 2 × MIC, 1 × MIC, and 1/2 × MIC of carvacrol or DMSO vehicle or 5 μM of CCCP, then incubated with 300 μM DiSO2 (3) for 24 hr. MIC=125 μg/mL. The fluorescence intensity of red and green was measured using FITC and PI channels in a FACS CytoFLEX flow cytometer. The intensity of red (left panel), green fluorescence (middle panel), and cells exhibiting both fluorescence intensities (right panel) are shown. Red dots represent CCCP, and blue dots represent carvacrol treatments in cytometry profiles (right panel). Representative fluorescence panels were shown for the *S. pyogenes* strain of ATCC 19615 suspension treated with carvacrol for 24 hr. The bar graph shows the membrane potential without (untreated control) or with DMSO vehicle or CCCP or different concentrations of carvacrol treatment for different *S. pyogenes* strains (B) ATCC 19615, (C) clinical isolate, and (D) *Spy* 1558. Flow cytometer data were collected with log amplifications, and red: green ratios were calculated using population mean fluorescence intensities (MFI) of carvacrol treatments in either presence or absence of CCCP. Results are expressed as the ratio of MFI of red: Green ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA, and the differences among means were compared using Tukey's test; $*P < 0.05$ and ***$P < 0.001$.

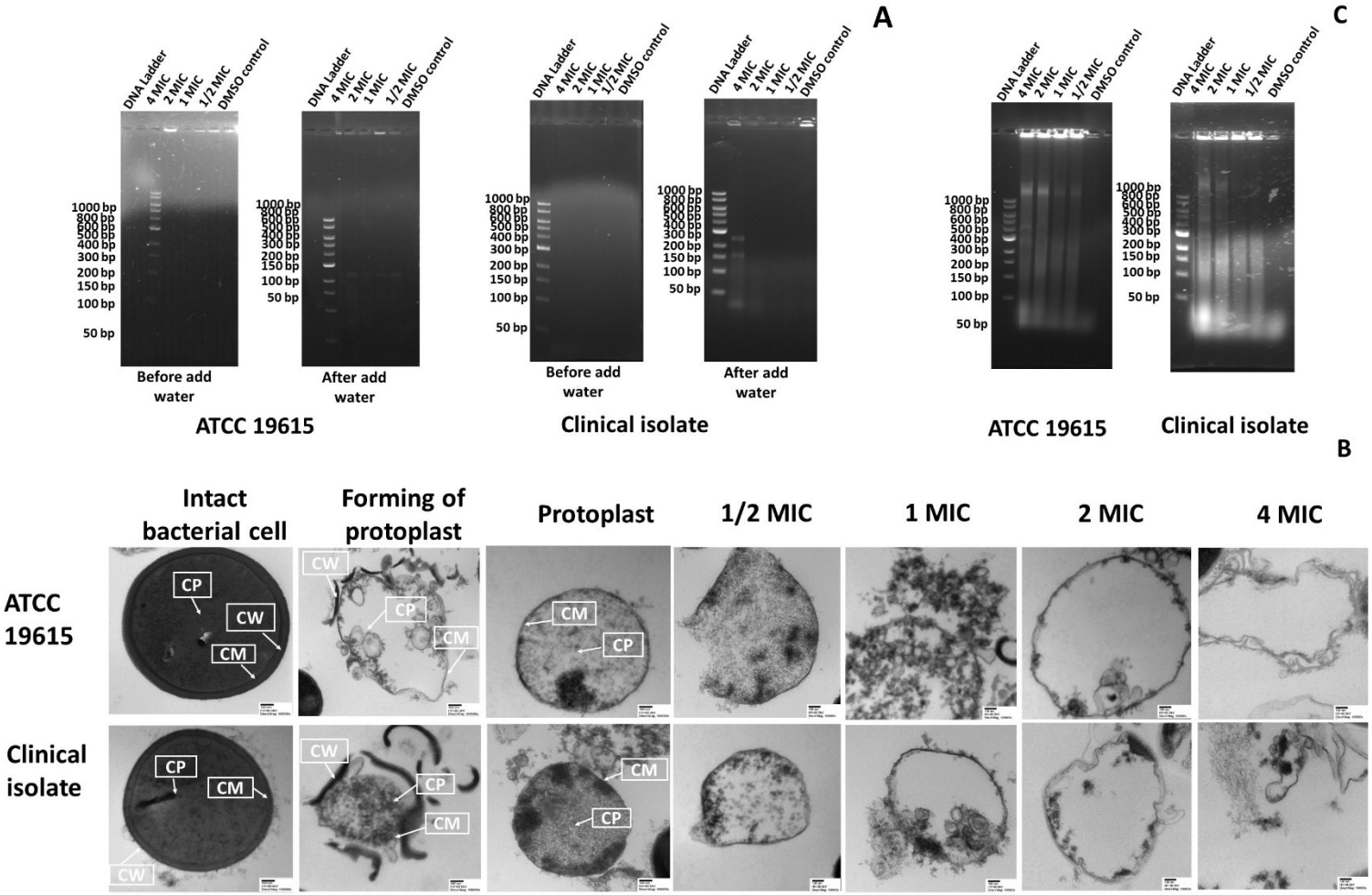
A
DNA Ladder
4 MIC
2 MIC
1 MIC
1/2 MIC
DMSO control
1000 bp
800 bp
600 bp
500 bp
400 bp
300 bp
200 bp
150 bp
100 bp
50 bp
Before add water
After add water
ATCC 19615
Clinical isolate
C
ATCC 19615
Clinical isolate
B
Intact bacterial cell
Forming of protoplast
Protoplast
1/2 MIC
1 MIC
2 MIC
4 MIC
ATCC 19615
Clinical isolate
CP
CW
CM

Figure 3.4 Confirmation of *Streptococcus pyogenes* protoplast formation, carvacrol effect on membrane damage of *S. pyogenes*-protoplast, and cytoplasmic nucleic acid leakage.

(A) Agarose gel (0.8 %, w/v) electrophoresis and gel red staining of centrifuged clinical isolate-protoplast pellets after formation and busted out by adding water. **(B)** Transmission electron micrographs of thin sections of intact bacteria without treatment, a representative example of a progressive stage of protoplast stage of the effect of mutanolysin in protoplast buffer (15 min, 37 °C), protoplast resulting from exposure in protoplast buffer with mutanolysin (30 min, 37 °C) and ATCC 19615 and clinical isolate protoplast cell suspensions (OD = 0.6) treated with DMSO or 4 × MIC, 2 × MIC, 1 × MIC, and 1/2 × MIC of carvacrol concentrations for 2 hr at 37 °C. Magnifications and bars: × 50,000 (200 nm) and × 100,000 (100 nm). CW: cell wall, CM: cytoplasmic membrane, CP: cytoplasm **(C)** Agarose gel (0.8 %, w/v) electrophoresis, and gel red staining of ATCC 19615 and clinical isolate-protoplast supernatants after exposure to the different carvacrol concentrations. 1 kb ladder as reference.

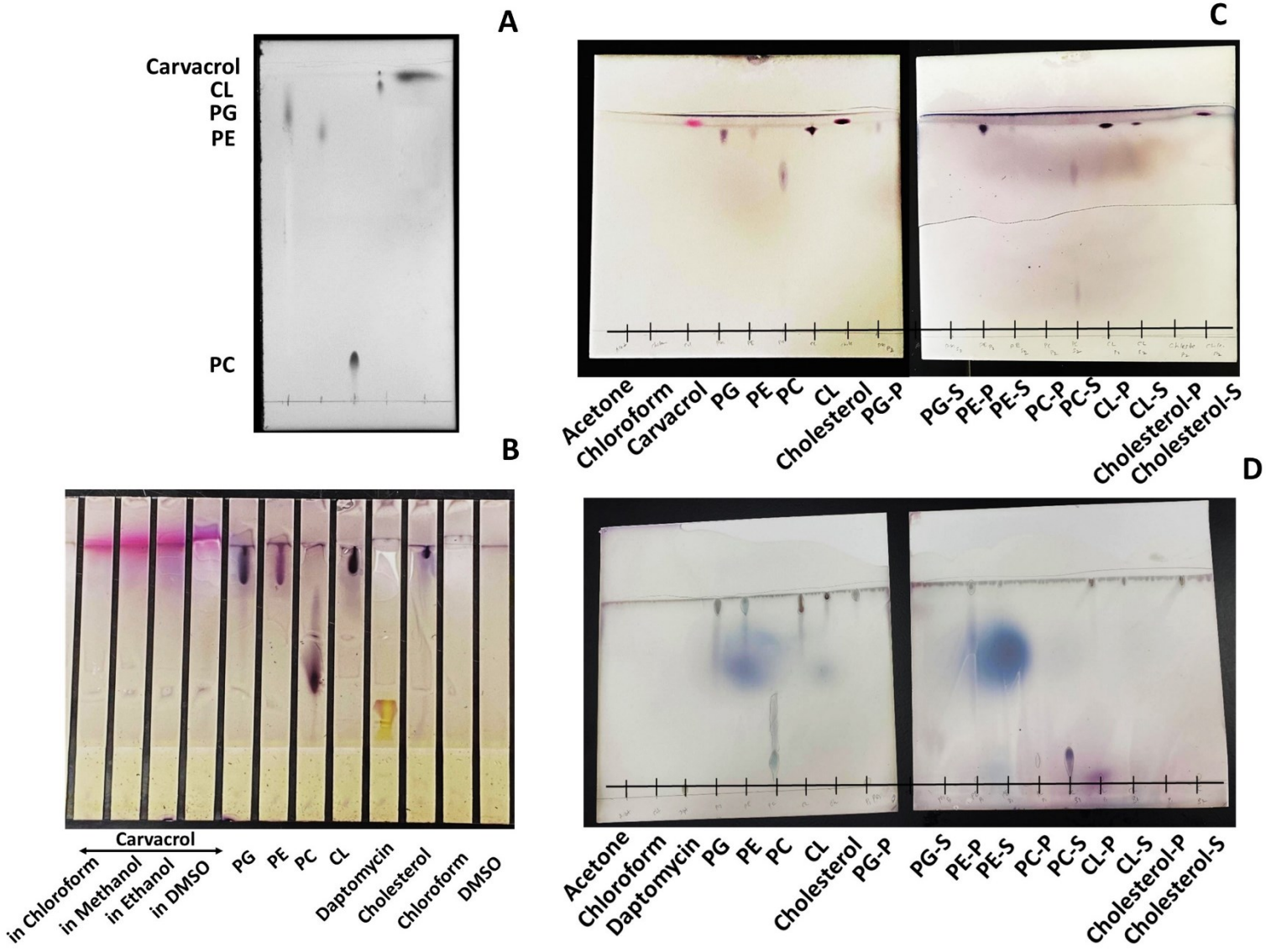
A
Carvacrol
CL
PG
PE
PC
B
Carvacrol
in Chloroform
in Methanol
in Ethanol
in DMSO
PG
PE
PC
CL
Daptomycin
Cholesterol
Chloroform
DMSO
C
Acetone
Chloroform
Carvacrol
PG
PE
PC
CL
Cholesterol
PG-P
PG-S
PE-P
PE-S
PC-P
PC-S
CL-P
CL-S
Cholesterol-P
Cholesterol-S
D
Acetone
Chloroform
Daptomycin
PG
PE
PC
CL
Cholesterol
PG-P
PG-S
PE-P
PE-S
PC-P
PC-S
CL-P
CL-S
Cholesterol-P
Cholesterol-S

Figure 3.5 Visualization of thin-layer chromatography (TLC).

(A) UV visualization of phospholipids standards (25 mg/mL, each) and carvacrol (12.5 mg/mL) and photographs of (B) selection of better solvents and detection method for all samples, (C) carvacrol, and (D) daptomycin interaction with phospholipids. Phospholipids: carvacrol/daptomycin (2:1 v/v, in chloroform) were incubated for 1 hr, and the phospholipids were precipitated using cold acetone. Precipitant (P) and supernatant (S) were collected by high-speed centrifugation (21,000 ×g), drying, and reconstituted with chloroform. The TLC plates were developed with the solvent system of chloroform-methanol-water (65:25:10 [vol/vol/vol]). TLC plates were then stained with alcoholic vanillin–sulphuric acid solution, heated in an oven and visualized under UV. Colors of the spots were compared and identified in photographs of TLC plates, and their mobilities (Rf) were compared with those of phospholipids. P.G.: phosphatidylglycerol, P.E.: phosphatidylethanolamine, P.C.: phosphatidylcholine, CL: cardiolipin, Ch: cholesterol.

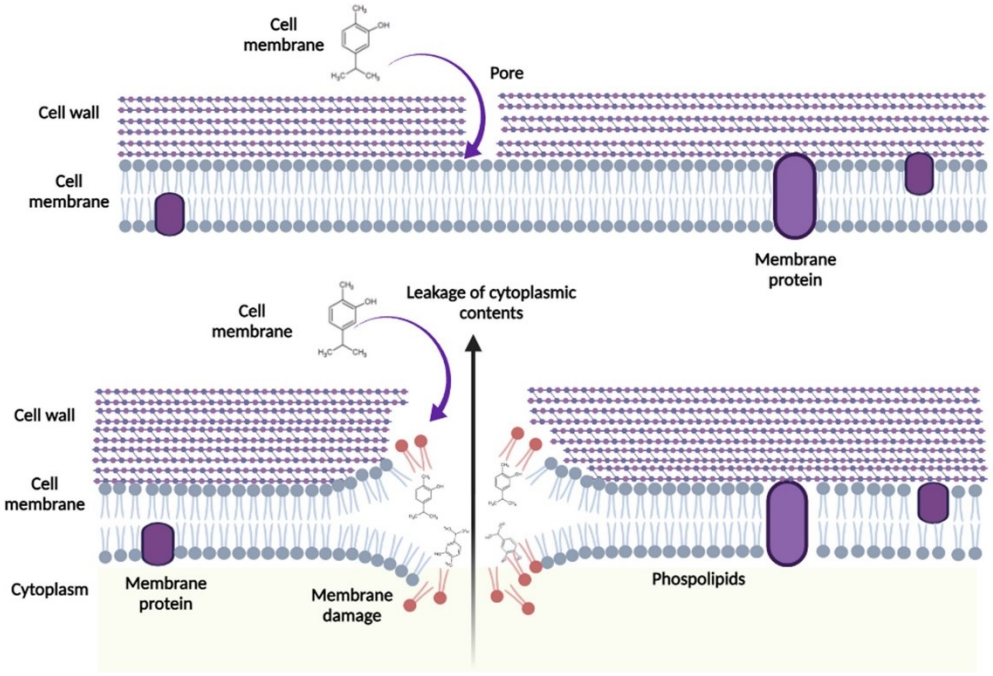
A
Cell membrane
Pore
Cell wall
Cell membrane
Membrane protein
B
Cell membrane
Leakage of cytoplasmic contents
Cell wall
Cell membrane
Cytoplasm
Membrane protein
Membrane damage
Phospolipids
C
Polar head
Nonpolar tail

Figure 3.6 Potential mechanism of action of carvacrol in damaging the cell membranes of *Streptococcus pyogenes.*

A) Due to its hydrophobic nature, carvacrol penetrates through cell wall pores to the cell membrane and **(B)** interacts with the bacterial cytoplasmic membrane lipid bilayer. Carvacrol may align between fatty acid chains of the membrane phospholipids, specifically PG, PE, and CL, causing the expansion and destabilization of the membrane structure by increasing the fluidity and permeability. As a result, cytoplasmic cell contents will leak from the cytoplasm, which ultimately causes cell death. **(C)** The accumulation of carvacrol in hydrophobic phases, which occupy more than the usual amount of space between fatty acyl chains, subsequently causes conformational changes in the phospholipid bilayer. PG: phosphatidylglycerol, PE: phosphatidylethanolamine, CL: cardiolipin,

3.8.SUPPLEMENTARY FIGURES

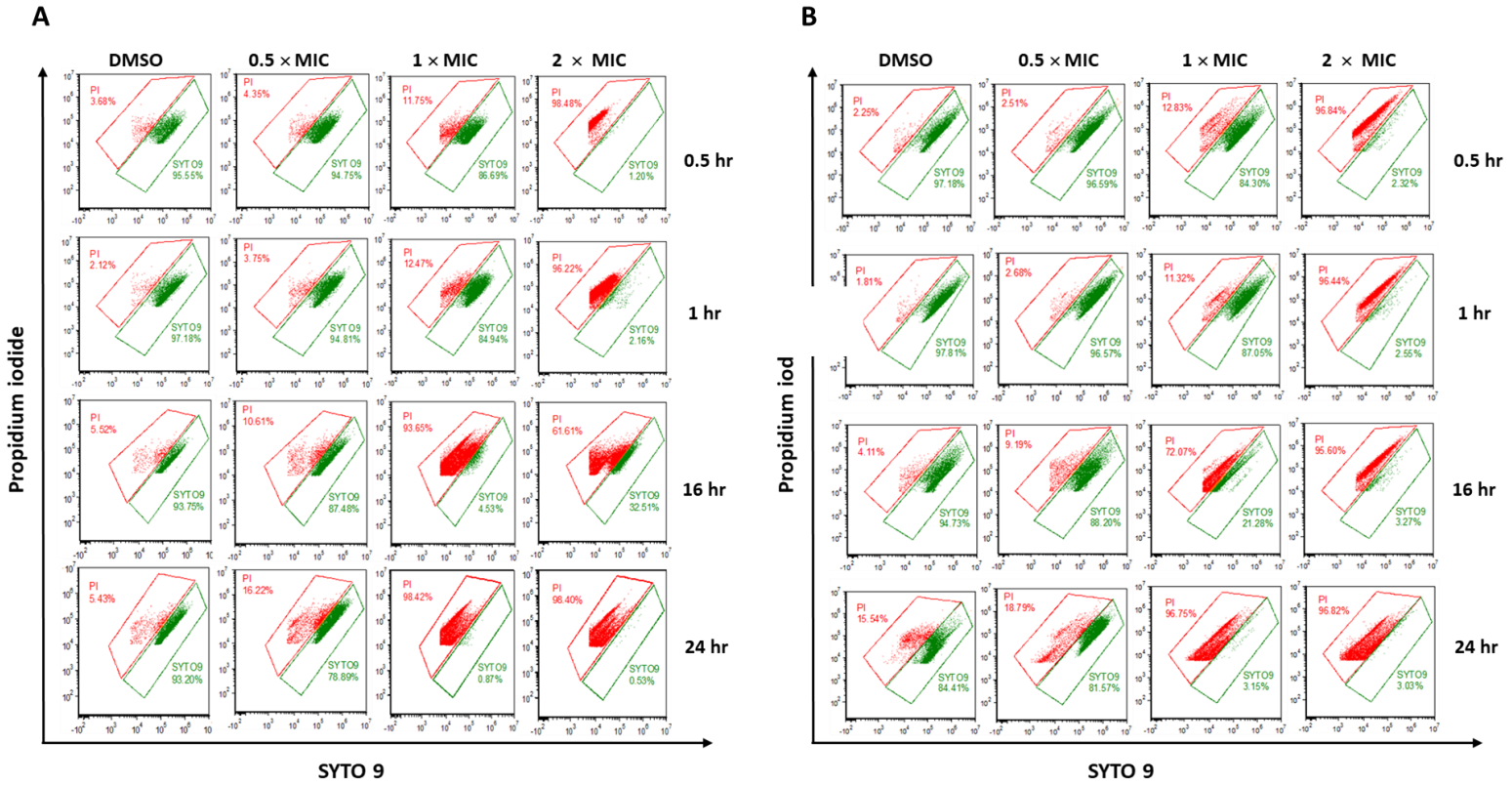

Figure 3.7.S2. Carvacrol increases the permeability of the (A) clinical isolate and (B) Spy 1558 *Streptococcus pyogenes* cell membrane in a concentration- and time-dependent manner.

Bacteria cells were treated with different carvacrol concentrations or vehicle control for 0.5, 1, 16, and 24 hr, stained with SYTO 9/PI and analyzed by flow cytometry. Red and green fluorescence intensity was measured using PC5.5 and FITC channels. Laser excitation/emission wavelengths of 485/542 nm for SYTO 9 and 485/610 nm for PI were used. FACS cytograms of vehicle control (0.25 % DMSO) or 0.5 × MIC, 1 × MIC, 2 × MIC of carvacrol treated *Spy* 1558 over four different incubation periods is summarized. The population of SYTO-9 positive cells PI-positive cells were shown in the green and red polygonal, respectively.

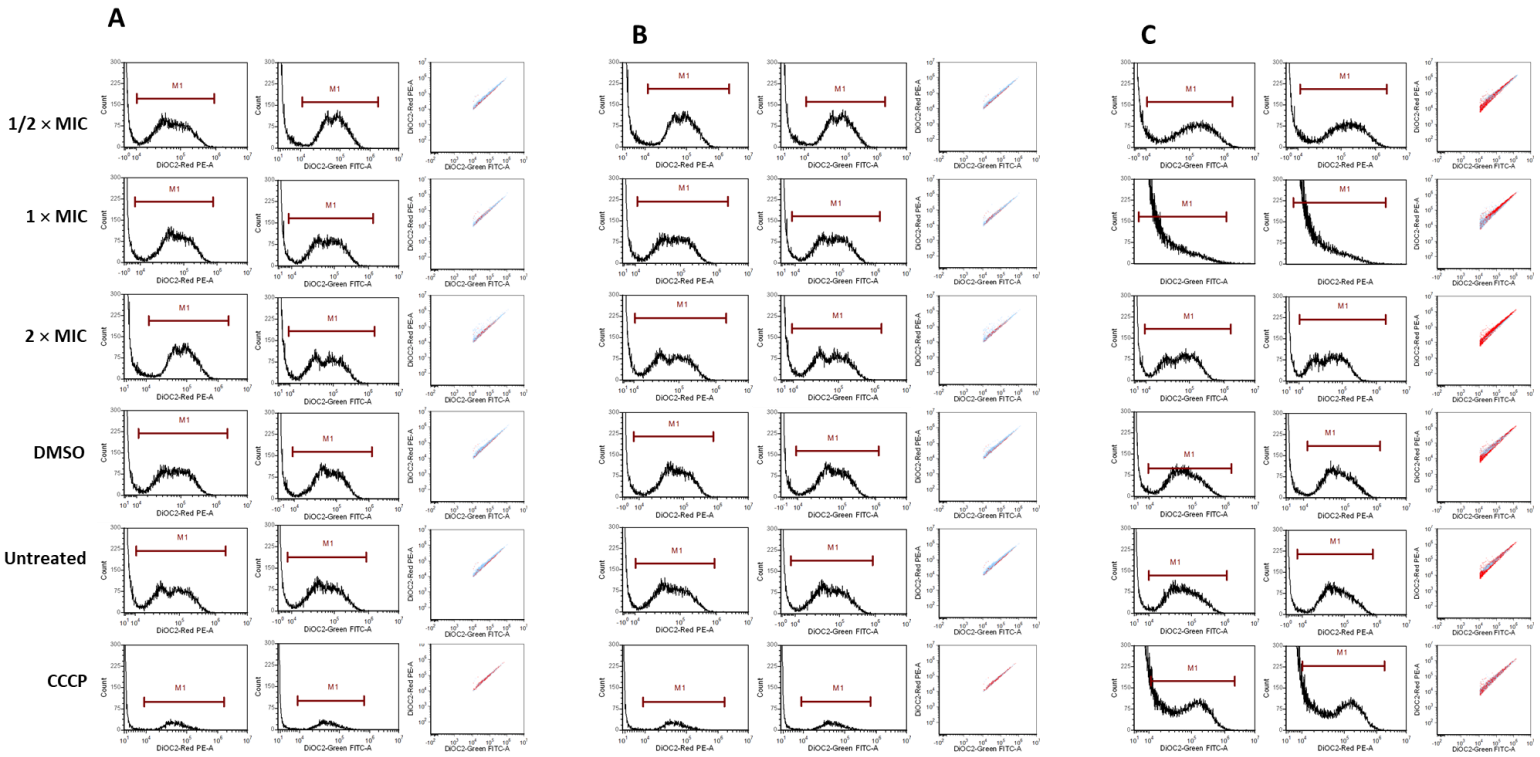
A
B
C
1/2 × MIC
1 × MIC
2 × MIC
DMSO
Untreated
CCCP
M1
Count
DiOC2-Red PE-A
DiOC2-Green FITC-A

Figure 3.8. S3. Time-dependent effect of carvacrol treatments on the *Streptococcus pyogenes* membrane potential.

S. pyogenes ATCC 19615 suspensions were treated with 2MIC, MIC, and 1/2 MIC of carvacrol, or DMSO vehicle or 5 μM of CCCP, then incubated with 300 μM DiSO2 (3) for (A) 30 min, (B) 1 hr and (C) 16 hr MIC = 125 μg/mL. The fluorescence intensity of red and green was measured using FITC and PI channels in a FACS CytoFLEX flow cytometer. The intensity of red (left panel), green fluorescence (middle panel), and cells exhibiting both fluorescence intensities (left panel) are shown. Red dots represent CCCP, and blue dots represent carvacrol treatments in cytometry profiles (left panel). The bar graph shows the membrane potential without (untreated control), DMSO vehicle, CCCP, or different concentrations of carvacrol treatment. Flow cytometer data were collected with log amplifications, and red: green ratios were calculated using population mean fluorescence intensities (MFI) of carvacrol treatments in either presence or absence of CCCP. Results are expressed as the ratio of MFI of red: Green ± SEM of three independent experiments. Statistical analysis was performed using one-way ANOVA, and the differences among mean were compared using Tukey's test; *$P < 0.05$.

A
Acetone
Chloroform
Carvacrol
PG
PE
PC
CL
Cholesterol
PG-P
PG-S
PE-P
PE-S
PC-P
PC-S
CL-P
CL-S
CL-S
Cholesterol-P
Cholesterol-S
Carvacrol
B
Acetone
Chloroform
Daptomycin
PG
PE
PC
CL
Cholesterol
PG-P
PG-S
PE-P
PE-S
PC-P
PC-S
CL-P
CL-S
CL-S
Cholesterol-P
Cholesterol-S
Daptomycin

Figure 3.9. S4. Visualization of thin-layer chromatography (TLC) (A) carvacrol and (B) daptomycin interaction with phospholipids (using the Bio-RadTM Gel Doc Imaging System).

Phospholipids: carvacrol/daptomycin (2:1 v/v, in chloroform) were incubated for 1 hr, and the phospholipids were precipitated using cold acetone. Precipitant (P) and supernatant (S) were collected by high-speed centrifugation (21,000 × g), drying, and reconstituted with chloroform. The TLC plates were developed with the solvent system of chloroform-methanol-water (65:25:10 [vol/vol/vol]). TLC plates were then stained with alcoholic vanillin–sulphuric acid solution, heated in an oven and visualized under UV. Colors of the spots were compared and identified in photographs of TLC plates, and their mobilities (Rf) were compared with those of phospholipids. P.G.: phosphatidylglycerol, P.E.: phosphatidylethanolamine, P.C.: phosphatidylcholine, CL: cardiolipin, Ch: cholesterol.

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CHAPTER 4 CARVACROL ALTERS THE MEMBRANE PHOSPHOLIPID IN ERYTHROMYCIN-RESISTANT STREPTOCOCCUS PYOGENES

4.1. ABSTRACT

Background: Phospholipids are significant constituents of bacterial membranes. Bacteria can alter their cytoplasmic membrane fatty acid composition in response to changing environmental stress conditions, including temperature, pH, or antimicrobial agents. Carvacrol, a mono-terpenoid phenol, has excellent antibacterial properties against *Streptococcus pyogenes.* **Objectives:** This study attempted to identify any modifications in the membrane phospholipid composition in the presence of sublethal carvacrol concentrations using an erythromycin-resist *S. pyogenes* model. **Methods:** The cell membrane isolates (CMI) was prepared from an erythromycin-resistant *S. pyogenes* strain (*Spy* 1558, erm). Transmission electron microscopy (TEM) was conducted for carvacrol-treated CMIs to assess their ultrastructural morphology. Carvacrol-treated CMIs were analyzed for fluidity changes using fluorescence-anisotropy. Lipids were extracted from untreated or carvacrol-treated CMIs, and changes in membrane lipid composition were assessed using gas chromatography-mass spectrometry (GC-MS). **Results:** Fluidity, a biophysical characterization of the bacterial membrane, increased time-dependently. The CMI isolated from cells treated with a sub-inhibitory concentration of carvacrol showed a change in cardiolipins (CL), phosphatidylglycerol (P.G.), and phosphatidylethanolamine (P.E.) percentages compared to the untreated control. **Conclusion:** Carvacrol induces modulation of specific membrane phospholipid and fatty acid composition of *S. pyogenes*. Further investigations are necessary to understand how fatty acid and phospholipid biosynthesis can be influenced by carvacrol.

Keywords: Carvacrol, *Streptococcus pyogenes*, cell membrane, mechanism of action, fluidity, phospholipid composition, anisotropy, cell membrane isolate

4.2. INTRODUCTION

Bacteria have evolved various stress-response mechanisms in response to external and intracellular challenges such as temperature, oxidative stress, food constraint, oxygen deprivation, and antibiotics (Fang et al., 2016). Changing the composition of phospholipids, a fundamental component of the cellular envelope, is critical for bacterial survival in response to environmental stress.

Antibiotics or antibacterial compounds in the host environment trigger bacteria to respond to that stress by affecting membrane integrity through the biosynthesis of new membrane phospholipids or altering the membrane lipid composition (Tang et al., 2018; Willdigg and Helmann, 2021). Bacterial cell membranes generally consist of bilayers of amphipathic phospholipids embedded with protein molecules (Singer and Nicolson, 1972; Sohlenkamp and Geiger, 2015). Phosphatidylethanolamine (P.E.), phosphatidylglycerol (P.G.), cardiolipin (CL), phosphatidylcholine (P.C.), phosphatidylserine (P.S.), and phosphatidic acid (P.A.) are the structural phospholipids in Gram-positive cytoplasmic membranes (Sohlenkamp and Geiger, 2015).

The lipid composition of membranes determines their functional properties, such as membrane curvature, fluidity, elastic free energy, and lipid packing (de Mendoza and Pilon, 2019). The importance of membrane integrity and how membrane-phospholipids affect cellular processes in Gram-positive bacteria have been investigated previously (Malanovic and Lohner, 2016a). However, due to the variability of the compositions and changes in membrane composition by bacteria, the impact of changes in phospholipid composition on bacterial physiology is unknown.

Furthermore, because each phospholipid has unique physical properties, its influence on bacterial membrane fluidity may differ (Tang et al., 2018). *Streptococcus pyogenes,* a leading human pathogenic bacterium, is a significant cause of global morbidity and mortality (Nelson et al., 2016; Pfoh et al., 2008).

S. pyogenes is responsible for a wide range of diseases, including pharyngitis, impetigo, cellulitis, necrotizing fasciitis, non-suppurative complications like scarlet fever or streptococcal toxic shock syndrome, and autoimmune post-streptococcal sequelae, such as rheumatic fever and glomerulonephritis (Carapetis et al., 2005; Greenberg and Kaskel, 2017).

β-lactam antibiotics, including penicillin, amoxicillin, and cephalosporins, are recommended as the drug of choice, and macrolides (erythromycin, spiramycin, and azithromycin) and lincosamides (clindamycin) were recommended as alternatives of treatment for *S. pyogenes* infections (Silva-Costa et al., 2015a; van Driel et al., 2016b; Zimbelman et al., 1999). Furthermore, the high prevalence of erythromycin-resistance of *S. pyogenes* has been reported worldwide (Friães et al., 2019; Lu et al., 2017), and the application of bioactive medicinal plant compounds against *S. pyogenes* infections has been emerging (Adil et al., 2018).

Carvacrol (Figure 4.1) is a mono-terpenoid phenol in several herbal plant extracts (Vinciguerra et al., 2018; Wijesundara and Rupasinghe, 2018b). Carvacrol was shown to be effective against an erythromycin-resistant strain of *S. pyogenes* in chapters 2 and 3. Furthermore, both chapters show that carvacrol impacts membrane permeability and integrity alterations at its bactericidal concentration. Therefore, this study aimed to understand the impact of sub-inhibitory doses of carvacrol on the fatty acid composition

and structure of the membrane lipid layer of *S. pyogenes*. We provide evidence that alterations of specific phospholipids in *S. pyogenes* have adverse effects on membrane fluidity, cell shape, and adaptability to antibacterial agent stresses.

4.3. MATERIALS AND METHODS

4.3.1. Media and chemicals

Brain heart infusion (BHI) and bacteriological agar were purchased from Oxoid Ltd (Nepean, ON, Canada), and media were prepared as per the manufacturer's instructions. Triton X-100 [10% solution (w/v)] was purchased from Thermo Fisher Scientific Inc. (M.A., USA). The phospholipid standards, L-α-phosphatidyl-DL-glycerol sodium salt, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine, L-α-phosphatidylcholine, and cardiolipin sodium salt were also purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). In addition, carvacrol, NaCl, penicillin G sodium salt, a common surfactant, Tween 20, (0.06 mM), daptomycin, dimethyl sulphide (DMSO) (≥ 99.8%), and 1, 6-diphenyl-1,3,5-hexatriene (DPH), were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

4.3.2. Bacterial strains maintenance and growth conditions

An erythromycin-resistant strain (*Spy* 1558, erm) was isolated previously and kindly provided by Dr. Song F. Lee. Bacterial culture was regularly grown at 37 °C, 5% CO_2 in BHI media (37 g/L, pH 7.0). Cultures were stored and maintained as previously described (Wijesundara and Rupasinghe, 2018b). The bacterial suspensions were diluted to OD_{600} = 0.6 in the BHI medium for the experiments.

4.3.3. Bacterial cell membrane isolation

4.3.3.1. Bacterial culturing

Overnight grown *Spy* 1558 was diluted 1:20 into 1L of pre-warmed sterilized BHI media ($OD_{600} \geq 0.6$) and grown to mid-exponential phase for 3-4 hr at 37 °C. Cells were

harvested by centrifugation (10 000 × *g*, 10 min at 4 °C) and were resuspended in 1-2 mL of phosphate-buffered saline (PBS, 8.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 1.45 M NaCl, pH 7.2) in 2 mL screw-cap centrifuge vials (SARSTEDT AG & Co., Nümbrecht, Germany) until suspended.

4.3.3.2. Cell fractionation

Glass beads (0.25 mL, 400 μm, VWR International, Deer Park, NY, USA) were added to the cell suspension (beads: suspension = 1:6 v/v), and the bacterial cells were broken by homogenization (7 bursts of 45s each with 1 min cooling between each burst, setting #4, 10,000 × *g*) using a Fast-Prep cell disrupter (Thermo Savant, FP120, CA, USA). Following homogenization, a density gradient centrifugation process was conducted. First, the suspensions were centrifuged at low speed (2000 × *g*, 3 min) to sediment the glass beads and intact cells. The translucent top layer of supernatants was then collected and pooled with the rest of the tubes. Then pooled samples were transferred to 25 mL centrifuge tubes and centrifuged at 20,000 × *g* for 20 min at 4 °C. Next, the cytoplasm-cell membrane fraction (cell membrane + cytoplasmic proteins) was separated by collecting the light-yellow supernatant. Finally, the membrane pellet (white color) was obtained by ultracentrifuge at 35,000 × *g* for 1 hr at 4 °C. Then, the pellet, the cell membrane isolate (CMI), was harvested, washed with 1 mL PBS to remove residual proteins and air-dried. The CMI stock solution was produced by resuspension in 1 mL PBS and stored at -80 °C.

4.3.4. Negative staining for transmission electron microscopy (TEM)

Carvacrol (1/4 × MIC, 1/2 MIC, 1 MIC, and 2 MIC), Tween 20 (equal volume to carvacrol from 0.06 mM solution) were treated and added to CMIs solution (1:10 ratio, v/v in PBS) for 5 min. Untreated CMI solution and 0.05 % DMSO were used as controls. Subsequently, samples were deposited on a glow-discharge TEM copper grid covered with a perforated carbon film. Then, samples were negatively stained as follows: 2% aqueous uranyl acetate for 10 min, distilled water rinse for 2 × 5 min, lead citrate for 4 min, and a rinse with distilled water for 1 min. Then they were blotted with a filter paper to form a thin liquid film (thickness of 100 – 250 nm) of the sample and air-dried. Samples were viewed using a transmission electron microscope (JEM 1230, JEOL USA, Inc., Peabody, MA, USA) with an acceleration voltage of 80 kV, and images were captured and recorded using a digital camera (ORCA-HR, Hamamatsu Photonics, Tokyo, Japan).

4.3.5. Fluorescence anisotropy

Measurement of anisotropy of DPH was used to examine the rotational diffusion of the fatty acyl chains in the CMIs upon the treatment with different inhibitory concentrations of carvacrol. Briefly, aliquots of *S. pyogenes* (*Spy* 1558) CMIs stock solution were diluted in PBS (1:10, v/v) and treated with carvacrol at the concentrations of 31.3 (1/4 × MIC), 62.5 (1/2 × MIC), (1 × MIC) 125 and (2 × MIC) 250 µg/mL after 5 min and 1 hr incubation time at 37 °C. Daptomycin (0.016 µg/mL) and two surfactants (Tween 20 and Triton X-100) were used as positive control. DPH was added to a final concentration of 2 µM. Then, mixtures were incubated for 10 min at 37 °C to allow the probes to be

incorporated into the membranes. For each time point, an unlabeled CMI suspension was utilized as a scattering reference, while DMSO-treated suspensions were employed as vehicle controls. Steady-state fluorescence anisotropy of DPH was measured at 37 °C with excitation at 356 nm and emission at 423 nm, nm (2.5-nm slit widths) using a PTI QuantaMaster-4-CW spectrofluorometer (Photon Technology International Inc., Birmingham, England). Data were processed using a PTI FeliX32 Analysis module (Photon Technology International Inc., Birmingham, England). Fluorescence anisotropy (r) is the ratio of fluorescence intensity of the polarized elements to the total intensity and is calculated as:

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$

"I" is measured fluorescence intensity is measured when the excitation and emission polarizers are fixed in the vertical (v) or horizontal (h) position.

The instrument determines "G" to correct for artifacts due to the optical components of the fluorimeter.

4.3.6. Lipid profile analysis

4.3.6.1. Culturing bacteria and membrane isolation

S. pyogenes (*Spy* 1558) cell suspensions were incubated in BHI at 37 °C for 24 hr until $OD_{600} \geq 0.6$, with or without carvacrol (31.3 and 62.5 μg/mL) in BHI at 37 °C. Cell membranes of each sample were harvested similarly to 4.3.3.

4.3.6.2. Lipid extraction and sample preparation for lipidomic

With some modifications, lipid extraction was performed using a Bligh-Dyer extraction method (Bligh and Dyer, 1959). Briefly, the CMIs samples (15 - 20 mg) were suspended

in 2 mL of phosphate-buffered saline (PBS), followed by the addition of 4 mL of a 2:1 (v/v) chloroform: methanol mixture. The mixture was incubated for 30 min at room temperature with intermittent mixing. Next, 1 mL of distilled water was added, and the mixture was vortexed for 1 min. Then the mixture was allowed to settle into a two-phase solution, then centrifuged at 3,500 × *g* for 10 min). Finally, the lower phase was recovered and dried under a nitrogen gas stream using the nitrogen evaporator (N-EVAP™, Organomation Association Inc., Berlin, NJ, USA). Dried lipid samples were stored at − 80 °C and were ready for analysis.

4.3.6.3. Lipidomics

Extracted lipid samples were analyzed to determine the percentage of different phospholipids and fatty acid composition by UHPLC-MS/MS (Thermo Vanquish LC-QEx-active MS/MS) (The Biological Mass Spectrometry Core Facility, Life Sciences Research Institute, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada). Samples from three independent repeats were measured per condition to technical triplicates. Data is analyzed and processed using Lipid-Search software, allowing the identification and quantification of the diverse and complex lipid spectra.

UHPLC method for lipid analysis. The Accucore C_{30} column (250 × 2.1 mm I.D., particle size: 2.8 µm) was obtained from ThermoFisher Scientific (ON, Canada). The mobile phase system consisted of solvent A (acetonitrile: H_2O 60:40 v/v) and solvent B (isopropanol: acetonitrile: water 90:10:1 v/v), both containing 10 mM ammonium formate and 0.1% formic acid. C_{30}-RPLC separation was carried out at 30°C (column oven temperature) with a flow rate of 0.2 mL/min, and 10 µL of the lipid extraction suspended in the mobile phase solvents mixtures (A: B, 70:30%) was injected into the

column. The following system gradient was used for separating the lipid classes and molecular species: 30% solvent B for 3 min; then solvent B increased to 50% over 6 min, then to 70% B in 6 min, then kept at 99% B for 20 min, and finally the column was re-equilibrated to starting conditions (30% solvent A) for 5 min prior to each new injection: high-resolution tandem mass spectrometry and lipidomics. Lipid analyses were carried out using a Q-Exactive Orbitrap mass spectrometer controlled by X-Calibur software 4.0 (ThermoScientific, MO, USA) with an acquisition HPLC system. The following parameters were used for the Q-Exactive mass spectrometer: sheath gas: 40, auxiliary gas: 5, ion spray voltage: 3.5 kV, capillary temperature: 250 °C; mass range: 200 – 2,000 m/z; full scan mode at a resolution of 70,000 m/z; top-1 m/z and collision energy of 35 (arbitrary unit); isolation window: 1 m/z; automatic gain control target: 1e5. The instrument was externally calibrated to 1 mg/L using ESI negative and positive calibration solutions (ThermoScientific, MO, USA). Tune parameters were optimized using a mixture of lipid standards (Avanti Polar Lipids, Alabama, USA) in both negative and positive ion modes.

Thermo Scientific™ LipidSearch™ software version 4.2 was used for lipid identification and quantitation. First, the individual data files were searched for product ion MS/MS spectra of lipid precursor ions. MS/MS fragment ions were predicted for all precursors adduct ions measured within ±5 mg/L. The productions that matched the predicted fragment ions within a ±5 mg/L mass tolerance were used to calculate a match score, and those candidates providing the highest quality match were determined. Next, the search results from the individual positive or negative ion files from each sample

group were aligned within a retention time window (±0.2 min), and the data were merged for each annotated lipid.

4.3.7. Statistical Analysis.

A complete randomized design was used. Experiments were performed in triplicate and three times independently. One-way variance analysis (ANOVA) analysis was performed for fluidity and quantitative microscopy analysis of CMI diameters. Two-way ANOVA was performed for the differences in lipid composition in bacteria under different concentrations of carvacrol treatment. Statistical differences were defined as (*, $p \leq 0.05$; ** $p \leq 0.01$; and *** $p \leq 0.001$), and mean separations among treatments were determined using Tukey's tests. Data analysis and graph design were done using GraphPad Prism (Version 5.0, L.A. Jolla, CA, USA). Results were expressed as mean ± standard error (S.E.).

4.4. RESULTS

4.4.1. *In vitro* model of membrane characterization

S. pyogenes membrane-carvacrol interactions were studied *in vitro,* and the isolated membrane was characterized. The procedure for preparing CMIs from *S. pyogenes Spy* 1558 is illustrated in Figure 4.2. A series of centrifugations were used for the isolation of bacterial membranes. A total yield of 135 ± 4.3 mg dry weight of CMI was collected, and the stock solution of CMI was prepared by resuspension in 1 mL PBS for further experiments.

4.4.2. TEM observations of CMIs

TEM micrograms of carvacrol or surfactant-treated CMI are shown in Figure 4.3. Membrane vesicles detected in the PBS control confirmed effective membrane isolation from the process (Figure 4.3 B). Liposomes are spherical vesicles in which a lipid bilayer membrane encloses an aqueous volume. The small unilamellar liposomes/vesicles (SUVs) have a size range of 20 – 100 nm. In the untreated sample, the diameters of the individual CMI vesicles ranged from 76 to 106 nm, and we can conclude that these liposomes are SUVs. Compared to the untreated vesicles, the diameter of the carvacrol-treated CMI suspension vesicles decreased in a concentration-dependent manner (Figure 4. 3C, 3D). Thus, carvacrol's tendency to cause size reduction damage in liposomes was significantly lower than that of liposomes treated singly with Tween-20.

Tween-20-treated CMI samples revealed irregular-shaped, small particles ranging in diameter from 7.5 to 18.7 nm (average of 13.2 nm). Similarly, CMIs treated with 2 × MIC carvacrol had morphologically similar particles to Tween-20-treated CMIs (average of 11.7 nm), but the sizes were significantly different.

4.4.3. Carvacrol-induced changes in membrane lipid composition

The alteration of membrane lipid composition is an adaptive mechanism of bacterial cells to external stress. Therefore, we analyzed the lipid profile of CMI in the presence of a subinhibitory concentration of carvacrol. Liquid chromatography-mass spectrometry (LC/MS) results of CMI isolated from carvacrol-treated cells suggested functional and structural alterations of phospholipids and fatty acid composition.

Fatty acids, phospholipids, neutral lipids, and glycolipids were analyzed by gas chromatography-mass spectrometry (GC-MS) (Table 4.1). In untreated and carvacrol-treated CMIs, the lipid profile revealed that the predominant membrane phospholipid class is phosphatidylethanolamine (P.E.) (Figure 4.5. S5 A). The other phospholipid classes are cardiolipins (CL), phosphatidylglycerol (P.G.), phosphatidylcholine (P.C.), phosphatidic acid (P.A.), and phosphatidylserine (P.S.) and their percentage in the CMI was different ($p \leq 0.05$) from each other (Table 4.1, Figure 4.5. S5). Other than phospholipids, we have also detected sphingolipids such as ceramides (Cer), simple Glc series [Glucosylceramide (GlcCer), galactosylceramide (LacCer), and Hexosylceramide (Hex1Cer)]. Furthermore, sphingosine (SPH) and neutral lipids such as diglyceride (D.G.), triglyceride (T.G.), deuterated triglyceride (D5TG), and fatty acyl and other lipids [N-acylethanolamine (AEA), O-acyl-(gamma-hydroxy) fatty acids (OAHFA) and other fatty acids (F.A.)] were also detected (Table 4.1 and Figure 4.5. S5).

4.4.4. Modulation of membrane fluidity

We measured the steady-state fluorescence anisotropy of DPH to observe the effect of carvacrol on CMI fluidity/micro-viscosity. The DPH probe detects the rotational diffusion of the fatty acyl chains in the membrane interior. The effect of different

carvacrol concentrations and daptomycin on the anisotropy value of the CMIs was observed (Figure 4.4). The results showed a significant decrease in anisotropy for the higher carvacrol-treated CMIs (62.5 – 250 μg/mL) compared with the untreated and DMSO control CMIs, indicating increased fluidity of the lipid core due to the carvacrol insertion into the membrane vesicles. Lower amounts of carvacrol, on the other hand, did not affect anisotropy. Furthermore, the anisotropy value was lesser in 5 min carvacrol-treated CMI than that of the 1 hr carvacrol-treated CMI ($P < 0.05$), suggesting that carvacrol decreases the membrane viscosity in a time-dependent manner.

4.5. DISCUSSION

The composition of membrane phospholipids, head groups, and fatty acyl chains of Gram-positive bacteria, including *S. pyogenes*, can alter rapidly in response to environmental conditions such as low pH, osmotic stress, or temperature extremes (Willdigg and Helmann, 2021). Membrane lipid content influences the membrane viscosity, which modulates membrane permeability and can influence solute transport and protein interactions (Mendoza, 2014). Membrane lipid homeostasis is critical; disrupting it can affect cell viability. Therefore, this study aimed to study the impact of carvacrol on bacterial membrane lipid compositional alteration upon exposure to sub-inhibitory concentrations of carvacrol for 24 hr.

Chapters 2 and 3 have shown quantitative indicators of carvacrol-induced increased membrane permeability leading to cell viability loss, cytoplasmic enzyme leakage (LDH enzyme), cytoplasmic nucleic material leakage (DNA and RNA) in four different strains of *S. pyogenes*, ATCC 19615, *S. pyogenes* strains, clinical isolates, and *Spy* 1558). In addition, we also studied the membrane potential and cell fluidity using intact cell models of *S. pyogenes* strains (ATCC 19615 and a clinical isolate from pharyngeal patients).

Though several studies exist on Gram-positive bacterial membrane compositions and compositional changes induced by antimicrobial agents, there is limited data for *S. pyogenes.* As far as we know, this is the first study revealing the membrane phospholipid compositional changes of erythromycin-resistant *S. pyogenes* in response to carvacrol. In addition, the CMI isolation procedures were optimized (Figure 4.2) and could be used to

extract membranes from other Gram-positive bacteria to assess the molecular mechanism of membrane-targeting antibiotics.

The bacterial cell membrane is a typical bilayer composed of different phospholipids, glycolipids, and proteins (Sohlenkamp and Geiger, 2015). Lipid analysis of streptococci has reported that most cell membranes are composed of glycolipids and two acidic phospholipids, CL and P.G. (Rosch et al., 2007). Our results showed that *S. pyogenes* bacterial membranes contain mainly phospholipids such as P.G., P.E., CL, P.C., P.S., and P.A. However, the composition of bacterial membranes differs among bacterial species and is also regulated by their environmental conditions (Sohlenkamp and Geiger, 2015). Membrane phospholipid and fatty acid compositions were determined by gas chromatography, which revealed that the two carvacrol concentrations [(62.5 µg/mL (1/2 × MIC) and 31.3 µg/mL (1/4 × MIC)] did not change the type of phospholipids and fatty acids but did change their proportions (Table 4.1).

Most Gram-positive bacteria (except Bacillus or Clostridium) lack P.E. in the cytoplasmic membrane and are composed of anionic (acidic) phospholipids such as P.G. and CL. In contrast, we identify a higher percentage of P.E. in all CMIs, which may be due to its compositional differences in erythromycin-resist strain, not recovering all lipids during extraction or detection sensitivity of the instrument. Furthermore, *S. pyogenes* genomic analysis revealed that they synthesize at least two anionic phospholipids, P.G. and CL, attributing a high negative charge to their membranes.

Substantial changes in membrane fatty acids composition of bacterial cells induced by thymol, carvacrol, limonene, cinnamaldehyde, and eugenol were reported previously (Di Pasqua et al., 2006b). The mechanism of action of thymol, another polar monoterpenoid like carvacrol, on cell membranes was investigated previously (Ferreira et al., 2016) and found to interact with membrane P.C. Therefore; carvacrol preferentially binds to *S. pyogenes* bacterial membranes due to their high content of the acidic phospholipids P.G. and CL could be similarly explained.

Two primary stress responses that can modify the cell membrane lipid composition are (i.) modulating the existing lipid fatty acyl chains (length, branching, or saturation state) and (ii.) altering membrane lipid composition, thereby replacing the existing lipids with new lipids with different properties (Willdigg and Helmann, 2021). For example, an increased degree of fatty acid unsaturation or decreased fatty acid chain length may be generally found when bacteria are exposed to extreme temperatures and oxidative stress (Wang et al., 2014). Moreover, when bacteria are exposed to external environmental fluctuations, changes in the membrane lipid composition may allow them to maintain proper biophysical and biochemical characteristics (Sohlenkamp and Geiger, 2015; Wang et al., 2014).

Previously, the affinity of membrane phospholipids for antimicrobial monocyclic terpenoids or monocyclic terpenoids-rich essential oils was investigated (Di Pasqua et al., 2007; Di Pasqua et al., 2006b; Siroli et al., 2015). They discovered that carvacrol's hydrophobicity and positive charge modify the structural remodeling of the bacterial membrane, primarily unsaturated fatty acids. Thus, we might speculate that the mechanism of action of carvacrol on *S. pyogenes* CMIs changes the membrane lipid

profile and fluidity, causing damage to the lipid packing density. Furthermore, our previous chapter 3 reported that carvacrol binds to the anionic P.G. and CL, partially anionic (or zwitterionic) P.E., but not to the zwitterionic P.C. highlighting the importance of the negative charge for the carvacrol-phospholipid interaction to occur.

Membrane phospholipid remodeling in Group A Streptococcus (GAS) and Group B Streptococcus (GBS) in response to human serum was investigated (Joyce et al., 2021). They have detected major lipids in GAS, such as mono-hexosyldiacylglycerol, di-hexosyldiacylglycerol, P.A., P.G., CL, and undecaprenyl phosphate. Furthermore, they have reported that all these streptococcus species, such as *S. pneumoniae*, *S. pyogenes,* and *S. agalactiae* synthesize zwitterionic P.C., which is more common in eukaryotes than in bacterial membranes. In contrast, we found no significant change in P.C. production after exposure to both carvacrol concentrations in the current investigation. However, we report the remodeling of lipids in *Spy* 1558 (erm) with another zwitterionic phospholipid, P.E., and changes in anionic phospholipids of P.G., CL, P.S., and P.A.

Another potential mechanism of bacterial adaptation changes in the external environment is modifying the existing membrane phospholipids to acquire a membrane with different properties (Zhang and Rock, 2008). For example, the anionic phospholipid P.G. can be modified to the cationic lipid lysyl-phosphatidylglycerol (LPG) by attaching a lysine residue to the glycerol head group (Slavetinsky et al., 2012; Zhang and Rock, 2008). Our study observed a significant concentration-dependent increment in the LPG, and these modifications permit rapid response to the new environmental conditions. On the other hand, altering the ionic membrane state may aid bacteria in increasing their

resistance to human innate immune products such as cationic antibacterial peptides and beta-defensins (Joo et al., 2016).

Bacteria maintain lipid homeostasis by regulating lipid composition in response to their internal and external environment. As a result, bacteria generally tolerate more severe alterations in membrane lipid composition while remaining viable than eukaryotic cells (Gohrbandt et al., 2019). On the other hand, Carvacrol has been shown to be cytotoxic to bacteria cells rather than human tonsil epithelial cells (Chapter 2). This selective cytotoxicity of carvacrol on bacterial cells may clarify that their membrane compositional changes contribute to the human cell membrane being rich in PC and cholesterol, as well as phospholipids containing long-chain and saturated fatty acids, which may have better carvacrol impermeability than bacterial membranes lacking PC and cholesterol.

Besides those compositional changes, it has been revealed that altering membrane fatty acid composition upon exposure to carvacrol affects membrane fluidity. The anisotropy value positively correlates with the lipid order parameter and is inversely proportional to cell membrane fluidity (Shinitzky and Barenholz, 1978). The findings revealed a distinct relationship between carvacrol concentration and membrane fluidity in *S. pyogenes* CMIs.

Fluorescent probes such as DPH, trimethylammonium-DPH (TMA-DPH), and 1-anilino-8-naphthalene sulfonate (ANS) are employed for the study of lateral diffusion, fluidity, placement of lipids and proteins in the lipid bilayer, and surface potential of membranes (Munishkina and Fink, 2007). DPH, the probe we used in the anisotropy experiments, is a highly hydrophobic molecule that can insert into the lipid core of the

bacterial membrane and sense the disordering of the fatty acyl chains in the lipid bilayer exposed to the drug (Gonzalez-Horta et al., 2013).

Daptomycin is a cyclic lipopeptide antibiotic that targets the cell membrane and is prescribed for Gram-positive bacterial infections (Heidary et al., 2017). The proposed mechanism for daptomycin against Gram-positive methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci revealed that it binds with membrane, thereby altering membrane fluidity through biosynthetic enzymes involved in cell wall separation (Miller et al., 2016).

Pathogenic Gram-positive bacteria such as streptococci and enterococci were found to inactivate daptomycin by releasing phospholipids (Ledger et al., 2017). In addition, the aggregation of daptomycin affects Gram-positive cytoplasmic membrane function, causing potassium ion leakage and, eventually, membrane potential loss and cell death (Pogliano et al., 2012; Silverman et al., 2003). We also observed some morphological curvature of carvacrol-treated CMIs in the TEM micrograms, which may be due to a similar mechanism.

Membrane permeabilization depends on the lipid profile of the membrane and is thereby responsible for the physical properties of the lipid bilayer, such as membrane fluidity, negative-curvature induction, surface charge, and lipid packing (Knobloch et al., 2015). Furthermore, a correlation of membrane fatty acid compositional changes with membrane fluidity changes was reported in other Gram-positive species. For example, *Staphylococcus aureus* and *Brochothrix thermosphacta* were induced by similar monoterpenes such as thymol, carvacrol, and eugenol (Di Pasqua et al., 2006a). Different explanations were reported that can affect membrane fluidity, such as the composition of

lipids, length of the phospholipid fatty acyl chain, and the saturation state of phospholipids (Fajardo et al., 2011).

4.5. CONCLUSION

In summary, our findings suggest that exposure to sub-inhibitory concentrations of carvacrol alters the composition of the membrane phospholipid in *S. pyogenes* thereby, increases the membrane fluidity. However, more research is needed to clarify whether the observed changes in membrane composition result from bacteria developing resistance to carvacrol or an adaptive response to the carvacrol concentration.

4.6. ACKNOWLEDGMENT

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Table 4.1 Lipid composition of cell membrane isolates from *Streptococcus pyogenes* exposed to carvacrol

Membrane composition	*% of total lipid		
	Untreated	Carvacrol exposure (24 hr at 37 °C)	
		1/4 × MIC (31.3 µg/mL)	1/2 × MIC (62.5 µg/mL)
Phospholipids			
Cardiolipin	1.9	3.7	7.6
Di-cardiolipin	0.7	3.3	1.4
Phosphatidic acid	0.4	0.4	3.0
Phosphatidylglycerol	4.6	12.8	9.5
Lyso-phosphatidylglycerol	0.1	0.4	3.9
Phosphatidylethanolamine	19.5	17.1	25.7
Phosphatidylcholine	2.9	1.4	2.0
Phosphatidylserine	1.9	1.8	4.5
Total phospholipids	**30.1**	**37.2**	**50.0**
Sphingolipids			
Ceramides	4.4	1.2	0.6
Simple Glc series	1.8	5.6	3.4
Sphingosine	12.2	5.3	7.3
Total Sphingolipids	**18.4**	**12.0**	**11.2**
Neutral lipids			
Diglyceride	4.9	1.2	3.9
Triglyceride	20.8	5.6	3.2
Deuterated triglyceride	1.4	0.5	0.2
Total Neutral lipids	**27.2**	**7.3**	**7.3**
Fatty acyl and other			
N-Acylethanolamine	2.6	4.2	3.9
OAcyl-(gamma-hydroxy) FA	17.8	26.9	19.7
Fatty acids	2.0	8.5	0.3

*Not all the lipids recovered. The values are given weight percentage to the total lipids recovered. These results represent the averages of three independent membrane isolation experiments from the erythromycin-resist *Streptococcus pyogenes* (*Spy* 1558, erm) cells with or without carvacrol exposure.

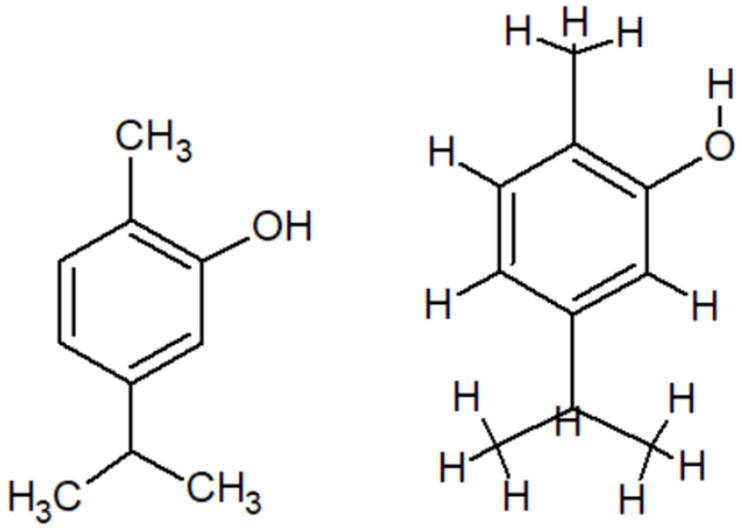
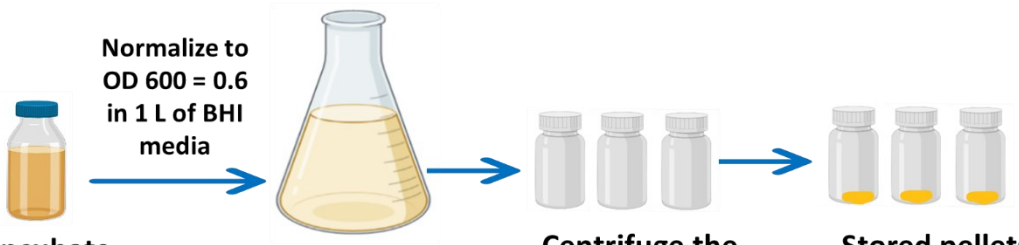


Figure 4.1 The chemical structure of carvacrol (Sketched using ACD/ChemSketch software).

Preparation of bacteria for membrane isolation



Incubate *S. pyogenes* bacteria in 50 mL BHI at 37 °C for 24 hr

Incubate at 37 °C for 3 - 4 hr

Centrifuge the centrifuge bottles at 4 °C for 10 min, 10,000 × g

Stored pellets at – 80 °C or continue the procedure

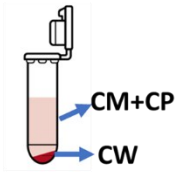
Preparation of bacteria cell membrane isolate (CMI)

Wash the pellet using PBS and resuspend in PBS

Fast centrifuge at 4 °C for 45 s, 7 times in glass beads (0.25 mL, 400 µm)

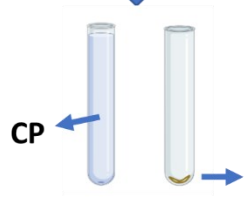
Low spin centrifuge at 4 °C for 3 min 2,000 × g

Centrifuge the pooled supernatants at 4 °C for 20 min, 20,000 × g



Collect the supernatants and volumize to the top of the ultracentrifuge tube (≈ 12 mL) with PBS

Ultracentrifuge the supernatants at 4 °C for 1 hr 35,000 × g



Wash with PBS to remove any leftover cytoplasmic proteins and stored pellets at – 80 °C

Figure 4.2 A schematic portrays the optimized procedure for Gram-positive bacterial membrane isolation (Figure was created using Biorender.com and Microsoft® PowerPoint®).

BHI: Brain heart infusion; PBS: Phosphate-buffered saline. CM: Cell membrane; CMI: Cell membrane isolate; CP: Cytoplasmic protein; CW: Cell wall.

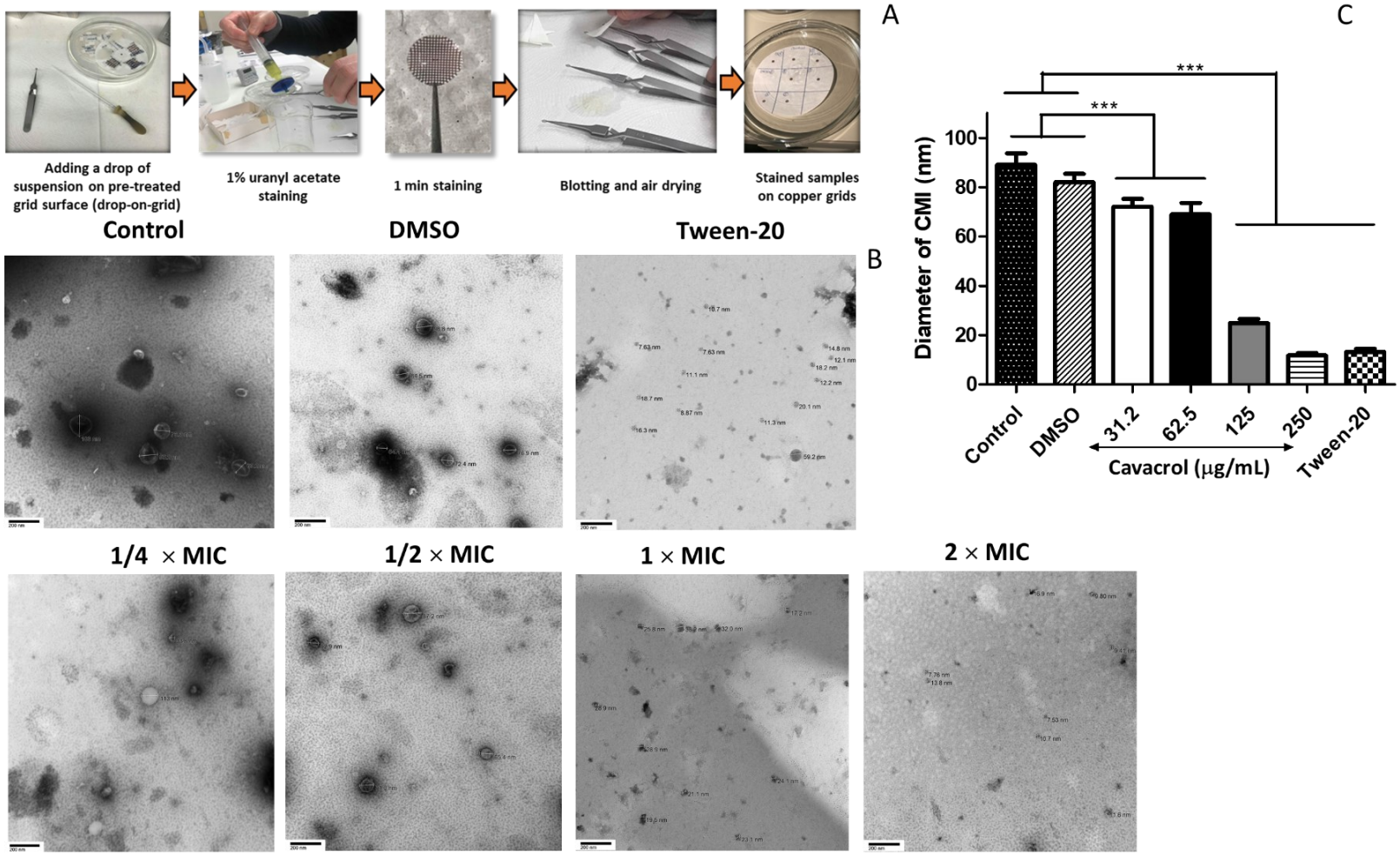

A
Adding a drop of suspension on pre-treated grid surface (drop-on-grid)
1% uranyl acetate staining
1 min staining
Blotting and air drying
Stained samples on copper grids
B
Control
DMSO
Tween-20
1/4 × MIC
1/2 × MIC
1 × MIC
2 × MIC
C
***
***
Diameter of CMI (nm)
100
80
60
40
20
0
Control
DMSO
31.2
62.5
125
250
Tween-20
Cavacrol (μg/mL)

Figure 4.3 Transmission electron microscopy (TEM) of cell membrane isolates (CMI) from erythromycin-resist *Streptococcus pyogenes* (*Spy* 1558, erm).
(A) Sample preparation procedure for TEM used in the experiment. Treated and untreated CMI aliquots were placed on grids and negatively stained with uranyl acetate. (B) Representative images of stained CMI treated without or with carvacrol (1/4 MIC, 1/2 MIC, 1 MIC, and 2 MIC) for 5 mins. MIC = 125 μg/mL. Tween-20 was used as a positive control. (Magnification and bar: × 100,000, 200 nm). (C) Carvacrol-induced changes of CMI vesicle diameter determined by TEM. An average of 10 cells per treatment from two independent experiments were analyzed. ***P < 0.001, compared among means (ANOVA, Tukey's test).

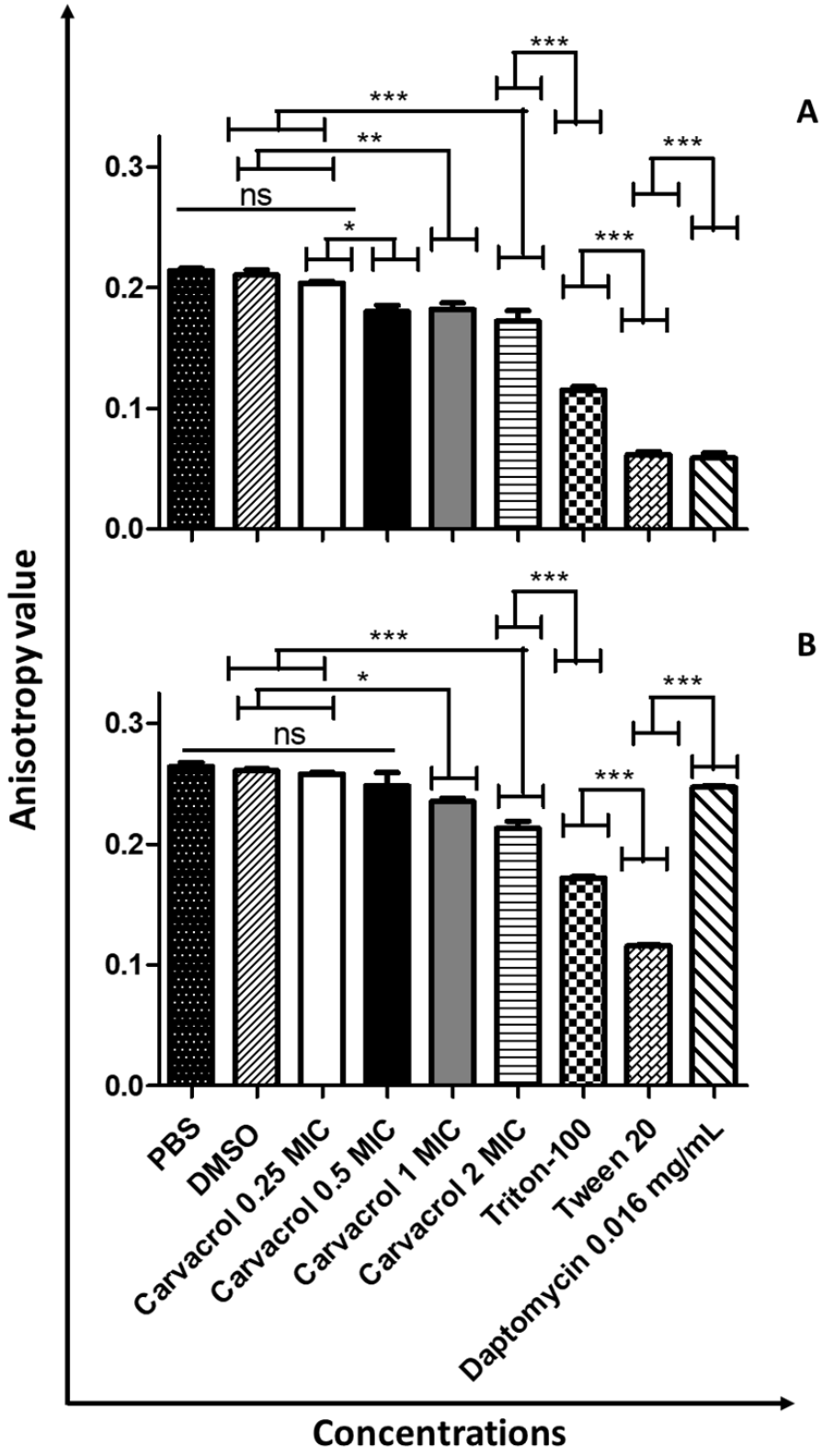

A
B
***
***
**
ns
*
***
***
0.3
0.2
0.1
0.0
Anisotropy value
PBS
DMSO
Carvacrol 0.25 MIC
Carvacrol 0.5 MIC
Carvacrol 1 MIC
Carvacrol 2 MIC
Triton-100
Tween 20
Daptomycin 0.016 mg/mL
Concentrations

Figure 4.4 The effect of carvacrol on the fluidity of the cell membrane isolates (CMIs) from *Streptococcus pyogenes.*

The fluorescence anisotropy values for the DPH fluorescence probe are presented as (A) 5 mins and (B) 1 hr exposure to the carvacrol concentrations (31.2 – 250 µg/mL). MIC = 125 µg/mL for carvacrol. Two surfactants (Tween-20 and Triton-100) and daptomycin (0.016 µg/mL) were used as positive controls. Untreated PBS and DMSO vehicle controls were used as negative controls. Values presented as average ± S.E. of three replicates. The symbol (∗) represents values statistically significant in comparison to the control erythrocytes, $P < 0.05$; (∗∗), $P < 0.01$ and (∗∗∗), $P < 0.001$.

4.7.SUPPLEMENTARY FIGURES

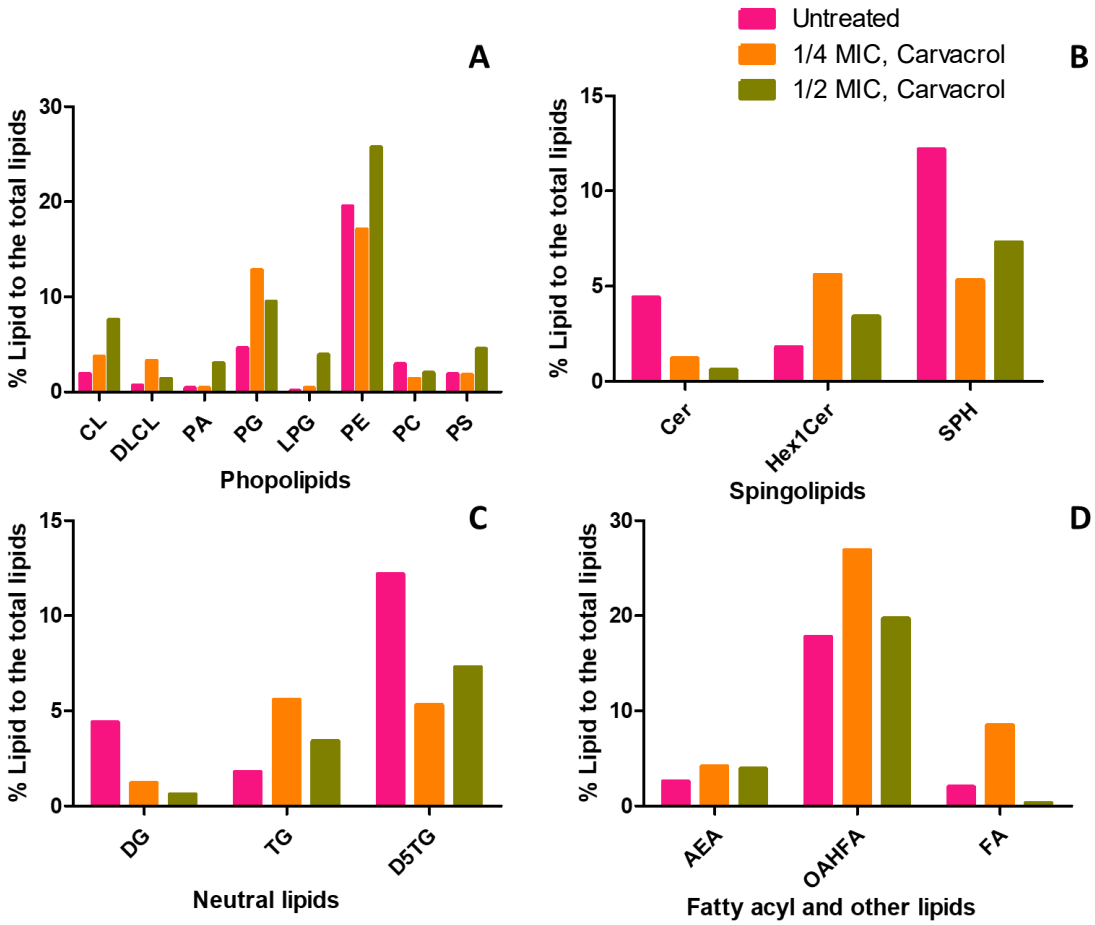


Figure 4.5. S5. Differences in lipids class composition in cell membrane isolate extracted from erythromycin resist *Streptococcus pyogenes* (*Spy* 1558, erm). Percentage of (A) phospholipids, (B) sphingolipids, (C) neutral lipids, and (D) fatty acyl and other lipids to the total lipids were expressed. Samples from three independent repeats were measured per condition as to technical triplicates. CL: cardiolipin, DLCL: di-cardiolipin, PA: phosphatidic acid, P.G.: phosphatidylglycerol, LPG: lyso-phosphatidylglycerol, PE: phosphatidylethanolamine, P.C.: phosphatidylcholine, P.S.: phosphatidylserine, Cer: ceramides, Hex1Cer: simple Glc series, SPH: sphingosine, DG: diglyceride, T.G.: triglyceride, D5TG: Deuterated triglyceride, AEA: N-Acylethanolamine, OAHFA: O-Acyl-(gamma-hydroxy) F.A. and F.A.: other fatty acids.

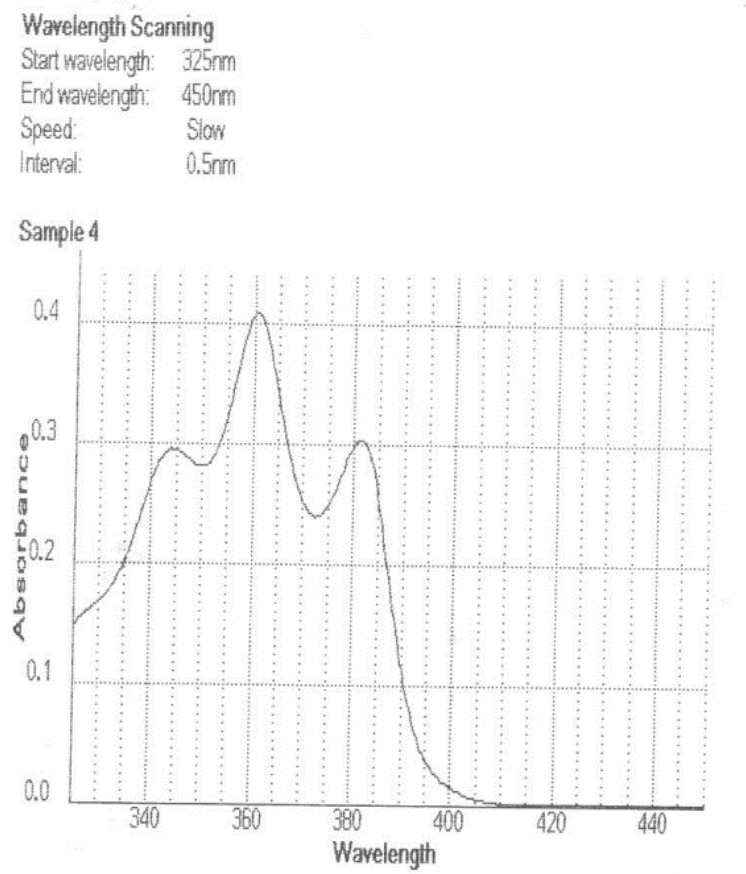


Figure S.6. Fluorescence emission spectra of 1.0 µM DPH (excitation at 325 nm), normalized by the intensity of the emission band at 423 nm, in three PBS, in cell membrane isolate. PBS: Phosphate-buffered saline.

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CHAPTER 5. CARVACROL INHIBITS BIOFILM FORMATION BY STREPTOCOCCUS PYOGENES BY SUPPRESSING THE EXPRESSION OF GENES ASSOCIATED WITH QUORUM-SENSING AND REDUCING *CELL* SURFACE HYDROPHOBICITY

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5.1. ABSTRACT

Streptococcus pyogenes is a leading cause of chronic and acute infections, including streptococcus pharyngitis. Biofilm formation by *S. pyogenes* can cause tolerance against antibiotics. Although penicillin remains the first choice of antibiotic for *S. pyogenes*, alternative approaches have gained interest due to treatment failures and hypersensitive individuals. Carvacrol is a monoterpenoid from herbal plants with selective biocidal activity on *S. pyogenes*. Therefore, the present study reveals the efficacy of carvacrol in inhibiting and eradicating *S. pyogenes* biofilm. The antibiofilm activities were investigated using colorimetric assays, microscopy, cell surface hydrophobicity, gene expression analysis, and *in-silico* analysis. Carvacrol also showed a minimum biofilm inhibitory concentration (MBIC) against *S. pyogenes* of 125 µg/mL. The electron microscopic and confocal microscopic analyses revealed a dose-dependent suppression of biofilm formation and a reduction in the biofilm thickness by carvacrol. Carvacrol also inhibited the biofilm-associated virulence factors such as cell surface hydrophobicity. Quantitative real-time polymerase chain reaction analysis showed the downregulation of *speB*, *srtB*, *luxS*, *covS*, *dltA*, *ciaH*, and *hasA* genes involved in biofilm formation. The results suggested the therapeutic potential of carvacrol against biofilm-associated streptococcal infections.

Keywords: antibiofilm activity, virulence gene, biofilm, Streptococcal pharyngitis, carvacrol

5.2. INTRODUCTION

Streptococcus pyogenes [Group A β-hemolytic streptococci (GAS)], an exclusively human pathogen, is responsible for significant morbidity and mortality worldwide (Sims Sanyahumbi et al., 2016). Moreover, *S. pyogenes* is the common cause of acute bacterial pharyngitis, accounting for 15-30% of children and 5-10% of adult cases (Shaikh et al., 2010a).

Biofilm formation is considered an adaptation strategy of pathogenic bacteria for survival in the host. Bacterial cells in biofilm become resistant or tolerant of antibiotics and immune responses (Wu et al., 2015). The development of the biofilm is essential to the pathogenesis of *S. pyogenes* (Baldassarri et al., 2006). It has been reported that up to 90% of *S. pyogenes* isolates were able to form a biofilm (Baldassarri et al., 2006). Although combination antibiotic treatments are needed to combat biofilm infections [4], such a treatment can be led to antibiotic resistance, therapeutic failures, or treatment difficulties (Lebeaux et al., 2014).

β-Lactams are the preferred antibiotics for treating streptococcal pharyngitis (van Driel et al., 2016a). Fortunately, despite potential changes in virulence, *S. pyogenes* have universally remained susceptible to penicillin over the decades (van Driel et al., 2016a). However, streptococci can survive, persist within human cells, and continue invulnerability to antibiotic treatment and innate immunity (Rohde and Cleary, 2016). Furthermore, the inactivation of penicillin by other β-lactamase -producing bacteria living on the surface of the tonsils may also result in treatment failures (Brook, 2017). Therefore, macrolides, such as erythromycin, azithromycin, and clarithromycin, are

recommended for patients with β-lactam allergy or penicillin failure to treat some *S. pyogenes* infections (Martin, 2015; Randel, 2013; van Driel et al., 2016a).

Given the challenges in combating streptococcal biofilm with antibiotics, alternative antibacterial agents with activities against biofilm are needed. Carvacrol, 5-isopropyl-2-methyl phenol, is a mono-terpenoid (Figure 5.1A) found in oregano and thyme (Wijesundara and Rupasinghe, 2018a; Wijesundara and Rupasinghe, 2019a). Carvacrol or carvacrol-containing extracts have been shown to possess antibacterial (Wijesundara and Rupasinghe, 2018a), anticancer (Dai et al., 2016), and anti-inflammatory (Wijesundara et al., 2017b) activities.

Antibiofilm activity of carvacrol on several other bacteria, such as *Listeria monocytogenes* and *Pseudomonas aeruginosa* biofilms, was reported previously (Ashrafudoulla et al., 2021). In addition, we have previously shown antibacterial and antibiofilm activities of carvacrol-rich plant extracts and essential oils against several strains of *S. pyogenes* (Wijesundara and Rupasinghe, 2019a, b). Furthermore, we have demonstrated carvacrol's dose- and time-dependent rapid bactericidal activity against *S. pyogenes* (Wijesundara et al., 2021). Furthermore, among the bactericidal characteristics of carvacrol, the instant-killing kinetics of *S. pyogenes* due to membrane disruption was observed (Wijesundara et al., 2021). Therefore, the present study investigated carvacrol's antibiofilm and antivirulence activities against *S. pyogenes*.

5.3. MATERIALS AND METHODS

5.3.1 Chemicals

Carvacrol (99%, v/w), dimethyl sulfoxide (DMSO), and Dulbecco's phosphate-buffered saline (PBS) were purchased from Sigma-Andrich, Oakville, ON, Canada. Carvacrol stock solution (100 mg mL^{-1} in DMSO) was prepared and stored at 4 °C. Brain heart infusion (BHI) and bacteriological agar were purchased from Oxoid (Nepean, ON, Canada).

5.3.2. Bacterial strains and culture conditions

Two biofilm-forming *S. pyogenes* strains, ATCC 19615 and ATCC 49399, from the American Type Culture Collection (ATCC) (Manassas, VA, USA), a clinical isolate derived from a pharyngeal patient and erythromycin resist *erm* gene incisional inactivated strain; *Spy* 1558 were used. All strains were stored at − 80 °C in BHI supplemented with 20% glycerol. Four strains were routinely grown and maintained on BHI agar plates at 4°C. A bacterial colony was inoculated to 10 mL BHI media (pH 7.0) under 5% CO_2 incubation at 37 °C overnight. Inoculum suspension (1×10^6 CFU/mL, $OD_{600} = 0.02$) was prepared using the early log phase of *S. pyogenes* as described previously (Wijesundara and Rupasinghe, 2018a).

5.3.3. Biofilm inhibition activity

5.3.3.1. MTT reduction assay

The metabolic activity of viable microbial cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Life Technologies, Burlington, ON, Canada) reductase assay as previously described (Wijesundara and Rupasinghe, 2018a). Briefly, a 100 µL of BHI containing carvacrol ranging from 0.195 to

1,000 µg/mL, and a 100 µL bacterial suspension (1×10^6 CFU/mL, $OD_{600} = 0.02$) of each strain was added to each well in a 96-well plate (Falcon, Corning Inc., NC, USA). Plates were incubated at 37 °C for 72 hr to facilitate biofilm growth. Then, planktonic bacteria were removed, and wells with biofilms were washed with sterile PBS. Fresh BHI broth (100 µL) supplemented with 10 µL of 12 mM MTT was added to each well and incubated for 3 hrs at 37 °C. After removing 85 µL of BHI broth, 50 µL of DMSO was immediately added to the wells to dissolve the insoluble purple formazan crystals formed in the wells by reducing MTT due to the activity of dehydrogenase enzymes in living *S. pyogenes* biofilms. The colorimetric change was measured at OD_{540} nm. The penicillin Vk, penicillin G, clindamycin, erythromycin, DMSO, bacterial without treatment, and carvacrol-free medium were used as the controls. The minimum biofilm inhibitory concentration (MBIC) was determined as the lowest concentration of carvacrol that showed a reduction (at least 90%) of biofilm formation compared to the DMSO control. The percentage of biofilm inhibition was calculated as $[(OD_{control} - OD_{treated})/ OD_{control}] \times 100$.

5.3.3.2. Crystal violet (CV) assay

The crystal violet assay was previously performed (Jalal and Lee, 2020). Biofilms of *S. pyogenes* were formed in 24-well plates with or without carvacrol at 37 °C for 24 hr. The planktonic cells were removed, and biofilms were washed with sterile PBS. Then, at room temperature, biofilms were stained with 200 µL of 0.1% aqueous CV solution for 10 min. The wells were washed thrice with sterile PBS to remove excess stains. Then, the dye was added to extract 200 µL of a mixture of 7.5% acetic acid and 10% methanol. The

absorbance was measured at 595 nm, and MBIC was determined as the lowest concentration required to inhibit biofilm formation.

5.3.4. Biofilm eradication activity

A 100 µL of carvacrol (1.95 – 1000 µg/mL), penicillin G (0.0002 – 1 µg/mL) or antibiotic controls [penicillin Vk (0.0002 – 1), clindamycin (0.002 – 0.5 µg/mL) and erythromycin (0.002 – 1 µg/mL)] were added to 24 hr preformed *S. pyogenes* biofilms separately in 96-well plates and incubated for another 2 hr at 37 °C. MTT and CV assays were conducted to determine carvacrol's minimum biofilm eradication concentration (MBEC), as described above. The minimum biofilm eradication concentration (MBEC) was determined as the lowest concentration of carvacrol that reduced the bacterial metabolic activity by 95 ± 5% compared to the DMSO control.

5.3.5. Scanning electron microscopic (SEM) for the visualization of biofilm morphology

S. pyogenes biofilms were formed in 96-well plates in the presence of carvacrol at 1 × MBIC and ½ × MBIC concentrations or absence of carvacrol as controls at 37 °C for 72 hr. Samples were prepared and examined as described previously (Wijesundara and Rupasinghe, 2018a).

5.3.6. Confocal laser scanning microscopic (CLSM) analysis: Live/dead assay

5.3.6.1. Visualizing biofilm inhibition by carvacrol

S. pyogenes biofilms were grown on the coverslips placed in 12-well microtiter plate wells (Nunc, Rochester, NY, USA) along with carvacrol (15.6 - 62.5 µL/mL), DMSO, and penicillin G for three days at 37 °C. The coverslips were washed with sterile saline and stained with SYTO^{TM} green and propidium iodide (PI) mixture in the LIVE/DEAD

biofilm viability kit (Molecular Probes Invitrogen, Nepean, ON, Canada) for 20 min in the dark. The biofilms were washed three times in sterile saline, air-dried, and sealed on a glass slide. Stained biofilms were visualized under the laser scanning confocal imaging system (Zeiss LSM 510 META, Heidelberg, Germany) using a 488 nm argon laser for stain excitation and a 500 nm emission filter for stain detection, attached with a water immersion dipping objective lens of 60 × oil. Image capturing of the cells was concluded with an inverted microscope attached to the CLSM imaging system (ZeissLSM 510 META, Heidelberg, Germany). Randomly selected, three positions of the slide were observed. Image processing and z-stack analysis were performed using ZEN image software (Version 3.1, Carl Zeiss Microscopy GmbH, Germany). The threshold value that presented the best fit for all the image stacks of a trial was chosen.

5.3.6.2. Visualization of biofilm eradication of *S. pyogenes*

Three-day-old *S. pyogenes* biofilms attached to the coverslip were treated with carvacrol at 1/4 × MBEC (31.25 µL/mL) and 1/2 × MBEC (62.5 µL/mL) and DMSO-treated as for vehicle control and penicillin G for the comparison. They were visualized and compared as in 2.6.1.

5.3.7. Microbial adhesion to hydrocarbon (MATH) assay

The effect of carvacrol on the *S. pyogenes* cell surface hydrophobicity was examined using microbial adhesion to hydrocarbon (MATH) assay as described previously (Adil et al., 2019). The adherence of *S. pyogenes* to the hydrophobic substrate, toluene, was determined as follows. Briefly, *S. pyogenes* cultures grown in the presence and absence of carvacrol (2 ×, 1 ×, 1/2, and 1/4 × of MBIC) were diluted to OD_{600} = 0.02. Then, 1 mL of toluene was added to the 1 mL of the diluted culture. The mixture was vortexed

vigorously for 2 min, followed by standing at room temperature for 10 min. The lower aqueous phase was carefully recovered and measured at 530 nm. Cultures grown in the presence of DMSO were included as the vehicle control. The hydrophobicity of the bacterial cell surface was determined as [1 – (OD $_{530\ nm}$ after vortex/ OD $_{530\ nm}$ before vortex)] × 100.

5.3.8. Quantitative real-time polymerase chain reaction (RT-qPCR)

5.3.8.1. RNA extraction

S. pyogenes cells from ATCC 19615 and clinical isolate were treated with carvacrol (1/2 MBIC = 62.5 µg/mL, or untreated (control) and were grown at 37 ˚C in BHI medium for 24 hr. Total RNA was isolated from cells of *S. pyogenes* overnight cultures grown with or without carvacrol using an RNase Mini kit (Qiagen, Toronto, ON, Canada). The total RNA concentration was determined using a TECAN^{TM} Nano-Quant Plate (Infinite M200 PRO, TECAN, Atlanta, GA, USA). The integrity of the isolated RNA was checked by running agarose gel electrophoresis. The RNA was treated with DNase I (Bio-Rad Laboratories Inc, MP, CA, USA) to remove any contaminated genomic DNA.

5.3.8.2. cDNA synthesis

cDNA was synthesized from the RNA using the antiScript gDNA clear cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada), following the manufacturer's instructions. The conditions included a cycle of 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C. The freshly prepared cDNA was used immediately for the RT-qPCR assay.

5.3.8.3. RT-qPCR

RT-qPCR reactions were performed as per the manufacturer's instructions for candidate genes (*covS, covR, ropB, speB, luxS, hasA, dltA, srtB*, and *ciaH;* Table 5.1) using SSO-Advanced Universal SYBR^{TM} Green PCR super-mix (Bio-Rad Laboratories Inc. ON,

Canada) in a CFX Connect System (Bio-Rad, Mississauga, ON, Canada). As listed in Table 5.1, primers were designed using the Primer quest tool (Integrated DNA Technologies, Coralville, IA, USA). The PCR cycles consisted of 2 min at 94 °C; 10 min at 95 °C; 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. *gyrA* was included as the control gene. The results were expressed $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008).

5.3.9. *In silico* Studies

5.3.9.1. Molecular modeling

Due to the three-dimensional structure of LuxS was not known, a homology modeling approach was taken to model the structure of this protein (Vyas et al., 2012). The amino acid sequence of *S. pyogenes* LuxS was retrieved from the UniProt database (UniProt id: P0C0C7). Its structure was generated using the known structure of LuxS from *Streptococcus suis* (pdb id: 4XCH) as a template. The two proteins shared 81% identity. Modeller9v14 was also used to generate a structure of *S. pyogenes* LuxS by homology modeling. The scores of discrete optimized protein energy (DOPE) were used to select the best of the five generated models. The structure was further subjected to validation using Procheck.

5.3.9.2. Molecular docking

The 3D structure of carvacrol was extracted from the PubChem compound database (Kim et al., 2016). A molecular docking study was performed to gain further insight into the binding of carvacrol to the active site of LuxS. Molecular docking of these compounds against LuxS was conducted by Autodock 4.0 using the Lamarckian genetic algorithm. LuxS was selected as it was commonly studied against biofilm studies of streptococcus species; hence comparative ideas could be derived from this initial molecular docking

study. The total number of runs was set at 15. The optimal docking was selected based on considerations of binding free energy.

5.3.10. Statistical analysis

A complete randomized design was used as the experimental design. All *in vitro* experiments were conducted independently three times in triplicates. Biofilm formation, biofilm eradication, and adhesion data were evaluated using a one-way analysis of variance (ANOVA) with a p-value of ≤ 0.05 using the GraphPrism software version 5.0 (La Jolla, CA, USA). For RT-qPCR, a one-tailed Student's t-test was used to determine the significance of the difference between the means ($p \leq 0.05$). The data were presented as mean values ± standard error.

5.4. RESULTS

5.4.1 Carvacrol shows concentration-dependent inhibition on biofilm formation of *S. pyogenes.*

The results showed that carvacrol inhibited biofilm formation by this bacterium in a concentration-dependent manner. At MBIC, percentage inhibition of more than 90% was observed (Figure 5.1B). MTT and CV assays showed similar results. All three strains were inhibited at 1 × MBIC, while the ATCC 49399 biofilm was most susceptible at ½ × MBIC. Other tested antibiotics showed concentration-dependent inhibition of biofilm formation, and their respective MBIC values are in Table 5.2.

5.4.2 Carvacrol eradicates performed mature biofilm of *S. pyogenes* in a concentration-dependent manner.

The effect of carvacrol in eradicating one-day-old, preformed biofilms by *S. pyogenes* is shown in Table 5.2 and Figure 5.1C. The results showed that carvacrol effectively eradicated preformed biofilms (percentage inhibition of 88-99%) at the carvacrol concentration of 250 µg/mL (Figure 5.1C, Table 5.2). In addition, we have previously reported MBEC for 3-day-old, preformed *S. pyogenes* biofilm by penicillin G 0.0016 µg/mL (Wijesundara and Rupasinghe, 2019a). In this study, we also found that four antibiotics tested eradicated mature biofilms at the range of MBICs of 0.016 – 0.25 µg/mL (Table 5.2).

5.4.3. Carvacrol decreases biofilm coverage and thickness and destroys the biofilm matrix.

SEM was employed to examine the effects of carvacrol on the structure of *S. pyogenes* biofilms treated with 1 × MBIC and ½ × MBIC of carvacrol compared to that treated

with penicillin G and the vehicle control (Figure 5.2). Biofilms treated with carvacrol had deformed and damaged cells and deteriorated extra-polymeric substances (EPS). Furthermore, very few intact biofilms or embedded bacterial cells were observed at 1 × MBIC of carvacrol, which agreed with that of the MBIC experiment above. Moreover, biofilms of the vehicle controls have intact spherical and regular-shaped cells, intact structure (EPS) surrounding bacteria, and a smooth surface. On the other hand, Penicillin G-treated cells were substantially damaged, leaving smaller cell debris.

In addition to SEM, the effect of carvacrol on biofilm was examined using CLFM. Figures 5.3 and 5.4 showed that carvacrol-treated biofilms reduced thickness and regions of low cell densities compared to the vehicle control. The results further confirmed that *S. pyogenes* did not form thick biofilm layers when treated with carvacrol at sub-MBIC. Moreover, carvacrol caused the death of most of the cells embedded in the mature biofilms of *S. pyogenes* and decreased cell viability compared to the vehicle control (Figure 5.4). Thus, during biofilm formation and the removal of mature biofilm processes, carvacrol may trigger the loss of intact cells and smoothness of biofilm surfaces, which leads to the disintegration of the formation of biofilm and the removal of matured biofilm.

At the ½ × MBIC of carvacrol, the biofilm layers were thoroughly disrupted, and at higher concentrations, the biofilm structures started to diminish. However, at ½ × MBIC and ½ × MBEC, there were a few detectable numbers of viable cells, suggesting that carvacrol could have a bacteriostatic effect during the formation of biofilm and eradication of mature biofilms. Furthermore, a lower concentration of carvacrol did not

cause a significant change in the thicknesses and viability of its mature biofilm, which is consistent with what was observed both by SEM imaging and the MTT reduction assay.

5.4.4. Effect of carvacrol on bacterial cell surface hydrophobicity

The effect of carvacrol on *S. pyogenes* adherence to hydrophobic substrates was performed using the MATH assay, and the results are reported as hydrophobicity index (Figure 5.5). At 125 µg/mL, carvacrol lowered the surface hydrophobicity by 84.2%, while at 62.5 µg/mL and 31.2 µg/mL, carvacrol treatment lowered it by 25.7% and 18.9%, respectively.

5.4.5. Gene expressions

The effect of carvacrol on the expression of several genes related to biofilm formation, quorum sensing, and virulence in *S. pyogenes* was assessed by RT-qPCR. The results showed that, at sub-MBIC levels, carvacrol increased the transcription of several genes but not the housekeeping gene, *gyrA* (Figure 5.6). Furthermore, s*peB*, *srtB, luxS, covS, dltA, ciaH*, and *hasA were* downregulated for both *S. pyogenes* strains at a sub-inhibitory concentration of carvacrol treatment. Besides that, the sub-inhibitory concentration of carvacrol-treated clinical strain showed a down-regulation of the *ropB* gene. Results suggest that carvacrol may affect the formation of biofilms by disturbing the production of EPS and down-regulate quorum sensing. The relative expression levels of the *covR* in bacteria treated with carvacrol at 62.5 µg/mL were higher in all three strains at 2.06 and 2.66 for ATCC 19615 and clinical isolate, respectively.

5.4.7. *In silico* studies of molecular docking on luxS protein with carvacrol

A molecular docking study of carvacrol was performed against the modeled structure of LuxS. Molecule binding free energy against LuxS was calculated and shown in Figure 5.7. The binding free energy that existed during the interaction between LuxS and

carvacrol was –4.33 kcal/mol, and hydrophobic interactions dominated the binding of carvacrol within the active site of LuxS. V17, S78, F80, and H87 were involved in carvacrol positioning within the binding pockets of LuxS.

5.5. DISCUSSION

S. pyogenes form biofilms as a part of their survival mechanisms to evade the host defence systems. Bacterial biofilm accumulates surface-associated microbial cells that are irreversibly attached to a substratum or interface or each other, enclosed in a self-produced EPS matrix, and exhibit an altered phenotype concerning growth rate and gene transcription (Vestby et al., 2020). This EPS matrix contains fibrin-like proteins, polysaccharides, and DNA (Vestby et al., 2020).

S. pyogenes biofilm plays a significant role in the pathogenesis of streptococcus pharyngitis (Vyas et al., 2019a). Therefore, its biofilm-forming ability has increased concern in identifying the disease, and it is difficult to treat compared to infections caused only by planktonic cells. Consequently, it is essential to obstruct the biofilm formation to prevent streptococcal pharyngitis (Vestby et al., 2020). The MTT assay results were compared with the bacterial viability staining images of SEM and CLFM. It is suggested that carvacrol significantly reduces the biofilm formation by S. *pyogenes* and disturbs the biofilm structure and individual cells.

Antibiofilm properties of several other natural compounds, such as psychorubrin (Lemos et al., 2018), naphthoquinones (Macé et al., 2017b), and fukugiside (Nandu et al., 2018) have been reported against *S. pyogenes* biofilms with similar mechanisms. For example, they have shown morphological damage to structures, involvement in the expression of virulence genes, enhancing extracellular cysteine protease production, and mitigating EPS and cell surface hydrophobicity.

Bacterial cells adhere to the host cell through solid hydrophobic interactions, and it is essential to interfere with this hydrophobic interaction to reduce bacterial adherence

and the infection cycle (Krasowska and Sigler, 2014). Our results showed that cells treated with carvacrol reduced cell surface hydrophobicity. Carvacrol is monohydric due to one hydroxyl group. The hydroxyl group of carvacrol is in the *ortho* position of the methyl group in the ring, thus enabling the hydroxyl (O.H) group to release quickly (Marchese et al., 2018). The presence and location of these O substituents are mainly responsible for carvacrol's chemical properties and biological activities (Marchese et al., 2018). The weak hydrophobicity of the bacterial cell surface possibly hinders the penetration of carvacrol into the EPS matrix of biofilms; hence carvacrol might not reach its target molecules.

Cell surface components, such as surface proteins and lipoteichoic acids, contributed to cell-surface hydrophobicity (Navarre and Schneewind, 1999). Hence, the possible explanation for this reduction in cell surface hydrophobicity by carvacrol (at its MBIC) could be due to the carvacrol-induced modification of the adhesin proteins on the cell surface. D-alanine incorporated lipoteichoic acid (LTA) is an amphiphilic polymer in the cell wall of *S. pyogenes,* which plays a vital role in the first step of biofilm formation (Fabretti et al., 2006). D-alanine-deficient LTA carries a negative charge. Therefore, D-alanine-deficient LTA triggers a higher repulsive force between the substratum and the bacterial cell surface, influencing the adhesion step of biofilm formation (Fabretti et al., 2006).

D-alanyl carrier protein ligase (DltA) is the enzyme that puts D-alanine on LTA. It has been reported that the *dltA* mutant *Staphylococcus aureus* failed to form biofilm on polar and non-polar surfaces (Gross et al., 2001). The findings are similar to the present study's hydrophobicity and RT-qPCR results (downregulation of *dltA*). Thus, suppression

of *dltA* may explain the reduced biofilm formation of *S. pyogenes* after carvacrol treatment.

The four main stages of the biofilm formation cycle are the initial attachment of bacteria to the cell surface, the formation of microcolonies, biofilm maturation, and planktonic bacteria dispersal (Vyas et al., 2019a). *S. pyogenes* cell surface proteins assist the initial adherence to the tonsil epithelial cell surface via hydrophobic interactions. The new findings suggest that carvacrol may reduce hydrophobic bond formation, thereby reducing the chances of bacterial adhesion. After attachment, the biofilm proliferates and produces EPS (Vyas et al., 2019a). Finally, a dispersal stage occurs, where some bacteria are released from the biofilm matrix to colonize new surfaces to start the cycle again (Vyas et al., 2019a). We found that carvacrol effectively reduces the expression of biofilm-associated virulence genes of quorum sensing (QS), hyaluronic acid capsule synthesis, class C sortase production involved in aggregation, etc.

Biofilm formation requires cell-cell signaling, called QS, which is essential for integrating bacteria into complex communities (Hawver et al., 2016; Jimenez and Federle, 2014). In addition, QS regulates the expression of several genes in response to population density (Cvitkovitch et al., 2003; Jimenez and Federle, 2014). The inter-communication of *S. pyogenes* uses oligopeptide signal molecules called autoinducers (AIs) to act via two-component phosphoryl cascades (Cvitkovitch et al., 2003).

LuxS enzyme is responsible for the last enzymatic step of autoinducer-2 (AI-2) synthesis, and this AI-2 has been proposed as a universal bacterial communication molecule in pathogenic bacteria (Jimenez and Federle, 2014; Sun et al., 2004). When the concentration of AIs (dihydroxy pentanedione, DPD) reaches a threshold, the QS is

stimulated, thereby regulating the transcription of target *luxS* (Hawver et al., 2016; Lyon et al., 2001). Therefore, the suppression of this *luxS* gene lowers bacterial communication, inhibiting associated virulence expressions during biofilm formation (Niazy, 2021; Zuberi et al., 2017).

LuxS protein, an essential protein that regulates pathogenic traits in *S. pyogenes,* which is responsible for the adhesion stage of biofilm development and regulates the QS system, significantly impacts virulence (Lyon et al., 2001). Downregulation of *luxS* may suppress biofilm formation through the QS mechanism based on AI-2 secretion. Similarly, the expression of *luxS* has been reduced by phloretin and citral, reducing biofilm development in *S. pyogenes* (Adil et al., 2019). Therefore, the downregulation of *luxS* gene expression by carvacrol in this study proposes that *luxS* of *S. pyogenes* may restrict the QS system (initial step of biofilm formation). Complementary to the gene expression, docking studies confirmed carvacrol interaction with LuxS protein, and results agree with previous studies of suppression QS mechanisms (Adil et al., 2019). However, further investigations are required to provide experimental evidence that carvacrol binds LuxS using surface plasmon resonance or isothermal titration calorimetry. In addition, advanced bioinformatics tools can be applied to further understand carvacrol's mechanisms of action.

S. pyogenes secretes several bacterial surface-associated virulence factors, including streptococcal pyrogenic exotoxin B (SpeB) (Loughman and Caparon, 2006). Furthermore, *SepB* is one of the most widely characterized Group A streptococcal virulence factors for its role in disease pathogenesis (Loughman and Caparon, 2006). Moreover, the global gene regulator, also known as a regulator of proteinase B (RopB),

forms an intracellular receptor and intercellular peptide signal pair with SpeB-inducing peptide (SIP), which controls *speB* expression (Do et al., 2019).

Among the analyzed genes in our study, *ropB* was found to be up-regulated in all three strains. Gene *ropB*, is a global transcriptional regulator controlling the expression of genes associated with metabolism, stress response, and virulence (Dmitriev et al., 2008). A previous study has shown that an intact RopB and efficient SpeB production are needed for systemic infection with *S. pyogenes* (Hollands et al., 2009). Although both strains show upregulation, ATCC 19615 *ropB* expression is considerably higher than the clinical strain may be due to differences in their virulence.

Expression of the *SpeB* cysteine protease in the growth phase of *S. pyogenes* has been reported previously as the function of *RopB* (Neely et al., 2003). Therefore, it was evident that down-regulation of *speB* expression in the present study upon carvacrol treatment.

The regulation of QS and biofilm adhesion mechanisms is not solely dependent on *ropB* in *S. pyogenes*. Instead, another critical regulator, CovR-CovS two-component regulatory system, also involves virulence gene expression (Treviño et al., 2009), and *ropB* may negatively influence the transcription of *covS/covR* (Friães et al., 2015b). Previous studies have revealed the influence of the sensor kinase *CovS* of the *CovRS* two-component system on the pathogenesis of *S. pyogenes* (Sugareva et al., 2010; Treviño et al., 2009). Furthermore, *CovS* is reported to be simultaneously activated and inhibit CovR-mediated repression (Treviño et al., 2009).

On the other hand, *CovS* could inactivate CovR and is required for growth under general stress conditions in *S. pyogenes* (Dalton and Scott, 2004). Furthermore, *CovR* has been

shown to act as a negative regulator of *hasA* by repressing the hyaluronic acid capsule, affecting its internalization strain. We observed the up-regulation of *CovR* and the down-regulation of *CovS*. Therefore, we suggest that may be a possible reason for the suppression of virulence genes and control of the initialization of biofilm formation upon carvacrol treatment.

Mode of action of carvacrol

Terpenes and their derivatives have been reported to have potent antimicrobial activity against pathogenic bacteria. Carvacrol, geraniol, linalool, linalyl acetrate, menthol, piperitone, and thymol are assessed for antibacterial activities. Although many terpenes' antibacterial mode of action remains largely unknown, several potential mechanisms were proposed for the modes of action of terpenes on antibiotic-resistant pathogens. For example, terpenes can be used in combination therapies because they can disrupt membranes, inhibit oxygen uptake, alter oxidative phosphorylation, reduce cell adherence ability, and suppress bacterial biofilm development (Álvarez-Martínez et al., 2021; Mahizan et al., 2019).

Phenol monoterpenes, including carvacrol, were reported to prevent or inhibit biofilm formation in other pathogenic bacteria such as *Staphylococcus aureus* and *Salmonella enterica* (Knowles et al., 2005), *Chromobacterium violaceum* (Burt et al., 2014), *S. mutans* (Ciandrini et al., 2014) and *Porphyromonas gingivalis* (Ciandrini et al., 2014).

The development of antimicrobial agents involves overcoming several limiting factors, such as cytotoxicity, side effects, bioavailability, absorption, distribution, metabolism, and excretion in the host body. We have previously assessed the cytotoxicity

of carvacrol using a tonsil epithelial cell model system (Wijesundara et al., 2021) and found that carvacrol selectively kills *S. pyogenes* compared to the host cells. In contrast to most synthetic drugs, higher doses (up to 125 μg/mL) of most phytochemicals are not cytotoxic to human oral epithelial cells (Wijesundara et al., 2021).

The lipophilic nature of carvacrol facilitates easy and quick absorption upon ingestion. Therefore, an appropriate delivery system must be identified to prevent them from being absorbed before reaching the target site of infection. Orally administrated carvacrol and thymol have been almost absorbed in the stomach, the proximal small intestine in rats (Austgulen et al., 1987) and piglets (Michiels et al., 2010), with maximum plasma concentration within 1.3 hr and a significant amount is eliminated through urine in rats within 24 hr of administration (Austgulen et al., 1987). Carvacrol was excreted unchanged, or glucuronide and sulphate conjugates by esterifying. However, its derivatives of benzyl alcohol and 2-phenyl propanol and corresponding carboxylic acids have been detected (Suntres et al., 2015b).

Carvacrol is also classified among the Generally Recognized as Safe (GRAS) substances by the US Food and Drug Administration (FDA) and approved by the Council of Europe and the FDA for use in food. On the other hand, carvacrol and many other natural monoterpenes are available at low prices. Therefore, carvacrol could be used in developing pharmaceutical or natural health products (lozenges, throat vapors, or herbal tea) to manage streptococcal pharyngitis.

The combination therapy containing an antibiotic and carvacrol or its derivative may be an alternative to inhibit biofilm or eradicate biofilm of an antibiotic-resist strain of *S. pyogenes*. However, the application of carvacrol as an antibiotic in the clinical phase

is yet to be explored. Clinical trials regarding terpenes were reported; however, if carvacrol is being used solely or as a combination therapy, further studies, including proper clinical trials, should be performed to employ the best of its functional potential. In addition, inventions can be explored to study the appropriateness of advanced delivery technologies, such as carvacrol-loaded nanoparticles and structural modifications of carvacrol for better antibacterial activities, greater efficiency, and fewer side effects.

5.6. CONCLUSIONS

Failure to treat biofilm-associated bacterial infections has promoted an interest in discovering alternative therapeutic agents. Here, we have demonstrated the ability of carvacrol to inhibit biofilm formation and eradicate the preformed biofilm of *S. pyogenes*. This study concludes that sub-inhibitory (62.5 µg/mL) or higher concentrations of carvacrol can effectively inhibit biofilm formation and development and eradicate preformed biofilms of *S. pyogenes*. Furthermore, carvacrol causes morphological alteration to the formed biofilm cells, loses biofilm integrity, and influences cell surface hydrophobicity and biofilm formation-related gene expression regulation. The basis of the structural and functional properties of LuxS protein involved in the quorum-sensing has been shown. However, we recommend conducting molecular docking studies against respective proteins from other down-regulated genes. The present investigation suggests that carvacrol has the potential to be applied as a safe alternative antibiofilm agent for *S. pyogenes* strains, including antibiotic-resistant strains.

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Table 5.1 List of genes and the primers used for the qRT-PCR to examine their expression concerning *Streptococcus pyogenes* virulence and biofilm formation.

Gene	Function (reference)	Primer sequence (5′-3′)	
		Forward	**Reverse**
covS	Regulates biofilm and virulence, sensor kinase gene of covRS (activates and inhibits the CovR-mediated repression) (Trevino et al., 2009).	GAGTGAGCGCGATATCACAA	GCAAGCCAGGAGATGATTCT
covR	Repressor gene of covRS two-component system and regulates stress (Friães et al., 2015a).	TGCGCGTGATTCTATTATGG	GGCGGAAAATAGCACGAATA
ropB	Global transcriptional regulator (Friães et al., 2015a).	TGATATGGATACGGCAAAACA	TTGACCAAGGCAAAAAGGTT
speB	Extracellular cysteine protease production (von Pawel-Rammingen and Björck, 2003).	CTAGGATACTCTACCAGCG	CAGTAGCAACACATCCTG
luxS	Involved in quorum sensing and biofilm formation (Lyon et al., 2001).	CTTTTGGCTGTCGAACAGGT	TCCAGGAACATCTTCCCAAG
hasA	Hyaluronic acid capsule synthesis (Crater and van de Rijn, 1995).	AGCGTGCTGCTCAATCATTA	AACATCGATCATCCCCAATG
dltA	D-alanylation of Lipoteichoic acid (Cox et al., 2009).	GCATTTGGACATCGACTCCT	GTTTTCGAGCCGTAGAAACG
srtB	Class C sortase production involved aggregation (Liang et al., 2016).	GCTGGTTTTGGTTTGTGGGA	CCCCGGGATATTTAACCAACC
ciaH	Acid and oxidative stress responses response (Tatsuno et al., 2014).	GGCGGTCTTACAGAATCGTC	CATGTTGCGAACCTCGTCTA
gyrA	Gyrase production (housekeeping gene in the present study)	CAACGCACGTAAGGAAGAAA	CGCTTGTCAAAACGACGTTA

Table 5.2 Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) (μg/mL) of carvacrol against *Streptococcus pyogenes.*

Test compounds	Strain of *Streptococcus pyogenes*							
	ATCC 19615		ATCC 49399		Clinical isolate		*Spy* 1558	
	MBIC[a]	MBEC[b]	MBIC[a]	MBEC[b]	MBIC[a]	MBEC[b]	MBIC[a]	MBEC[b]
Carvacrol	125	250	125	250	125	250	125	250
Penicillin G	0.008	0.016	0.008	0.016	0.008	0.016	0.008	0.016
Penicillin Vk	0.008	0.016	0.008	0.016	0.008	0.016	0.008	0.016
Clindamycin	0.031	0.0625	0.031	0.0625	0.031	0.0625	0.25	0.5
Erythromycin	0.125	0.25	0.125	0.25	0.125	0.25	15.625	31.25

[a]MBIC was determined as the lowest concentration of the test compound that inhibited biofilm formation.

[b]MBEC was identified as the lowest concentration of the test compound that eradicates already formed biofilm.

Data are representative of three independent experiments, each conducted in triplicate.

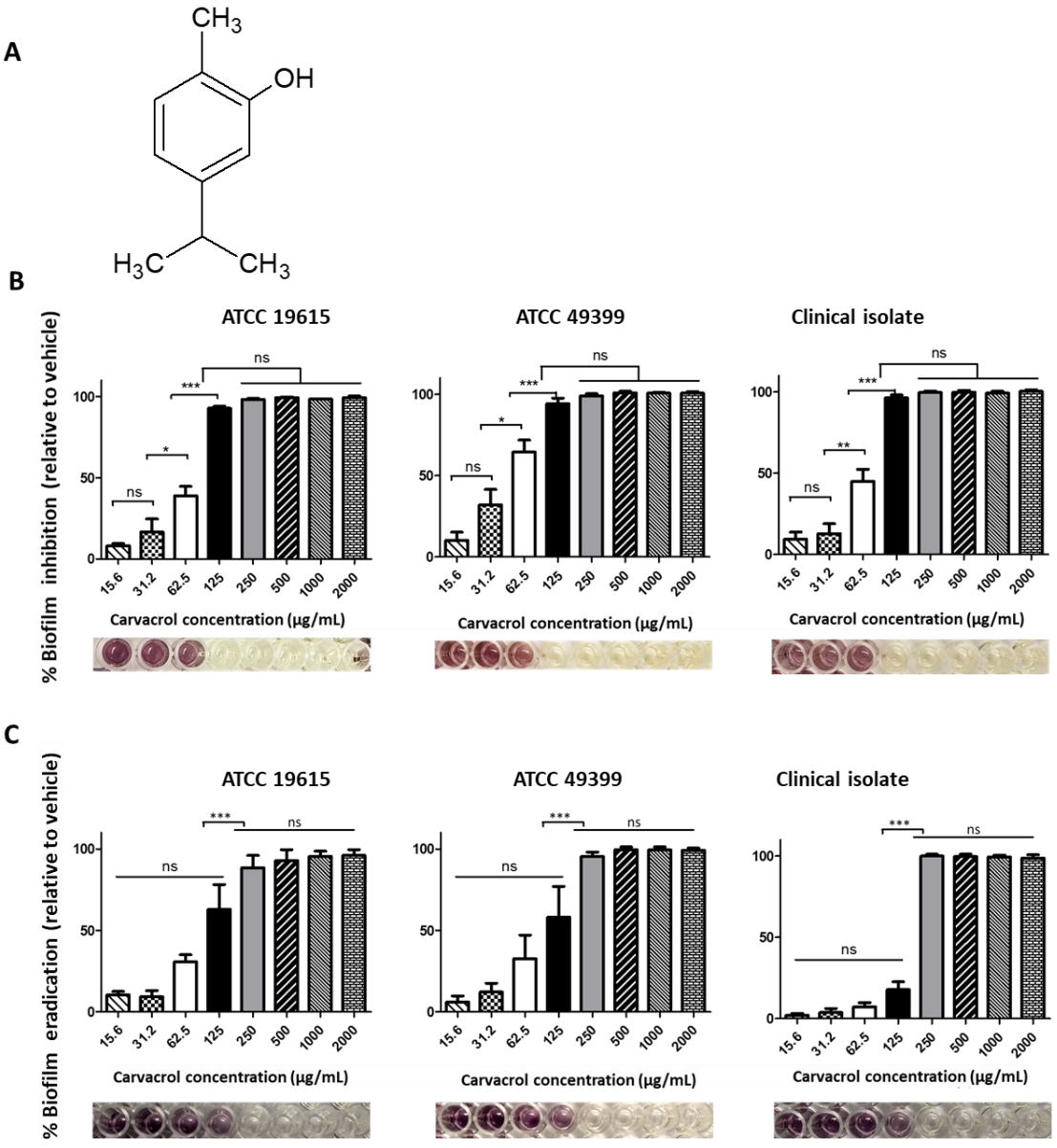
A
CH3
OH
H3C
CH3
B
ATCC 19615
ATCC 49399
Clinical isolate
% Biofilm inhibition (relative to vehicle)
Carvacrol concentration (µg/mL)
15.6
31.2
62.5
125
250
500
1000
2000
ns
*
**
***
C
% Biofilm eradication (relative to vehicle)

Figure 5.5 Carvacrol inhibits biofilm formation and eradicates the preformed biofilm of *Streptococcus pyogenes* in a concentration-dependent manner.
(**A**) The chemical structure of carvacrol (5-isopropyl-2-methylphenol). (**B**) Quantitative analysis of inhibition of *S. pyogenes* biofilm by carvacrol using MTT assay. Graphs indicate the percentage of biofilm formation in the presence of carvacrol relative to biofilm in DMSO. The bottom panels are representative biofilms stained with MTT. (**C**) Percentage eradication of preformed *S. pyogenes* biofilm by carvacrol relative to the eradication of preformed biofilm of DMSO vehicle control. Inserted panels below the graphs are representative biofilms stained with MTT. Results are means ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

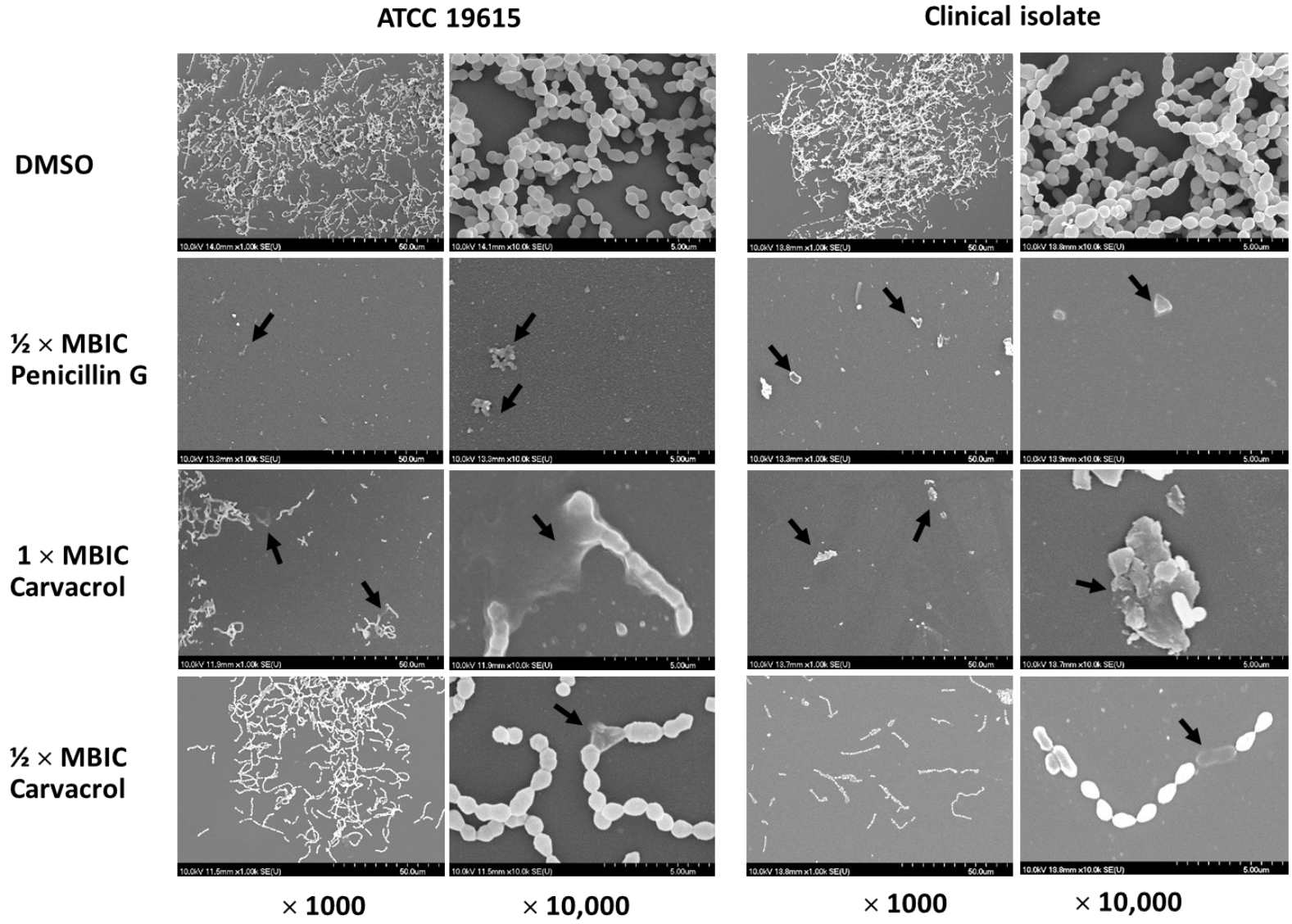
ATCC 19615
Clinical isolate
DMSO
½ × MBIC Penicillin G
1 × MBIC Carvacrol
½ × MBIC Carvacrol
× 1000
× 10,000
× 1000
× 10,000

Figure 5.6 Scan electron micrographs of *Streptococcus pyogenes* biofilms formed on the 96-well polystyrene microplates.

Biofilms were grown in the presence of carvacrol, penicillin G, or DMSO (vehicle control) for 78 hr. The black arrows indicate the damaged bacterial cells and altered biofilm structures. Micrographs were captured at 1,000, and 10,000 magnifications.

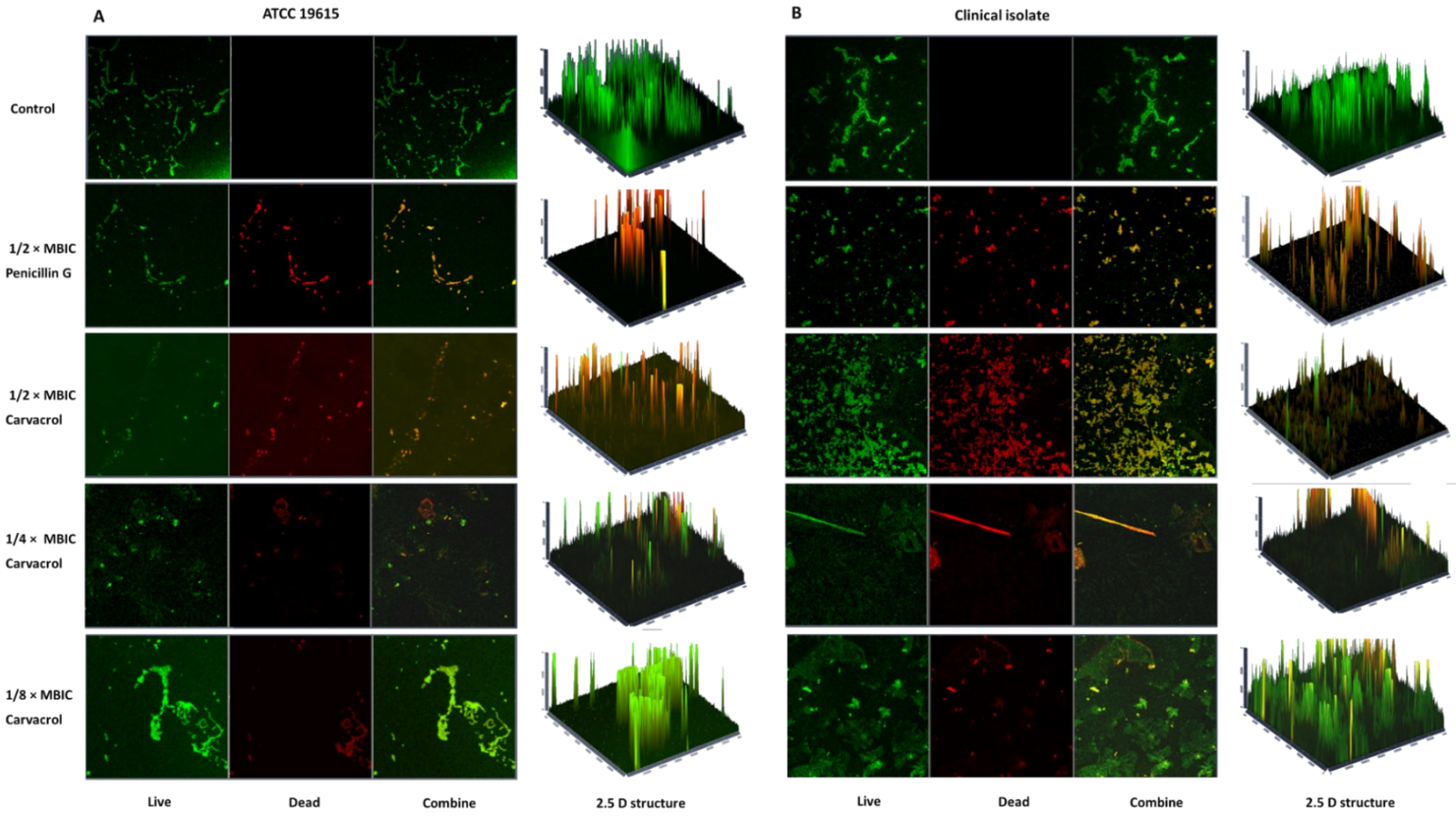
A
ATCC 19615
B
Clinical isolate
Control
1/2 × MBIC
Penicillin G
1/2 × MBIC
Carvacrol
1/4 × MBIC
Carvacrol
1/8 × MBIC
Carvacrol
Live
Dead
Combine
2.5 D structure
Live
Dead
Combine
2.5 D structure

Figure 5.7 Confocal laser scanning microscopy of *Streptococcus pyogenes* biofilms. *S. pyogenes* biofilms were grown in the presence of DMSO (vehicle control), penicillin G (positive control) or carvacrol and stained with the LIVE/DEAD BackLight Viability Kit. Live and dead cells were stained with SYTO 9 (green) and propidium iodide (red/yellow). The third and fourth panels show representative overlapping of live and dead images, and the 2.5-dimensional structural organization of the biofilms is shown in the third and fourth panels. The images are representative of three independent experiments.

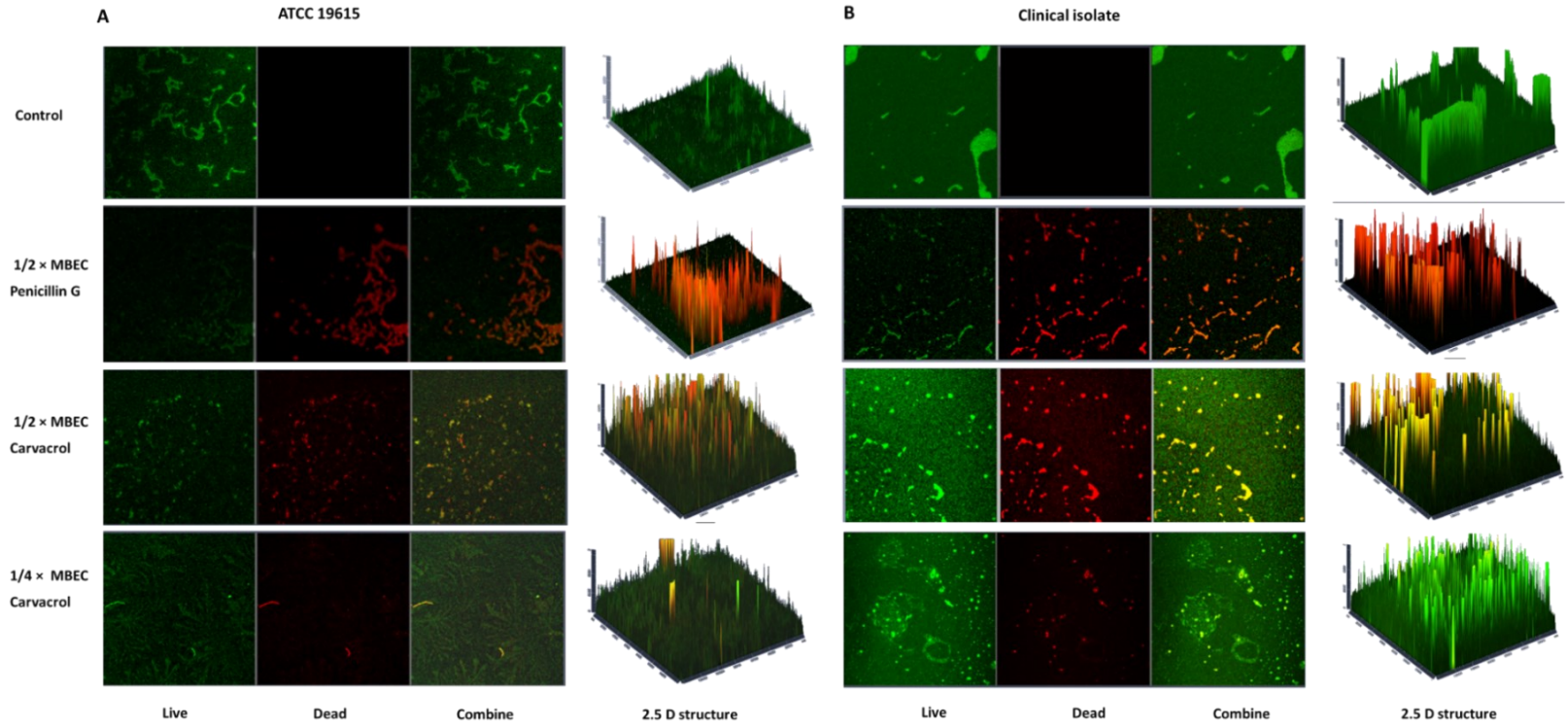
A
ATCC 19615
B
Clinical isolate
Control
1/2 × MBEC
Penicillin G
1/2 × MBEC
Carvacrol
1/4 × MBEC
Carvacrol
Live
Dead
Combine
2.5 D structure
Live
Dead
Combine
2.5 D structure

Figure 5.8 Confocal laser scanning microscopy of preformed *Streptococcus pyogenes* biofilms eradicates.

Confocal laser scanning microscopy images of three days of *S. pyogenes* biofilms treated with DMSO (vehicle control), 1/2 × MBIC of penicillin G (positive control) and upon treated with carvacrol at 1/2 × MBIC (62.5 μg/mL), 1/4 × MBIC (31.25 μg/mL) and 1/8 × MBIC (15.63 μg/mL). All the biofilms were stained using the LIVE/DEAD BackLight Viability Kit. Live and dead cells were stained with SYTO 9 (green) and propidium iodide (red/yellow). The third and fourth panels show representative overlapping of live and dead images, and the 2.5-dimensional structural organization of the biofilms is shown in the third and fourth panels. The images are representative of three independent experiments.

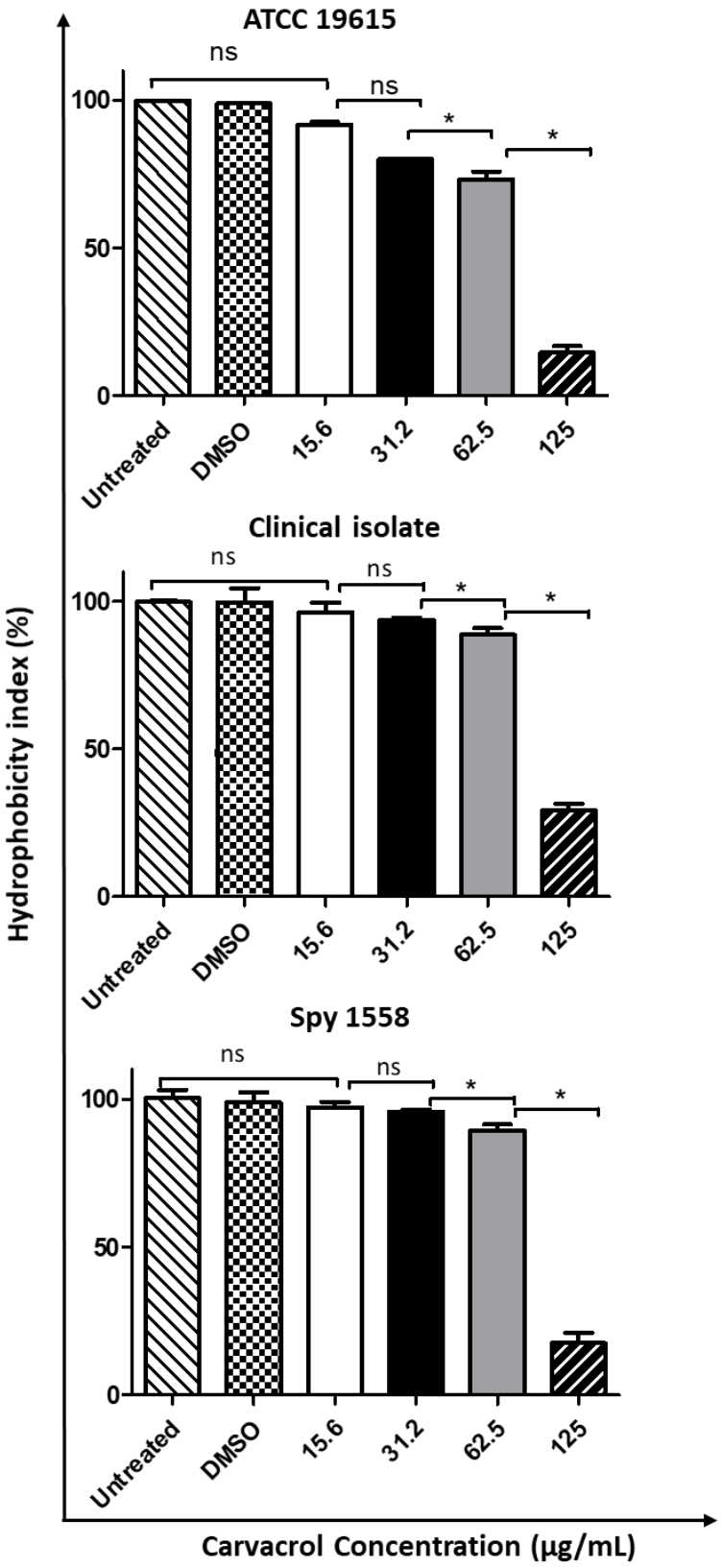
ATCC 19615
ns
ns
*
*
100
50
0
Untreated
DMSO
15.6
31.2
62.5
125
Clinical isolate
ns
ns
*
*
100
50
0
Untreated
DMSO
15.6
31.2
62.5
125
Spy 1558
ns
ns
*
*
100
50
0
Untreated
DMSO
15.6
31.2
62.5
125
Hydrophobicity index (%)
Carvacrol Concentration (µg/mL)

Figure 5.9 Effect of carvacrol on *Streptococcus pyogenes* cell surface hydrophobicity. *S. pyogenes* were grown in carvacrol, DMSO (diluent control), or no treatment (untreated, bacteria control), standardized to an OD_{600} = 0.02, and tested for cell surface hydrophobicity. The experiments were carried out in triplicate. *$P < 0.05$, and data presented as means ± SEM.

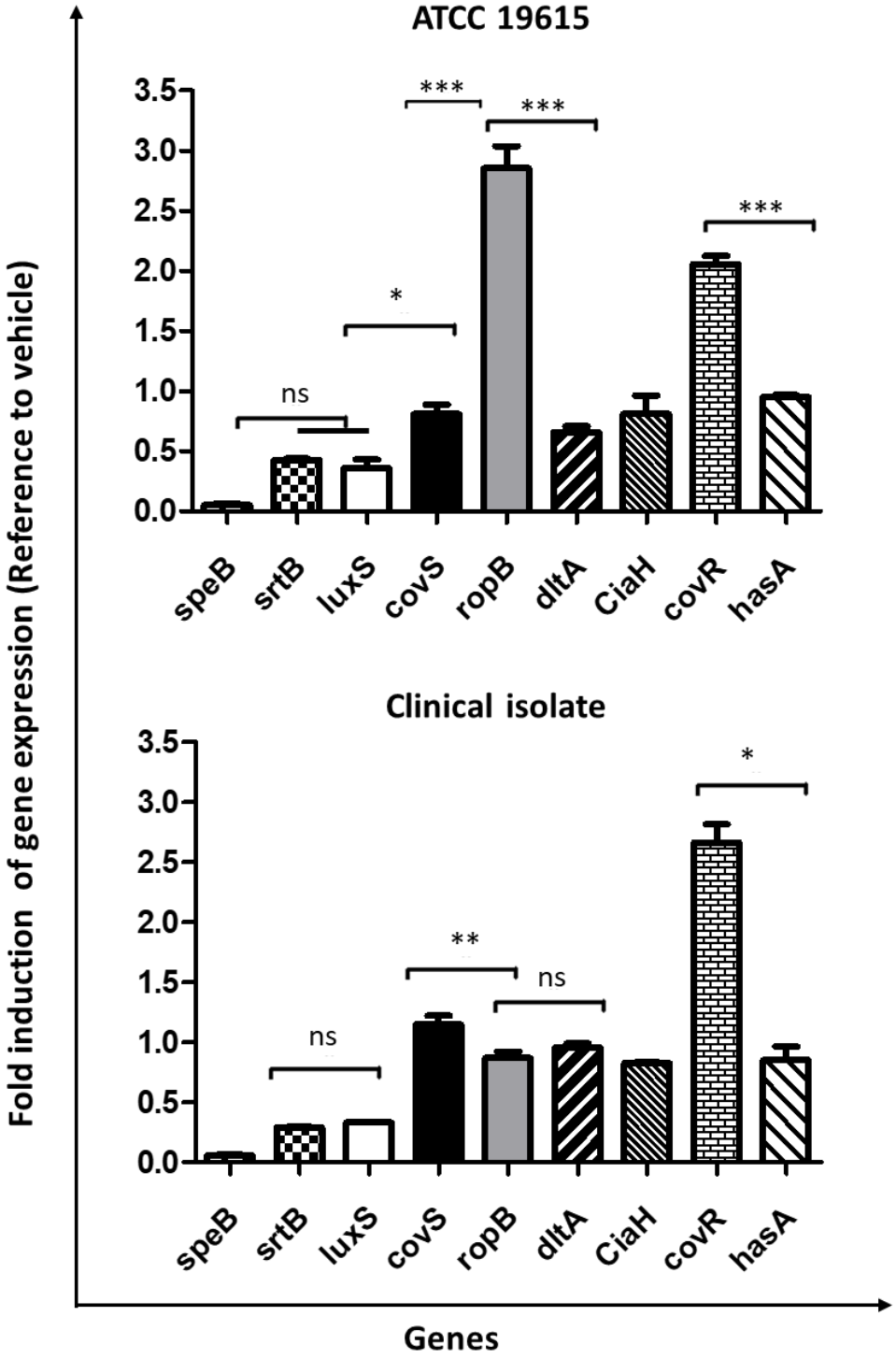
ATCC 19615
Clinical isolate
Fold induction of gene expression (Reference to vehicle)
Genes
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
***
*
ns
***
**
ns
*
speB
srtB
luxS
covS
ropB
dltA
CiaH
covR
hasA

Figure 5.10 The expression profile of candidate genes is associated with biofilm formation and virulence factors of *Streptococcus pyogenes* in the presence of carvacrol.

Two different *S. pyogenes* strains were grown under similar conditions with 62.5 µg/mL of carvacrol. The group without a test compound is considered a control. Values represent mean fold change expression differences compared to untreated control cells. Three independent experiments present data as mean ± standard deviation (SEM). *P < 0.05, and data presented as means ± SEM.

A

Enzyme	Compound	Binding free energy (Kcal/mol)	Residues Involved	
			Hydrogen Bond	Hydrophobic Interaction
LuxS	Carvacrol	-4.33	G81	V17, S78, F80, H87

B

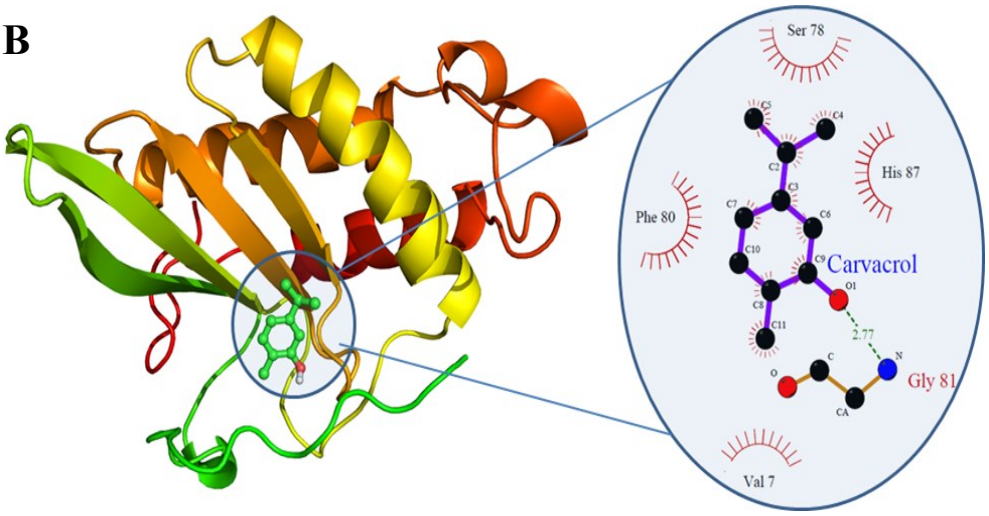

Figure 6.11 ***In-silico*** **study of carvacrol binding against LuxS.**

(A) The binding properties from an *in-silico s*tudy of carvacrol binding against LuxS protein. **(B)** The binding of carvacrol within the active site of LuxS and the important binding site residues involved in the orientation of carvacrol within the active site of LuxS.

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CHAPTER 6. CARVACROL SUPPRESSES INFLAMMATORY BIOMARKERS PRODUCTION BY LIPOTEICHOIC ACID- AND PEPTIDOGLYCAN-STIMULATED HUMAN TONSIL EPITHELIAL CELLS

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6.1. ABSTRACT

Pharyngitis is an inflammation of the pharynx caused by viral, bacterial, or non-infectious factors. In the present study, the anti-inflammatory efficacy of carvacrol was assessed using an *in vitro* model of streptococcal pharyngitis using human tonsil epithelial cells (HTonEpiCs) induced with *Streptococcus pyogenes* cell wall antigens. HTonEpiCs were stimulated by a mixture of lipoteichoic acid (LTA) and peptidoglycan (PGN) for 4 hr, followed by exposure to carvacrol for 20 hr. Following exposure, interleukin (IL)-6, IL-8, human beta defensin-2 (HBD-2), epithelial-derived neutrophil-activating protein-78 (ENA-78), granulocyte chemotactic protein-2 (GCP-2), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), and prostaglandin (PGE_2) were measured by enzyme-linked immunosorbent assays (ELISA). The levels of pro-inflammatory cytokines IL-6, IL-8, ENA-78, and GCP-2 were decreased in a carvacrol dose-dependent manner. The production of HBD-2 was significantly suppressed over 24 hr carvacrol treatments. PGE_2 and COX-2 levels in the cell suspensions were affected by carvacrol treatment. TNF-α was not detected. The cell viability of all the tested carvacrol concentrations was greater than 80%, with no morphological changes. The results suggest that carvacrol has anti-inflammatory properties, and carvacrol needs to be further assessed for potential clinical or healthcare applications to manage the pain associated with streptococcal pharyngitis.

Keywords: anti-inflammatory; carvacrol; streptococcal pharyngitis; cytokines; LTA; PGN; inflammation; *Streptococcus pyogenes*

6.2. INTRODUCTION

Inflammation is a natural defense mechanism of the body designed to eliminate harmful stimuli, including exogenous pathogens, and initiate the healing process through various chemical mediators and signaling pathways (Chen et al., 2017). Pharyngitis is an inflammatory process of the posterior oropharynx (Wolford et al., 2020). Pharyngitis is one of the most common upper respiratory tract syndromes (URT) infections. Although pharyngitis is usually self-limiting and a minor health concern, it is one of the leading causes of physician visits worldwide.

Bacterial pharyngitis is primarily caused by *Streptococcus pyogenes,* also known as group A Streptococcus (GAS). In North America, adults account for 5–15% of cases of streptococcal pharyngitis, while children account for 15–35% of the cases (Mustafa and Ghaffari, 2020). *S. pyogenes* first colonizes the tonsil epithelial cell*s*, and these cells stimulate an innate immune response (Soderholm et al., 2018). The tonsillar epithelial cells recognize *S. pyogenes* cell wall antigens through Toll-like receptors (TLR), such as TLR-2. These receptors identify pathogen-associated molecular patterns, including lipoteichoic acid (LTA) and peptidoglycan (PGN) (Lange et al., 2009; Mogensen, 2009). Upon TLR recognition, *tonsil epithelial cells produce* pro-inflammatory cytokines, chemokines, prostaglandins, and tumor necrosis factor-alpha (TNF-α) (Lange et al., 2009; Ricciotti and FitzGerald, 2011a). Several inflammatory and infectious complications are associated with Streptococcal pharyngitis, such as tonsillar hypertrophy, scarlet fever, and peritonsillar abscesses. In addition, post-infectious sequelae, such as acute rheumatic fever and post-streptococcal glomerulonephritis, have also been described.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, aspirin, ketorolac, diclofenac, and nimesulide have been widely recommended to manage acute inflammatory conditions (Moilanen and Vuolteenaho, 2019), including streptococcal pharyngitis. It was found that cyclooxygenase (COX)-1 and -2, enzymes responsible for the synthesis of pro-inflammatory prostaglandins E_2 (PGE_2), are targets of NSAID (Ricciotti and FitzGerald, 2011a).

The pharmaceutical and nutraceutical industry has a growing interest in novel sources of antimicrobial and anti-inflammatory agents. Bioactive compounds from medicinal plants have been explored as a potential source for developing novel autoimmune-modulating, analgesic, and anti-inflammatory products. For example, we have previously shown that the ethanol and aqueous extracts of several herbal plants possess anti-inflammatory activities, decreasing the production of tonsil cell-associated inflammatory mediators, including cytokines, such as interleukin (IL)-8, human beta defensin-2 (HBD-2), granulocyte chemotactic protein-2 (GCP-2), and epithelial-derived neutrophil-activating protein-78 (ENA-78) (Wijesundara et al., 2017a).

Carvacrol (5-isopropyl-2-methyl phenol (Figure 6.1), is a small monoterpene phenolic compound naturally found in the essential oils rich in the plants of the Lamiaceae family, including *Oregano* L., *Thymus* L., and *Salvia* L. (Pirintsos et al., 2020; Wijesundara and Rupasinghe, 2018b). Several studies have reported anti-inflammatory (Ezz-Eldin et al., 2020b; Somensi et al., 2019; Xiao et al., 2018) and analgesic (Ezz-Eldin et al., 2020b) properties of carvacrol using different infectious inflammatory cell/animal

models. Furthermore, the potential mechanisms of carvacrol were reported as inhibiting the synthesis of *TNF-α,* PGE_2, and nitric oxide (Arigesavan and Sudhandiran, 2015; Lima et al., 2013; Somensi et al., 2019) and decreasing the levels of cytokines and COX-2 (Lima et al., 2013; Xiao et al., 2018). However, the effect of carvacrol on *S. pyogenes* antigen-induced tonsil epithelial cells and its inflammatory mediator production has not been reported. Therefore, the present study investigated the impact of carvacrol on cytokine production by the tonsil epithelial cells (HTonEpiCs) following LTA and PGN stimulation.

6.3. MATERIALS AND METHODS

6.3.1. Chemicals and reagents

Carvacrol (≥98%, food-grade), Dulbecco's phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), lipoteichoic acid (LTA), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), and phenazine methosulfate (PMS) were obtained from Sigma-Aldrich (Oakville, ON, Canada). Peptidoglycan (PGN) was obtained from Cedarlane Laboratories (Burlington, ON, Canada). Human tonsil epithelial cells (HTonEpiCs), tonsil epithelial cell medium, tonsil epithelial cell growth supplement, poly-L-lysine (PLL), trypsin neutralization solution (TNS), and trypsin-ethylenediamine tetra acetic acid (0.25%) solution (TE) were purchased from ScienCell Research Laboratory (San Diego, CA, USA). Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). 7-Aminoactinomycin D (7-AAD) stain was purchased from BioLegend, Inc. (San Diego, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from different manufacturers: IL-6 and TNF-α ELISA kits were purchased

from BD Biosciences (Mississauga, ON, Canada); human ENA-78, GCP-2, and human COX-2 ELISA kits were purchased from RayBiotech, Inc. (Norcross, GA, USA); a human BD-2 ELISA kit was purchased from PromoCell GmbH (Sickingenstraße, Heidelberg, Germany); and PGE_2 and IL-8 kits were purchased from Invitrogen (Nepean, ON, Canada).

6.3.2. HTonEpiC cell culturing and maintaining

The HTonEpiCs were cultured and maintained according to the manufacturer's guidelines. Briefly, HTonEpiCs were cultured in a PLL-coated flask (2 μg/cm^2 T-75 flask) with a complete growth medium (CGM) and incubated at 37 °C in a 5% CO_2 humidified atmosphere. CGM was a mixture of growth supplement, penicillin/streptomycin solution, and tonsil epithelial cell medium [1:1:100 (*v*/*v*/*v*)]. Cells (approximately after 3–4 days of growth) were used for the *in vitro* assays and consistently showed a 95–100% viability, as determined by trypan blue staining. Cells were recovered from the flask using TE solution and then neutralized with TNS and washed with PBS prior to use.

6.3.3. Cell viability assay

6.3.3.1. Spectrophotometric MTS cell viability assay

Cell viability was measured using a standard MTS assay. Briefly, HTonEpiC cells were seeded at 10,000 cells/well in PLL-coated 96-well plates and incubated for 24 hr. The cells were washed with PBS and fresh CGM (100 μL/well) containing each treatment, carvacrol (4, 8, 15.6, 31.2, 62.5, 125, and 250 μg/mL), nimesulide (a positive control at 4, 8, 16 μg/mL), and LTA + PGA (5 μg/mL, each), or DMSO (a negative control, 0.05%, *v*/*v*), were added. Then, the plates were incubated for 24 hr (5% CO_2 at 37 °C). After 24

hr of incubation, the media were discarded, and fresh CGM was added (100 µL/well). Then, 10 µL of MTS reagent (MTS: PMS (20:1)) was added to each well, and the plates were incubated at 37 °C for 2.5 hr. The absorbances were measured at 490 nm using a microplate reader (Tecan Infinite™ M200 PRO, Tecan US Inc., Morrisville, NC, USA). The percentage of cell viability is calculated as (absorbance of treated well/absorbance of control well) × 100.

6.3.3.2. Flow cytometric cell viability using the 7-AAD assay

The HTonEpiCs were seeded at 200,000 cells/well in PLL-coated six-well plates at 37 °C. The next day, media was discarded, cells were washed with PBS, and CGM (1 mL/well) was added. Then, cells were incubated in the absence or presence of each treatment as carvacrol (8–250 µg/mL), nimesulide (4, 8, 15.6 µg/mL), LTA + PGN mixture (5 µg/mL, each), and DMSO (0.05%, *v*/*v*) for 24 hr. Afterwards, 5 µL of lysis buffer (9% Triton X-100) (positive control) was treated for 5 min before cell separation. Then, the cell monolayer was washed with PBS and detached by incubation with TE solution (1 mL/well) for 3 min. The cell suspension was neutralized with TNS (1.5–2 mL/well), and the cells were harvested by centrifugation (1000 × *g*, 5 min). The cells were resuspended in PBS (300 µL/pellet) and incubated with 5 µL of 7-AAD (1 mg/mL) at room temperature (RT) for 5 min. Effects of carvacrol on cell viability (proliferation) were analyzed by flow cytometry using an Attune™ NxT acoustic focusing flow cytometer (AFC2, Thermo Fisher Scientific Inc., San Jose, CA, USA) and Attune™ NxT flow cytometry analysis software (v3.1.1234.0., Thermo-Fisher Scientific Inc., San Jose, CA, USA).

6.3.4. Treatment of carvacrol and controls: HTonEpiCs inflammatory cell model

The LTA- and PGN-stimulated tonsil epithelial cell inflammatory model system used in the study is shown in Figure 6.2. HTonEpiCs were cultured (density of 35,000 cells/well) in PLL-coated 24-well plates for 24 hr. The cells were stimulated with a mixture of LTA and PGN (each at 10 µg/mL) at 37 °C. After 4 hr, the cells were exposed to carvacrol (4–125 µg/mL) and nimesulide (4 µg/mL) for 20 hr. DMSO (0.05%, *v*/*v*) was included as the experimental control. After incubation, the cultures were centrifuged (1000 × *g*, 10 min), and the supernatants were saved at −80 °C to determine pro-inflammatory biomarkers. The concentrations of pro-inflammatory cytokines were measured using ELISA kits according to the manufacturer's instructions.

6.3.5. Pro-inflammatory biomarker ELISA

The concentrations of pro-inflammatory cytokines were measured using ELISA kits according to the manufacturer's instructions.

6.3.5.1. IL-6 assay

According to the manufacturer's instructions, the protein concentration of IL-6 was measured by the ELISA kit supplied by BD Biosciences (Mississauga, ON, Canada). Briefly, 96-well Nunc-Immuno™ polystyrene Maxisorp ELISA flat-bottom plates (Thermo-Fisher Scientific Inc., Nepean, ON, Canada) were coated with anti-human IL-6 monoclonal antibody (100 µL/well) and incubated overnight at 4 °C. Uncoated spaces were blocked using assay diluent (1 hr, RT), and wells were washed. Then, 100 µL of each standard (4.7–200 pg/mL), samples (2–250 µg/mL concentrations of carvacrol-treated cell supernatants), and controls were pipetted into wells. Plates were sealed and incubated for 2 hr at RT with gentle shaking. Then, five well-washing steps were

conducted, and 100 µL of the working detector (detection antibody + streptavidin-hoarse reddish peroxidase (HRP)) was added to each well. The covered plates were incubated for 1 hr at RT. Following another seven washing steps, 100 µL/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate solution was added and incubated for 30 min at room temperature in the dark (without the sealer). Then, the stop solution was added (50 µL/well), and the absorbance readings were obtained at 450 nm. The concentration of IL-6 levels was calculated using a standard curve and expressed as a percentage of IL-6 concentration on the 24 hr model.

6.3.5.2. IL-8 assay

The levels of IL-8 were determined by using the IL-8 ELISA kit according to the manufacturer's guidelines. Briefly, 50 µL of each 2-fold diluted standard (0–1000 pg/mL), carvacrol (2–250 µg/mL), LTA + PGN (4 and 24 hr), nimesulide (4, 8, and 16 µg/mL), and DMSO control were added, and 50 µL of human IL-8 biotin conjugate solution was also added. The plates were incubated for 1.5 hr at RT with gentle shaking. After the supernatant was discarded, the plates were washed (4 times) after discarding the supernatant. Followed by four washing steps, streptavidin–HRP solution (100 µL per well) was added to each well except for the chromogen blanks, which were incubated (30 min, RT) with gentle shaking. The wells were thoroughly aspirated and washed four times, and subsequently, 100 µL of TMB substrate reagent was added to each well and incubated for 30 min at RT with gentle shaking in the dark. Then, 100 µL stop solution per well was added to stop the reaction. The solution in the well was changed from blue to yellow, which was measured at an absorbance of 450 nm. The IL-8 concentration was

calculated using a standard curve, and the data were expressed as percentage of ENA-78 production.

6.3.5.3. GCP-2 assay

The assay was performed according to the manufacturer's instructions. In the assay, 100 µL of each standard (0, 2, 500 pg/mL), carvacrol (2 – 250 µg/mL), LTA + PGN controls (4 and 24 hr), nimesulide (4 – 16 µg/mL) and vehicle control were pipetted into the appropriate wells. Plates were incubated for 2.5 hr at RT with gentle shaking. Then, the solution was discarded and washed with a wash buffer. Then, a 100 µL of biotinylated antibody was added to each well and incubated for 1 hr at room temperature with gentle shaking. Another washing step followed this, and streptavidin solution (100 µL/well) was added. After incubation (45 min, RT) with gentle shaking, the wells were washed, and a 100 µL of TMB One-Step Substrate Reagent (TMB) was added to each well. The planes were covered and incubated for 30 min at RT in the dark. The absorbance at 450 nm was immediately measured after stopping the reaction by adding 50 µL of the stop solution. The protein concentration was calculated using a standard curve, and the data were expressed as a percentage of GCP-2 concentration.

6.3.5.4. ENA-78 assay

The human ENA-78 ELISA kit was used to measure the protein production of ENA-78 according to the instructions provided by the manufacturer. The 96-well plates coated with anti-human ENA-78 were used, and the assay procedure was similar to the description in the GCP-2 assay in section 6.5.5.3.

6.3.5.5. TNF-α assay

The TNF-α secretion in the cell culture supernatant was measured according to the manufacturer's guidelines. Briefly, a 50 µL of ELISA diluent was pipetted into each well, and then, 100 µL of six standards (7.8 – 250 pg/mL), samples, and controls were added into the wells. The plates were sealed and incubated for 2 hr at room temperature with gentle shaking. After the wells were aspirated and washed (3 times), 100 µL of the working detector (enzyme concentrate: detection antibody = 1:250) was added. Then, the plates were incubated (1 hr, RT), aspirated, and washed (7 times). Then, 100 µL of substrate reagent, TMB, was added and further incubated for 30 min at RT in the dark. After that, 50 µL of the stop solution was added to each well, and the absorbance was measured at 450 nm. The TNF-α concentrations were calculated using a standard curve and expressed as pg/mL.

6.3.5.6. HBD-2 assay

The concentration of HBD-2 was measured according to the guidelines provided by the HBD-2 ELISA kit. Briefly, anti-HBD-2 antibody-coated 96-well plates (Nunc-Immuno™ polystyrene Maxisorp, Thermo-Fisher Scientific Inc., Nepean ON, Canada) were prepared using capture antibody (0.5 µg/mL) incubation and then block buffer incubation according to the manufacturer's guidelines. First, 100 µL of standards (0 – 2 ng/mL), carvacrol, or controls were added to each well and allowed to incubate for 2 hr (RT). Next, the wells were washed four times with washing buffer and incubated for another 2 hr (RT) with the detection antibody (0.5 µg/mL). After aspiration and washing, 100 µL of the avidin–HRP conjugate was added to each well and incubated for 30 min (RT). Then,

2, 2′-azino-bis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) solution was added to the washed and dried wells. After the development of color, the absorbance of the wells was read at 405 nm. A standard curve was used to calculate the HBD-2 concentrations.

6.3.5.7. PGE_2 assay

The PGE_2 levels in the cell supernatants were measured using the human PGE_2 ELISA kit according to the protocols and plate plan provided. Briefly, 100 µL of each diluted standard (0 – 4 ng/mL) and samples were added to the appropriate wells. Then, 50 µL of PGE_2-alkaline phosphate (PGE2-ACE) tracer and 50 µL of PGE_2-monoclonal antibody was added to the wells as described in the assay protocol provided by the manufacturer. The blanks, the total activity, the non-specific binding, and the maximum binding wells were maintained. The plate was covered and incubated for 2 hr at RT with orbital shaking. The wells were thoroughly aspirated and were washed (5 times) with wash buffer. Then, 200 µL of pNPP solution (5 nNPP tablets in 25 mL diethanolamine [DEA] buffer) was added to the wells, and the covered plates were incubated for 1 hr at RT in the dark. Then, the absorbance was measured between 405 and 420 nm. The PGE_2 concentration in each sample was calculated using the standard curves and equations provided in the kit protocol.

6.3.5.8. COX-2 assay

The COX-2 protein concentration of in cell culture supernatant was measured using the human COX-2 ELISA kit (Ray Biotech, Inc., Norcross, GA, USA) as described in the manufacturer's assay protocol. Briefly, the standards (0 – 300 ng/mL), carvacrol (2–250 µg/mL), and controls were added into the appropriate wells. The plate was sealed and incubated for 2.5 hr at RT. The wells were emptied and washed using the wash buffer

seven times. Then, 100 µL of biotin antibody solution was pipetted into each well and incubated for another 1 hr at RT. The wells were aspirated, washed (7 times), and 100 µL of streptavidin solution was added. After an incubation period of 45 min at RT, the wells were rewashed four times, and 100 µL of TMB substrate was added to each well. The reaction was facilitated for 30 min incubation at RT in the dark and was stopped by adding 100 µL of stop solution. The absorbance was read at 450 nm immediately, and the concentrations of COX-2 concentration were calculated using a standard curve and expressed in ng/mL.

6.3.6. Morphology assessment of cells in the inflammatory model

Cell morphology was examined under an inverted microscope (ECLIPSE TS 100/TS 100-F, Nikon Instruments Inc., Melville, NY, USA) with 400 X magnification. The images were captured using a Lumenara infinity camera (1-2 USB, 2.9 Megapixel) and processed using NIS elements software (v4.0, Lumenara Corporation, Ottawa, ON, Canada).

6.3.7. Statistical analysis

The experiments were designed using a completely randomized design. All the experiments were conducted in triplicates (experimental triplicates) and repeated three independent times on different days and using different cell passages for three biological replicates. Results were expressed as a mean ± standard error of the mean. Statistical analysis and designs of the graphs/figures were performed using Graph-prism™ software (v5.0, Informer Technologies, Inc., Los Angeles, CA, USA). One-way analysis of variance was used to determine the significant differences between means of pairs, which were resolved by Tukey's tests at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

6.4 RESULTS

6.4.1. Effect of carvacrol on cell viability and morphological changes of human tonsil epithelial cells.

We have recently demonstrated that carvacrol is not cytotoxic (3.9–250 μg/mL) to HTonEpiCs using the MTS assay. To further assess whether carvacrol has a harmful effect on HTonEpiCs, the cells were incubated with carvacrol (8–250 μg/mL) or controls (DMSO as vehicle control, nimesulide as positive control), and the cell viability was analyzed by flow cytometry. The results showed that carvacrol at the tested concentrations was not cytotoxic (>80% cell viability) to HTonEpiCs by flow cytometric cell viability assay (Figure 6.1 B). A set of representative histograms, scatter plots of the controls, and carvacrol-treated samples are shown in Figure 6.1. C and 6.6. S7. In addition, cells treated with carvacrol did not show significant morphological changes compared to those treated with DMSO (Figure 6.1 D). In addition, nimesulide, a known anti-inflammatory agent, showed no cytotoxicity to the cells at all the concentrations tested. Therefore, the concentrations of 15.6, 31.2, 62.5, 125, and 250 μg/mL carvacrol (in DMSO) and 4 μg/mL of nimesulide were used in the subsequent inflammation experimentation.

6.4.2. Effect of carvacrol on morphological changes of HTonEpiCs inflammation model.

No cell morphology or density changes were observed in untreated and vehicle (DMSO) controls-treated HTonEpiCs. However, after the 4 hr incubation with LTA + PGN, some cells in the population showed morphological changes such as irregular shape, reduced

size, and reduced cell density (Figure 6.2). The morphological changes were evident after 24 hr incubation with LTA + PGN.

6.4.3. Inhibitory effects of carvacrol on the secretion of pro-inflammatory cytokines in LTA + PGN stimulated HTonEpiCs

HTonEpiCs secreted pro-inflammatory cytokines, IL-6, IL-8, TNF-α, ENA-78, and GCP-2, when stimulated with LTA + PGN. In the absence of carvacrol, IL-6 and IL-8 at the 4 hr (baseline level) stimulation were 24 and 35 pg/mL, and at the 24th hr were 106 and 78 pg/mL, respectively. However, the levels of IL-6, IL-8, ENA-78, and GCP-2 were significantly decreased when the cells were treated with carvacrol in a concentration-dependent manner (Figure 6.3). It was observed that there was a decrease of IL-6 and IL-8 to 55% and 42% ($p < 0.0001$), respectively, in this cell model with the presence of 125 µg/mL carvacrol. Interestingly, at higher concentrations of carvacrol (125 and 62.5 µg/mL), the suppression of pro-inflammatory cytokines is similar to that of nimesulide (4 µg/mL) ($p < 0.05$).

Significant concentration-dependent suppression of GCP-2 (also known as chemokine ligand 6, CXCL6) production by carvacrol compared to the antigen control was observed (Figure 6.3 C). Carvacrol at 62.5 and 125 µg/mL showed a 63.2% and 51.1%, respectively, reduction in GCP-2 production compared to the control, which is a level similar to 4 µg/mL of nimesulide. In addition, the production of ENA-78 was suppressed by 3.36-fold when the cells were treated with 125 µg/mL of carvacrol compared to the 24 hr-model, whereas nimesulide (4 µg/mL) showed only a 2.8-fold reduction in the ENA-78 (Figure 6. 5D). However, the analysis indicated that carvacrol did not affect the secretion of TNF-α.

6.4.4. Inhibitory effects of carvacrol on the secretion of HBD-2, COX-2, and PGE_2.

The effects of carvacrol on modulating the antimicrobial peptide HBD-2 production by HTonEpics were assessed. A decrease in the production of HBD-2 in a carvacrol-concentration-dependent manner was observed compared to the 24 hr-model control (Figure 6.4). Furthermore, the anti-bacterial minimum inhibitory concentration (MIC) of carvacrol (125 μg/mL) reduced the release of HBD-2 by over 50%, while the positive control of nimesulide (4 μg/mL) also showed a similar reduction.

Next, we investigated the effects of carvacrol on COX-2 and PGE_2 productions by LTA- and PGN-stimulated HTonEpiCs. After a 24 hr incubation with LTA and PGN, the cells produced an increased level of COX-2 and PGE_2 compared to the control ($p < 0.05$) (Figure 6.4). Interestingly, when LTA- and PGN-stimulated cells were incubated with carvacrol, significantly decreased levels of COX-2 (at and above 31.5 μg/mL carvacrol) and PGE_2 (at and above 8 μg/mL carvacrol) were observed. The effects of carvacrol on COX-2 and PGE_2 production were concentration-dependent.

6.5. DISCUSSION

The use of phytochemicals, herbal extracts, or herbal formulas in traditional medicine has been reported to manage inflammatory diseases, including those associated with the respiratory tract (Hostanska et al., 2011; Wijesundara et al., 2017b). The anti-inflammatory activities of carvacrol have been demonstrated in various cells, such as RAW 264.7 macrophages (Lima Mda et al., 2013; Silva et al., 2012; Somensi et al., 2019), and animal models, such as paw inflammation in mice (Lima M et al., 2013) and colitis-associated colon cancer of male Fischer 344 rats (Arigesavan and Sudhandiran, 2015). Anti-inflammatory activities of carvacrol were reported, along with the suppression of pro-inflammatory cytokines. The suppression of interleukin IL-8 and HBD-2 secretion in LPS-stimulated airway epithelial A549 cells by an herbal formulation against respiratory infections was reported (Hostanska et al., 2011). However, the anti-inflammatory activity of carvacrol in the upper respiratory tract, mainly when the inflammation results from an *S. pyogenes* infection, has not been reported.

This study used a mixture of LTA and PGN, two Gram-positive bacterial antigens, to stimulate HTonEpiCs, representing an *in vitro* model of streptococcal pharyngitis. Tonsil inflammation is initiated by complex processes that are triggered by streptococcal cell wall antigens such as LTA and PGN. When bacteria colonize the tonsil, various innate immunity-associated cells, including pharyngeal and tonsillar epithelial cells, neutrophils, and macrophages (White, 1999a), begin to secrete inflammatory mediators, such as cytokines and chemokines, antimicrobial peptides (AMPs), and eicosanoids, including PGE_2 (Soderholm et al., 2018). After exposure to *S. pyogenes* or its cell wall components, the epithelial cells and macrophages act as the first line of

defense (Soderholm et al., 2018). Other inflammatory cells migrate to the site of inflammation at a later stage.

S. pyogenes adheres to tonsillar epithelial cells during infection, triggering these cells to release cytokines (Figure 6.5). Our results demonstrated that tonsil epithelial cells produced a spectrum of inflammatory biomarkers upon stimulation by LTA and PGN. Furthermore, we showed that carvacrol could suppress the production of pro-inflammatory cytokines and theoretically reduce inflammation. Our results are in agreement with previous studies demonstrating the anti-inflammatory activity of carvacrol, its derivatives, or carvacrol-rich plant extracts that inhibit the production of pro-inflammatory cytokines and inflammatory mediators such as COX-2, PGE2, nitric oxide (NO), and inducible nitric oxide synthase (iNOS) during respiratory tract inflammations (Bonfim et al., 2014; Ezz-Eldin et al., 2020b; Landa et al., 2009; Wijesundara et al., 2017a).

The significance of Gram-positive bacteria in the induction of IL-6 and IL-8 release was studied previously, and dose-dependent IL-6 and IL-8 release were demonstrated from both macrophages and respiratory epithelial cells after stimulation with bacteria (Larsson et al., 1999). IL-6 is primarily a pro-inflammatory cytokine-mediated by trans-signaling during an infection**;** it is also responsible for many immunoregulatory and anti-inflammatory activities mediated by classical signaling (Scheller et al., 2011). In addition, IL-8 is a chemotactic agent (chemokine), facilitating the migration and recruitment of neutrophils and T-cells and priming eosinophils (Eckmann et al., 1993). A recent meta-study reported a positive effect of carvacrol on the reduction of IL-1β, IL-4, and IL-8; however, there was no effect on IL-6 and TNF-α,

which was probably due to the methodological quality and heterogeneity of the studies (de Carvalho et al., 2020a). A recent study found that lipopolysaccharide (LPS) stimulated J774.1 mouse macrophages expressed the genes of IL-1β and TNF-α and carvacrol and thymol could significantly reduce the production of both IL-1β and TNF-α at the protein and mRNA levels (Gholijani et al., 2016). However, we did not detect TNF-α in our experiments, and it could be because HTonEpiCs are not specialized to produce TNF-α, or the antigen concentration used was not high enough to generate detectable levels of TNF-α.

The host's innate immune system provides the first line of defense against bacterial infection. Host cells recognize pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs), such as TLRs (Sardari et al., 2013). Among these TLRs, TLR2 recognizes LTA and PGN, whereas TLR4 recognizes LPS (Sardari et al., 2013). Respiratory tract epithelial cells express antimicrobial peptides such as HBDs to inhibit bacterial proliferation during infections, and they are important to innate host defenses (Travis et al., 2001; Yanagi et al., 2007). HBD-2 is upregulated in epithelial cells and mononuclear phagocytes in response to bacterial infection and associated pro-inflammatory cytokine release (Tsutsumi-Ishii and Nagaoka, 2002). The upregulation of HBD-2 by cell wall components such as LTA and PGN in Gram-positive bacteria (Harder et al., 1997) and LPS in Gram-negative bacteria (Tsutsumi-Ishii and Nagaoka, 2002) has been reported. The present study confirmed that an antigen mixture of LTA and PGN induces HTonEpiCs and increases HBD-2 production. Interestingly, a concentration-dependent reduction of HBD-2 production was observed in carvacrol-treated cells.

Our results further showed the suppression of PGE_2 and COX-2 production, which are also significant mediators of inflammation. PGE_2 plays a vital role in the inflammatory processes of pharyngitis and is synthesized from arachidonic acid through the COX- 1 and COX-2-involved biosynthesis of eicosanoids (Ricciotti and FitzGerald, 2011a). Therefore, blocking or inhibiting the COX-2 enzyme could potentially decrease PGE_2 production and thus reduce pharyngeal inflammation. COX-2 inhibitors suppress PGE_2 production, which ultimately reduces inflammation (Ricciotti and FitzGerald, 2011a). Several studies have proposed the role of PGE_2 during streptococcal pharyngitis (Ricciotti and FitzGerald, 2011a). Landa et al. suggested that the effect of carvacrol is mediated by inhibiting COX-2, which decreases PGE_2 production (2009).

Many of these inflammatory markers act interconnectedly during inflammation (Figure 6.5). It was reported that PGE_2 and COX-2 increase IL-8 expression in human pulmonary epithelial cells during inflammation (Peng et al., 2008). Our study demonstrated that carvacrol suppressed PGE_2, COX-2, and IL-8 production. Furthermore, neutrophil chemotactic chemokines such as GCP-2 and ENA-78 may play a role in IL-8 production. According to Frick et al. (2008), the cells that produce IL-8 have also affected the secretion of ENA-78 and GCP-2. Our results also showed that IL-8 has a similar secretion pattern to ENA-78 and GCP-2 by the tonsil epithelial cells after antigen stimulation. There are several categories of chemokines based on the position of conserved cysteine residues where GCP-2, ENA-78, and IL-8 are considered C-X-C chemokines (CXC) containing one amino acid between the two NH_2-terminal cysteine residues (Zlotnik and Yoshie, 2000). GCP-2 (CXCL6) is more structurally related to ENA-78 (CXCL); however, it functionally utilizes both of the IL-8 (CXCL8) receptors to

chemoattract neutrophils (Wuyts et al., 2003). The relationship between LPS-induced GCP-2 and IL-8 production in fibroblasts was studied (Wuyts et al., 2003), and our current study showed the interconnected production of GCP-2, ENA-78, and IL-8 in LTA- and PGN-stimulated HTonEpiCs. Carvacrol treatment suppressed the production of all three of the cytokines mentioned above, suggesting the potential of carvacrol in inflammation reduction.

The previously reported *in vitro* and *in vivo* studies showed that carvacrol exerted anti-inflammatory effects at one or multiple cellular targets. It has been suggested that the interaction of transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and pro-inflammatory mediators such as IL-6, TNF-α, and COX-2 during inflammation is a major concern (Guimarães et al., 2012). Furthermore, it has been reported that carvacrol blocks the NF-κB nuclear translocation and transcriptional activation in LPS-induced pro-inflammatory activation in RAW 264.7 macrophages (Somensi et al., 2019). Interestingly, a few other recent studies have also reported that carvacrol and carvacrol-rich plant extracts ameliorate the suppression of NF-κB signaling pathway and thereby downregulate the pro-inflammatory genes such as iNOS, IL-6, IL- 1β, COX-2, and TNF-α under various inflammatory conditions (Ezz-Eldin et al., 2020b; Gholijani et al., 2016; Guimarães et al., 2012). Therefore, we suggest that the anti-inflammatory activity of carvacrol on HTonEpiCs could also be due to the suppression of NF-κB signaling pathways. Further studies are required to assess the precise mode of action of carvacrol against HTonEpiCs inflammation induced by Gram-positive bacterial antigens.

Our findings indicate that carvacrol-containing lozenges or throat sprays for pharyngitis patients can be developed. Here, we provide evidence that carvacrol also possesses anti-inflammatory properties. More importantly, carvacrol is not cytotoxic to human epithelial cells and has been approved by the Food and Drug Administration (FDA) for use in foods (Code for Federal Regulation: 21CFR172.515). Therefore, carvacrol could be an excellent candidate for an oral throat pain-relieving natural health product.

6.6. CONCLUSION

In conclusion, the present study indicates that carvacrol suppresses the production of pro-inflammatory mediators such as IL-6, IL-8, HBD-2, GCP-2, ENA-78, PGE_2, and COX-2, suggesting that it has anti-inflammatory properties. Therefore, further investigations can be continued to develop carvacrol as a natural health additive for inclusion in products such as lozenges or throat sprays for pain management associated with streptococcal pharyngitis.

6.7. ACKNOWLEDGMENTS

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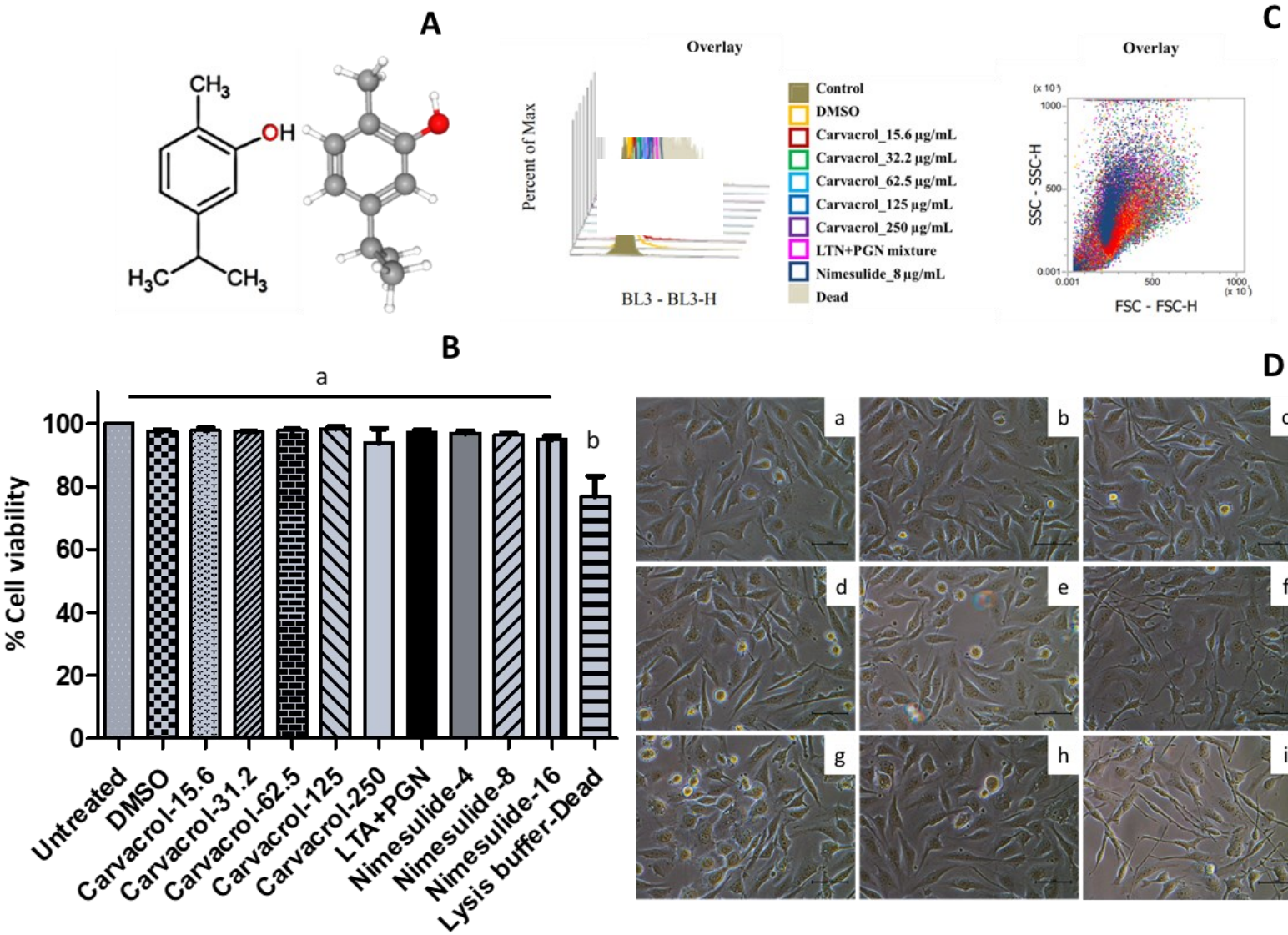
A
CH3
OH
H3C
CH3
Overlay
Percent of Max
BL3 - BL3-H
Control
DMSO
Carvacrol_15.6 µg/mL
Carvacrol_32.2 µg/mL
Carvacrol_62.5 µg/mL
Carvacrol_125 µg/mL
Carvacrol_250 µg/mL
LTN+PGN mixture
Nimesulide_8 µg/mL
Dead
C
Overlay
SSC - SSC-H
FSC - FSC-H
B
a
b
% Cell viability
100
80
60
40
20
0
Untreated
DMSO
Carvacrol-15.6
Carvacrol-31.2
Carvacrol-62.5
Carvacrol-125
Carvacrol-250
LTA+PGN
Nimesulide-4
Nimesulide-8
Nimesulide-16
Lysis buffer-Dead
D
a
b
c
d
e
f
g
h
i

Figure 6.1 The effect carvacrol on the viability of human tonsil epithelium cells. (A) chemical structure of carvacrol. (B) Cell viability changes with the 15.6, 31.2, 62.5, 125, and 250 µg/mL of carvacrol in DMSO, 4, 8, and 15.6 µg/mL of nimesulide, LTA + PGN (10 µg/mL each) and 0.05% DMSO for 24 hr was determined with 7-AAD staining followed by flow cytometry (FCM) analysis in human tonsil epithelium cells (HTonEpiCs). Absorbance was measured at 488 nm. Cell viability (%) was calculated relative to the control of 0.05% DMSO. Values are shown as mean ± SE from three independent experiments, each in triplicate. The different letters above the columns show that the means of different groups were significantly different ($p < 0.05$) by one-way analysis of variance using Tukey's test. (C) The overlay of histograms and scatter plots of the controls and samples given by FCM analysis. (D) The morphological changes of the HTonEpiCs cells after the treatments were examined under an inverted microscope at 10 × 40 magnification. Representative photographs that were taken 24 hr after the treatment in three independent experiments are presented. (a) untreated; (b) vehicle control (0.25% DMSO) and (c) 15.6 µg/mL carvacrol, (d) 32 µg/mL carvacrol, (e) 62.5 µg/mL carvacrol, (f) 125 µg/mL carvacrol, (g) 250 µg/mL carvacrol, (h) LTA + PGN (10 µg/mL, each) and (i) 8 µg/mL Nimesulide. Scale bar = 1 mm. 7-AAD: 7-amino-actinomycin D; DMSO: Dimethyl Sulfoxide; LTA: Lipoteichoic acid; and PGN: Peptidoglycan.

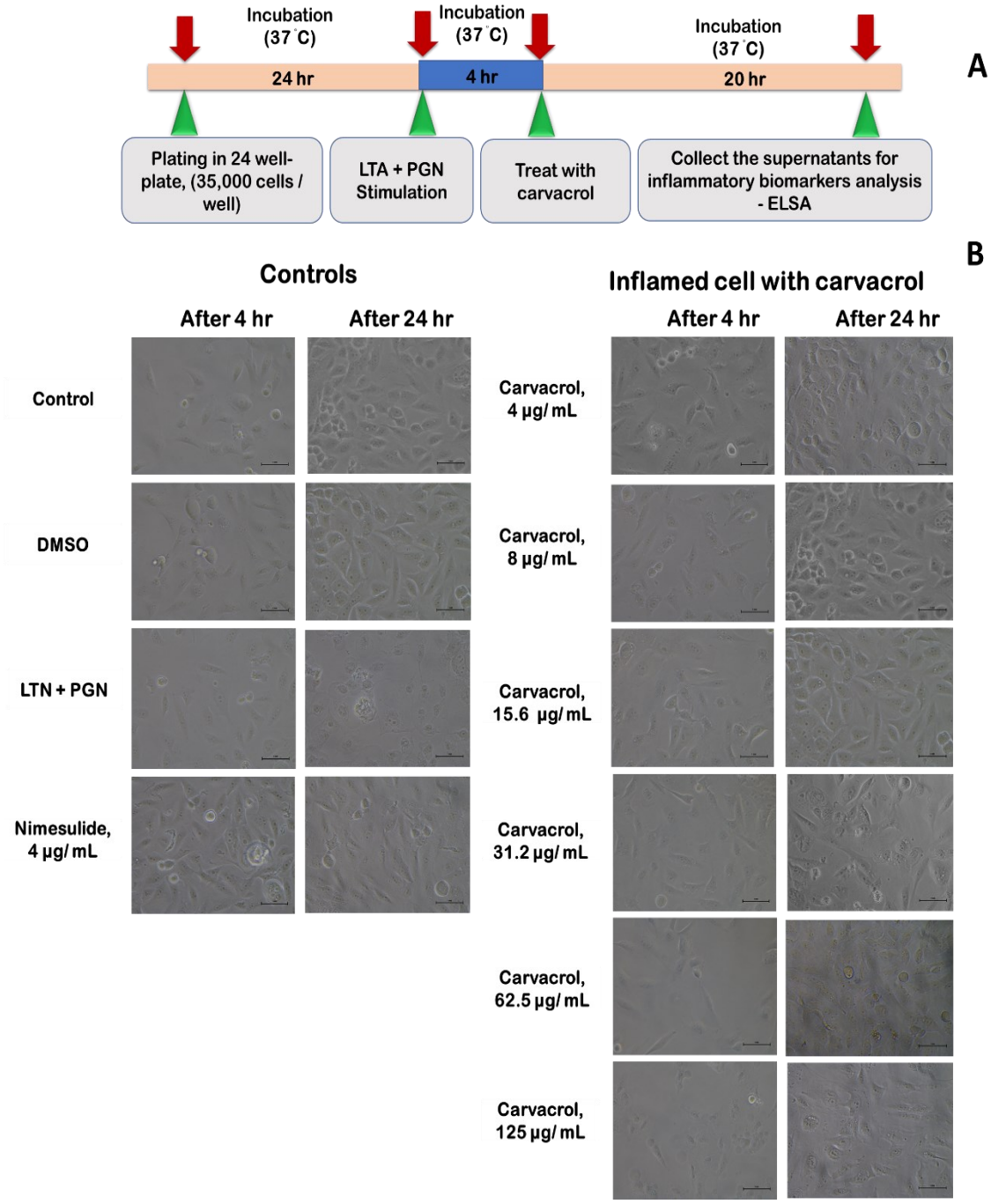
A
Incubation (37 °C)
Incubation (37 °C)
Incubation (37 °C)
24 hr
4 hr
20 hr
Plating in 24 well-plate, (35,000 cells / well)
LTA + PGN Stimulation
Treat with carvacrol
Collect the supernatants for inflammatory biomarkers analysis - ELSA
B
Controls
Inflamed cell with carvacrol
After 4 hr
After 24 hr
After 4 hr
After 24 hr
Control
DMSO
LTN + PGN
Nimesulide, 4 µg/ mL
Carvacrol, 4 µg/ mL
Carvacrol, 8 µg/ mL
Carvacrol, 15.6 µg/ mL
Carvacrol, 31.2 µg/ mL
Carvacrol, 62.5 µg/ mL
Carvacrol, 125 µg/ mL

Figure 6.2 Inflammatory cell model system and morphological changes of cells after carvacrol treatment.

(A) The LTA- and PGN-stimulated tonsil epithelial cell inflammatory model system used in the study. (B) Morphology of human tonsil epithelial cells treated with carvacrol. Controls include cells of untreated growth medium control, DMSO control, LTN + PGN control, nimesulide (4 μg/mL). LTN+PGN-induced cells (4 hr) were treated with carvacrol (4–125 μg/mL) and incubated for an additional 20 hr. All images were obtained at a magnification of 10 × 40: scale bar, 1 μm. LTA: Lipoteichoic acid; PGN: Peptidoglycan; ELISA: Enzyme-linked immunosorbent assay.

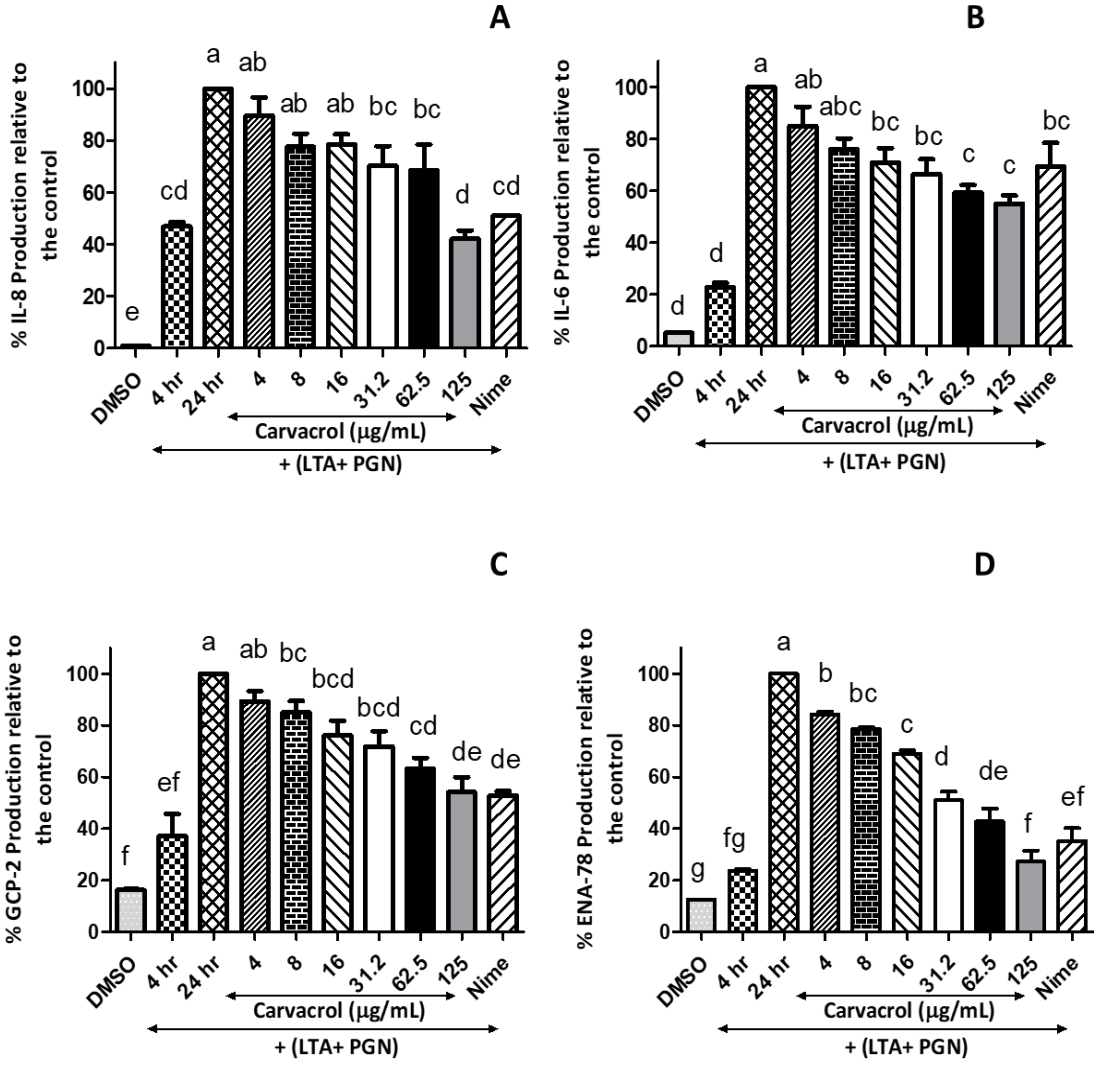
A
% IL-8 Production relative to the control
e
cd
a
ab
ab
ab
bc
bc
d
cd
DMSO
4 hr
24 hr
4
8
16
31.2
62.5
125
Nime
Carvacrol (µg/mL)
+ (LTA+ PGN)
B
% IL-6 Production relative to the control
d
d
a
ab
abc
bc
bc
c
c
bc
C
% GCP-2 Production relative to the control
f
ef
a
ab
bc
bcd
bcd
cd
de
de
D
% ENA-78 Production relative to the control
g
fg
a
b
bc
c
d
de
f
ef

Figure 6.3 Inhibitory effects of carvacrol on the production of pro-inflammatory cytokines by LTA + PGN-stimulated human tonsil epithelial cells.

Tonsil cells were treated with carvacrol (4 - 125 μg/mL) for 20 hr after 4 hr stimulation with LTA + PGN mixture (5 μg/mL, each). Supernatants were harvested 20 hr after stimulation. Concentrations of (A) IL-8, (B) IL-6, (C) CGP-2, and (D) ENA-78 in the culture supernatants were determined by ELISA. Results are shown as the mean ± SE, and the experiment was conducted in triplicate. Data are representative of three independent experiments. The different letters above the columns show that different groups' means were significantly different ($p < 0.05$) by one-way analysis of variance. IL-8: interleukin-8; IL-6: interleukin-6; ENA-78: epithelial-derived neutrophil-activating protein-78; GCP-2: granulocyte chemotactic protein-2; LTA: lipoteichoic acid; and PGN: peptidoglycan.

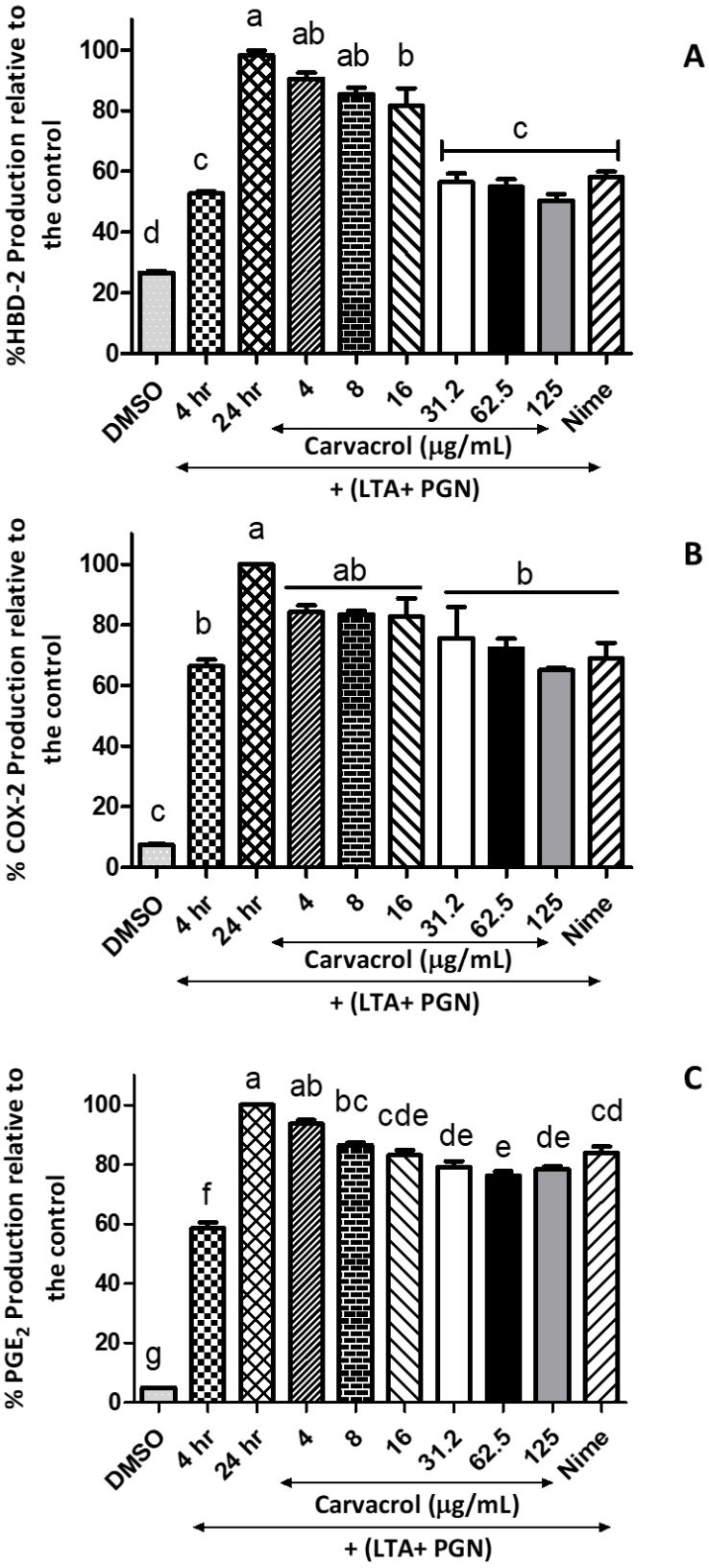
A
%HBD-2 Production relative to the control
100
80
60
40
20
0
a
ab
ab
b
c
c
d
DMSO
4 hr
24 hr
4
8
16
31.2
62.5
125
Nime
Carvacrol (µg/mL)
+ (LTA+ PGN)
B
% COX-2 Production relative to the control
a
ab
b
b
c
DMSO
4 hr
24 hr
4
8
16
31.2
62.5
125
Nime
Carvacrol (µg/mL)
+ (LTA+ PGN)
C
% PGE2 Production relative to the control
a
ab
bc
cde
de
e
de
cd
f
g
DMSO
4 hr
24 hr
4
8
16
31.2
62.5
125
Nime
Carvacrol (µg/mL)
+ (LTA+ PGN)

Figure 6.4 Inhibitory effects of carvacrol on the production of HBD-2, COX-2, and PGE_2 in LTA- and PGN-stimulated human tonsil epithelial cells.

Tonsil cells were treated with carvacrol (4 – 125 μg/mL) for 20 hr after 4 hr stimulation with LTA- and PGN mixture (5 μg/mL, each). Supernatants were harvested 20 hr after stimulation. Production of (A) HBD-2, (B) COX-2, and (C) PGE_2 in the culture supernatants was determined by ELISA. Results are shown as the mean ± SE; the experiment was done in triplicate. Data are representative of three independent experiments. The letters above the columns show that the means of different groups were significantly different ($p < 0.05$) by one-way analysis of variance using Tukey's test. HBD-2: human beta defensin-2; GCP-2: COX-2: Cyclooxygenase 2; PGE_2: Prostaglandin E2; LTA: Lipoteichoic acid; and PGN: Peptidoglycan.

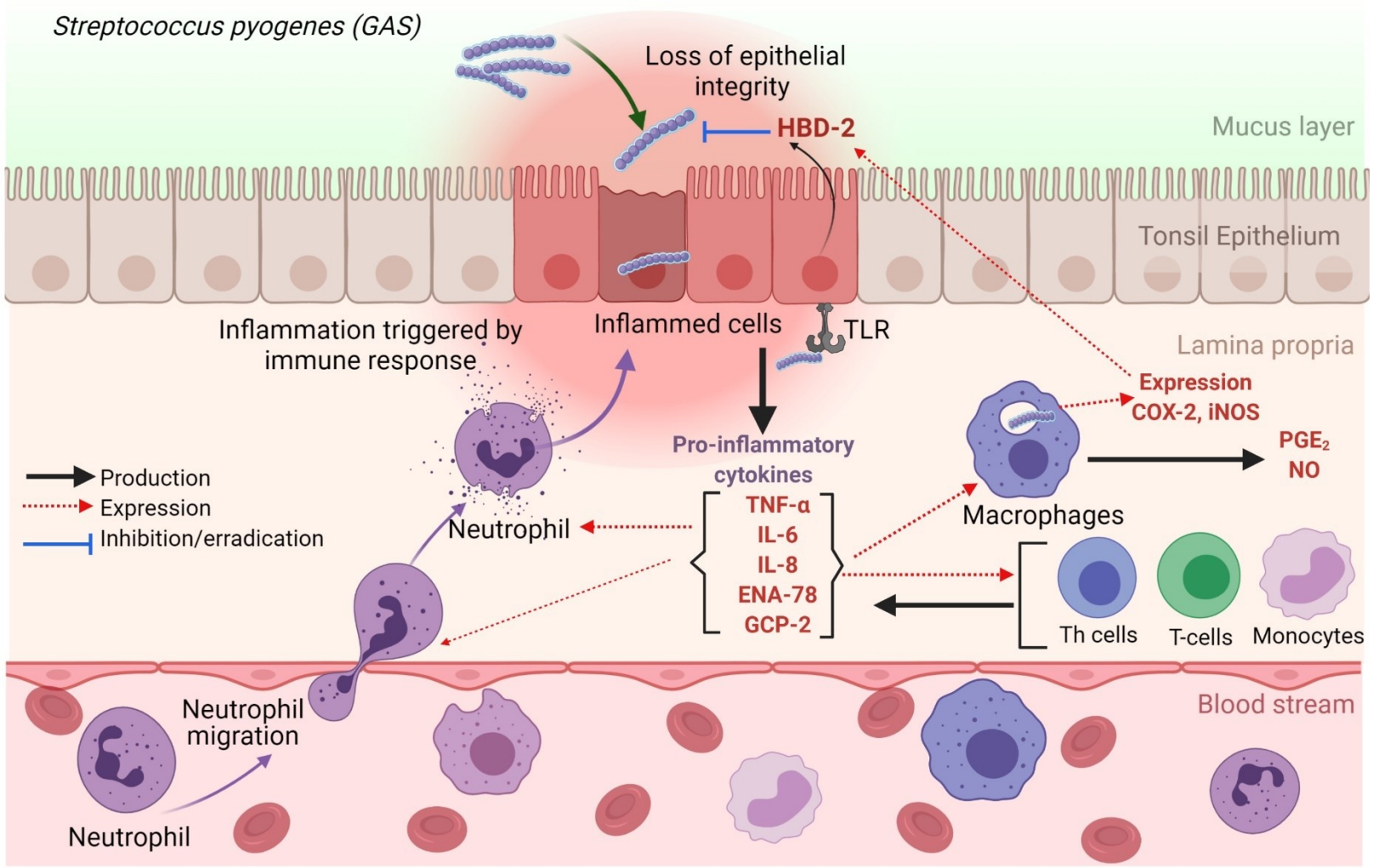
Streptococcus pyogenes (GAS)
Loss of epithelial integrity
HBD-2
Mucus layer
Tonsil Epithelium
Inflammation triggered by immune response
Inflammed cells
TLR
Lamina propria
Expression
COX-2, iNOS
PGE2
NO
Pro-inflammatory cytokines
TNF-α
IL-6
IL-8
ENA-78
GCP-2
Production
Expression
Inhibition/erradication
Neutrophil
Macrophages
Th cells
T-cells
Monocytes
Neutrophil migration
Neutrophil
Blood stream

Figure 6.5 Possible mechanisms of releasing pro-inflammatory cytokines in tonsil epithelial cells during *S. pyogenes* infection.

TLRs recognize *S. pyogenes* during the invasion, which results in the release of inflammatory cytokines by inflamed epithelial cells. The secretion of pro-inflammatory cytokines (IL-6 and IL-8) potently promotes the recruitment of neutrophils to the site of inflammation. TNF-α activates the nuclear factor κB pathway, which facilitates the expression of pro-inflammatory, the recruitment of neutrophils and macrophages to the site of infection and cell survival genes. GAS: Group A streptococcus; IL-6: interleukin-6; IL-8: interleukin-8; HBD-2: human beta defensin-2; GCP-2: granulocyte chemotactic protein-2; ENA-78: epithelial-derived neutrophil-activating protein-78; TLR: Toll-like receptors, TNF-α: tumor necrotic factor-alpha. The figure is created using BioRender.com.

6.8.SUPPLEMENTARY FIGURES

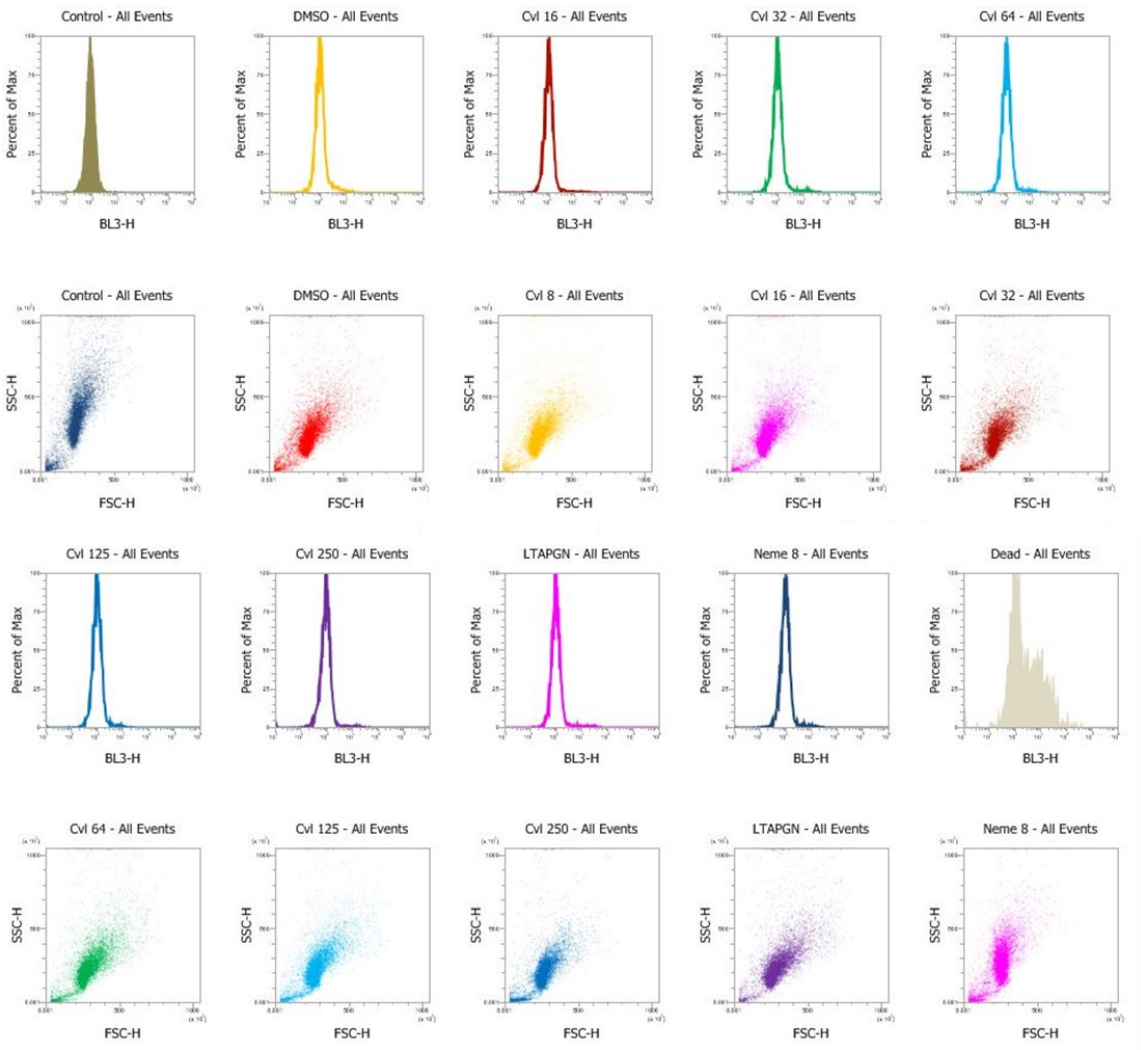

Figure S.7 A set of representative histograms and scatter plots of the controls and carvacrol samples given by FCM analysis in human tonsil epithelium cells (TonEpiCs).

Cell viability of cells harvested after 24 hr treatment of controls (untreated, DMSO, Nimesulide, LTA + PGN mixture) and carvacrol (16, 32, 64, 125, and 250 µg/mL) was determined using the 7-AAD staining for 5 min. Forward scattering (FSC) and side scattering (SSC) plots were also included.

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CHAPTER 7: GENERAL CONCLUSIONS

7.1. Overview of the thesis and major findings of the research

Streptococcal pharyngitis, the infection of interest in this study, causes systemic symptoms such as fever, tender anterior cervical lymphadenopathy, tonsillar exudates, sore throat, inflammation, and pain. In order to prevent disease transmission, proper antibiotic therapy is recommended. In addition, anti-inflammatory medications are also advised to treat the symptoms of streptococcal pharyngitis. However, research into natural anti-microbial agents derived from plants as complementary or alternative treatments to synthetic counterparts has received much attention due to some treatment challenges associated with antibiotic courses, such as poor patient compliance and significant adverse side effects. Carvacrol, the compound of interest in this study, was investigated for its anti-bacteriostatic, anti-bactericidal, anti-biofilm, and anti-inflammatory activities against *S. pyogenes*, and the concluding remarks of each technical chapter are summarized below.

In Chapter 2, I investigated whether carvacrol is effective against *S. pyogenes* and its synergistic interactions with commonly prescribed antibiotics such as penicillin G, penicillin VK, erythromycin, and clarithromycin. I found that carvacrol inhibited the planktonic growth of *S. pyogenes* through membrane leakage of cytoplasmic contents and instant bactericidal activities. However, no significant synergistic effect with any antibiotics tested was observed; instead, I discovered marginal additive synergism between carvacrol and clindamycin against *S. pyogenes*.

In Chapter 3, I expanded chapter 2 objectives by investigating how carvacrol interacts with bacterial membranes, leading me to conclude that carvacrol's primary mode

of action is membrane destruction and permeability changes. I showed that carvacrol kills *S. pyogenes* by altering cell membrane integrity, increasing permeability, decreasing membrane potential, and fluidizing the membrane, resulting in concentration-dependent cytoplasmic content leakage and, ultimately, bacterial cell lysis. Furthermore, I discovered that carvacrol-induced membrane disruption of *S. pyogenes* is suggested to be one of the carvacrol's potential significant modes of action by binding carvacrol with specific membrane phospholipids P.G., P.E., and CL.

Exposure to the minimum inhibitory and minimum bactericidal concentrations of carvacrol over a short duration and its effect on the bacteria membrane properties have been investigated in the previous two chapters. However, we considered what modifications might occur if carvacrol was treated for an extended period. Alterations in bacterial membrane lipid composition and changes in fluidity have been widely investigated in antibiotic studies. In addition, individual phospholipids have different physical properties, such as their charge, structure, and interaction with phytochemicals, making it crucial to consider how each one affects membrane fluidity. Therefore, a discussion of 24 hr treatment of sub-inhibitory concentrations of carvacrol's involvement in altering the phospholipid composition and membrane fluidity of *S. pyogenes* cell membrane isolates has been attempted in Chapter 4.

Biofilm formation is one of the adaptations and survival mechanisms of *S. pyogenes* in the human host. The virulence factors involved in *S. pyogenes* biofilm formation and pathogenesis have previously been studied and reviewed (Vyas et al., 2019b). However, difficulties in eradicating *S. pyogenes* biofilms from patients make treatment challenges to currently available drugs. Therefore, novel biofilm-inhibition and

eradication agents are expected to be developed to prevent the early stages of biofilm formation and disrupt the biofilm cell community, respectively. Therefore, the clinical relevance of carvacrol on *S. pyogenes* biofilms requires further investigation before it can be used as a treatment agent. With *in vitro* and *in silico* studies, in Chapter 5. I discussed carvacrol's ability to inhibit biofilm formation and eradicate the preformed biofilm of *S. pyogenes*.

The expression of several virulences of biofilm formation-related genes has been examined by qPCR. Moreover, I discovered molecular suppression of specific genes associated with bacterial attachment to the host, bacterial communication (quorum sensing), biofilm formation, aggregation, capsule biosynthesis, and acid and oxidative stress response. For example, bacteria grown with a sub-inhibitory concentration of carvacrol for 24 hours showed downregulation of *speB*, *luxS*, *covS*, *dltA*, *ciaH*, and *hasA*. Moreover, carvacrol triggers a morphological alteration of biofilm cells and reduces biofilm thickness. Carvacrol also affects concentration-dependently the development of biofilms by changing the host micro-environment (cell surface hydrophobicity).

It is important to investigate the potential practical applications of these findings of carvacrol's anti-bacterial activity in managing streptococcal pharyngitis and associated pain. With the growing interest in identifying natural compounds for developing NHPs, such as throat vapors, throat lozenges, or cough syrups, carvacrol has been incorporated into natural health products (NHP) intended to soothe a sore throat as a pain reliever. Therefore, an attempt has been made in Chapter 6 to address carvacrol's role in pain relief associated with streptococcal pharyngitis. Production of pro-inflammatory mediators such as IL-6, IL-8, GCP-2, ENA-78, PGE2, and COX-2 is suppressed by carvacrol in the

S. pyogenes antigen-stimulated human tonsil epithelial cells *in vitro*. Furthermore, in this preliminary study, I also demonstrated the safety of carvacrol by measuring the viability of human tonsil epithelium cells.

Finally, the findings of the research studies provide better evidence for carvacrol's anti-bacterial activity against *S. pyogenes*. Therefore, the findings of this study suggest that its mechanism of action inhibits multiple virulence factors, including membrane integrity destruction, biofilm inhibition, and suppression of pro-inflammatory markers. Therefore, this study demonstrates carvacrol's potential as a natural therapeutic agent for streptococcal pharyngitis and pain relief.

Given the anti-bacterial, antibiofilm, and anti-inflammatory effects discovered through this thesis research, I believe that future research can be aimed at developing NHPs and pharmaceuticals using carvacrol alone or combined with others for the management of streptococcus pharyngitis.

7.2. Limitations of the research and future directions

Animal Model and clinical studies

This thesis research has established the mode of action of carvacrol and host cell-pathogen interactions during the infection using several *in vitro* models (human and bacterial cells) and an *in silico* model. However, future human subjects investigations employing human subjects are required for a better understanding of the host defense mechanisms against carvacrol's action.

Even though I initially intended to conduct a mice trial, according to recent reports, using an oropharyngeal colonization animal model to study streptococcal pharyngitis infection has many drawbacks (Gogos and Federle, 2019). Humans are the

sole known host of *S. pyogenes*, and the posterior oropharynx, particularly the mucosal-associated lymphoid tissue known as Waldeyer's tonsillar ring (pharyngeal lymphoid ring), serves as the main reservoir for the pathogen. However, due to the absence of a Waldeyer's ring homolog in rodent pharynx, colonization of *S. pyogenes* strains in rodent oropharynx to precisely mimic *in vivo* streptococcal pharyngitis model has proven difficult (Gogos and Federle, 2019).

Additional drawbacks include the relatively short duration of infection in rodents and only the limited number of *S. pyogenes* strains capable of causing infection. Furthermore, the virulence factors such as M proteins, which are known to be significant in the development of pharyngitis, were not expressed in mice/rodents conducted previously (Tuomanen et al., 1999). Because the rodents/murine pharyngeal colonization models have been shown to be ineffective in inducing significant oropharyngeal colonization with *S. pyogenes*, we suggest adopting non-human primate models if carvacrol's therapeutic efficacy needs to be investigated.

Primate species such as the monkey, chimpanzee, baboon, and cynomolgus macaque have been studied for their ability to colonize the upper airway and oropharyngeal *S. pyogenes* over the last decade. Although non-human primate models of *S. pyogenes* pathogenesis are relatively expensive and challenging, we may be able to precisely mimic the human condition of pharyngitis. In addition, despite the high cost of the experiment, the non-human primates experimental model has many advantages over the rodent model, such as more accessibility to the infection site, extended persistence of *S. pyogenes* in their pharynx, larger collectible blood volumes for biochemical analyses, and the collectible of using multiple different strains of *S. pyogenes*.

Although clinical safety and tolerability of carvacrol have been studied recently at the phase-I stage (Ghorani et al., 2021), it is recommended to conduct clinical studies to assess the clinical efficacy of orally administrated carvacrol or carvacrol-incorporated NHP in Streptococcal pharyngitis patients as subjects.

Inflammation study

Inflammatory responses involve a highly coordinated network of many cell types, such as activated macrophages, monocytes, and other cells that mediate local responses to tissue damage. Damaged endothelium and epithelial cells also release chemokines and growth factors that create the inflammatory cascade that attract neutrophils and monocytes to the site of tissue injury (Chen et al., 2018a). Inflammatory markers predict inflammatory disease symptoms and assess responses to therapeutic interventions. Therefore, in the present inflammatory study, I assessed inflammatory markers released from the antigens-induced tonsil epithelial cells. However, the study did not extend to the other typical principal inflammatory mediators, such as IL-1β, interferon-γ, and NF-κB, nitric oxide, and TNF-α released specifically by macrophages during inflammation are shortcomings.

Development of a natural health product

We propose that carvacrol can be used as an NHP or for therapeutic purposes, with oral or inhalation administration against streptococcal pharyngitis infection and associated symptom relief. However, before any commercialization of carvacrol-incorporated NHPs, such as lozenges in pain management associated with streptococcal pharyngitis, the sensory attributes of carvacrol must be considered. Carvacrol has a specific strong, pungent aromatic taste. Therefore, incorporating carvacrol in NHPs, such as throat lozenges, may be challenging because some patients, including children, may not like its

flavor. Thus, carvacrol's spicy solid flavor and odor need to be masked, neutralized or altered using other appealing flavors or flavor enhancers.

When developing carvacrol-incorporated NHPs, natural flavor extracts or their bioactive compounds from citrus plants such as lime, lemon, and orange could be investigated for sensory features and consumer preferences along with carvacrol. On the other hand, those flavor-enhancing natural compounds may also have anti-bacterial activity against *S. pyogenes* solely or synergistically with carvacrol. Therefore, I have investigated the bactericidal properties of commercial flavor extracts from lime, lemon, and orange and their synergistic activity with carvacrol, even though the results were not included in the thesis. Moreover, I have assessed carvacrol's anti-bacterial synergy with several bioactive substances, including cinnamaldehyde, citral, and alpha-pinene. However, further research will be required to identify natural flavoring agents that can be combined with carvacrol to enhance flavor and anti-bacterial effectiveness.

Carvacrol can be incorporated in NHPs (lozenge/ throat vapor) to relieve cough, sore throat, and congestion quickly and effectively. However, one of the associated challenges is that the NHP manufacturing process conditions (temperature, pressure) may alter the effectivity of the incorporated carvacrol. Therefore, pre- and post-manufacturing carvacrol concentrations, stability, and effectiveness in NHPs should be investigated.

Safety of carvacrol as an NHP

Another concern associated with using carvacrol as a natural anti-bacterial agent or NHP is the safety of carvacrol and its adverse effects. Therefore, there must be adequate safety, allergy, and adverse effect testing before developing NHPs such as throat vapor, throat lozenge, or throat spray for people with streptococcal pharyngitis. However, carvacrol

has been designated as GRAS for use by humans by the US Food and Drug Administration. In addition, previous investigations have assessed the safety of carvacrol on food products.

Since the dosages employed in the anti-bacterial studies are substantially lower than the LD_{50} of carvacrol studied *in vivo*, which ranges from 310 – 2,700 mg/kg of human body weight, it is acceptable to assume that carvacrol can be generally safe within its anti-bacterial concentration (Suntres et al., 2015a). However, most of the previous investigations on the safety of carvacrol focused on foodborne pathogens, while the anti-bacterial effectiveness and associated processes of carvacrol against drug-resistant bacteria had not been as thoroughly studied. Nevertheless, more studies should be conducted to ensure the acceptability and safety of using carvacrol for young children, pregnant women, and individuals who have allergies or hypersensitivity to essential oils and their constituents.

Phytochemical-antibiotic conjugates

Anti-bacterial combination therapies involving phytochemicals and conventional antibiotics have been heavily investigated (Jayaraman et al., 2010; Kyaw et al., 2012). Therefore, future insights explore phytochemical-antibiotic conjugates that may have multiple applications. Moreover, carvacrol-antibiotic conjugates may also contain multitarget *S. pyogenes* inhibitors. Since phytochemical purification is one of the primary issues at industrial and commercial levels, exploring novel compounds or their conjugates is both economical and expensive. However, carvacrol can be produced in bulk quantities cost-effectively using the precursor of carvacrol (caravcone), synthetic analogs, or carvacrol derivatives. Therefore, future studies may explore the anti-bacterial activity of

conjugates against *S. pyogenes* compared to the synthetic and naturally extracted carvacrol. Additionally, the conjugation or combination of antibiotics with carvacrol may require further investigation for cellular uptake, stability in the body, and time of excreta.

7.3. Significance of the research and concluding remarks

Streptococcal pharyngitis, the infection of interest in this study, is a frequent and highly contagious infection reported worldwide. This thesis has emphasized that despite its widespread use for decades, *S. pyogenes* has remained susceptible to penicillin and other β-lactams. However, due to the high tolerance of *S. pyogenes* biofilms toward antibiotics, alternative or additional therapeutics are being investigated, primarily sourced from natural origins.

The overall objective of the research was to explore carvacrol's anti-bacterial, antibiofilm and anti-inflammatory activities and then study its potential modes of action to inhibit the growth or eradicate *S. pyogenes*. To the best of my knowledge, this thesis reports for the first time: the mode of action of carvacrol as a bactericidal agent, synergistic interactions between carvacrol and antibiotics, anti-biofilm activities, and anti-inflammatory activities against *S. pyogenes* stains related to streptococcus pharyngitis. In this study, I used mainly *in vitro* (bacterial model and human tonsil epithelial cell model) and one *in silico* model, thereby revealing a significant mode of action by which carvacrol inhibits the growth of planktonic bacteria and their biofilm formation, as well as initial anti-inflammatory activity-associated with infection development.

The findings provide new insight into bridging the gap between the anti-bacterial mechanisms of carvacrol against *S. pyogenes* caused by streptococcal pharyngitis,

evaluated at the molecular level and observations at the organism level. Therefore, the results of the current study can serve as fundamental new knowledge for further transformative research focused on the development of NHPs or pharmaceutical applications with carvacrol on streptococcal pharyngitis and associated respiratory complications. However, further research should emphasize *in vivo*, clinical studies, and sensory evaluations of carvacrol-incorporated products. Moreover, I have proposed the anti-bacterial mechanisms, target sites, and genes affected by carvacrol against *S. pyogenes* planktonic and biofilm bacteria; however, its regulated signaling pathways still need to be addressed.

The findings imply that carvacrol is a promising candidate for complementary and alternative medicine applications on biofilm-forming *S. pyogenes* strains. Nevertheless, more efforts are required in developing treatment approaches to prevent extensive and recurrent antibiotic treatment in patients with biofilm-associated recurrent *S. pyogenes* infections. At its sub-inhibitory concentration, Carvacrol downregulated genes responsible for the critical pathogenesis phases of the infection development, such as biofilm formation and quorum sensing of *S. pyogenes*.

Many recommended non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, provides rapid relief of painful symptoms. However, there is a risk of causing systemic adverse reactions, although the concentration of the active ingredient in the target tissue is low. Carvacrol suppressed pro-inflammatory biomarkers, suggesting that it has the potential of being orally administrative NHP or pharmaceutical as a stand-alone or adjuvant treatment of streptococcal pharyngitis. Therefore, carvacrol could be

developed to manage streptococcal pharyngitis by incorporating locally acting forms of NHPs such as throat lozenge, vapors rinses, or oral/throat sprays.

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