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The inhibitory effect of the methanolic extracts of *Origanum syriacum* and *Rosmarinus officinalis* and their major components on the biofilm formation of clinical isolates of *Staphylococcus aureus*

By

Reeda Shawkat Harb

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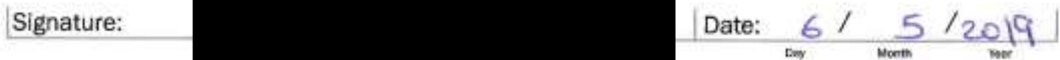

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Dedication

All my work, this thesis and every other achievement I did or will ever do in my life will always be dedicated to:

My father, *Shawkat Harb* who supported me by all means all through out my career and was always present to motivate me, guid me and provide me with his immense knowledge in life and career.

The most kindhearted and tender woman, my beloved mother, *Nada Harb* who taught me from her patience and perseverance the way to overcome all the obstacles I might face throughout my life and career.

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The inhibitory effect of the methanolic extracts of *Origanum syriacum* and *Rosmarinus officinalis* and their major components on the biofilm formation of clinical isolates of *Staphylococcus aureus*

Reeda Shawkat Harb

Abstract

Control of bacterial pathogenesis is becoming more difficult by the day. The emergence of bacterial resistance has made treatment of infections more difficult, a reason why a twist towards traditional medication was made as part of the efforts to control such infections. It is well known that bacterial biofilm formation is directly associated with initiation of bacterial infections, so a new trend in research is to try to inhibit biofilm formation and thus prevent infections. This study aimed at detecting whether the methanolic extract of two natural herbs, *Rosmarinus Officinalis* (Rosemary) and *Origanum syriacum (*Zaatar) have the ability to inhibit the biofilms that are produced by a serious pathogen, *Staphylococcus aureus,* using standard methods. The 21 clinical isolates *S. aureus*, included in the study, were defined phenotypically and genotypically. Six isolates were found to be methicillin resistant (MRSA) while the remaining 15 were methicillin susceptible (MSSA). The PFGE pulsotypes of the strains determined, allowed for segregation of the isolates into one major clade that was subdivided into two subclades, each, of which, contained a set of more closely related isolates. The rest of the isolates were each individually related to others. Notably, the methicillin susceptible *S. aureus* strains were more closely related to each other as compared to the methicillin resistant strains, which were either grouped in pairs or individually. Interestingly, few methicillin susceptible

isolates obtained from one hospital were more closely related to isolates obtained from a health center, than to other methicillin susceptible isolates from the same hospital. The methanolic extract of each of the two plants (0.1 g/ml) revealed an antibacterial effect at volumes equal or higher than 200 µL for *Rosmarinus Officinalis* and 150 µL for *Origanum syriacum* against the *S. aureus* isolates. It was, however, found that the volumes of 100 and 150 µL of the *Rosmarinus Officinalis* extracts and 50 and 100 µL of the *Origanum syriacum* extracts at the lower concentrations of 0.02 g/ml (five times diluted extract) and 0.01 g/ml (ten times diluted extract), of the two plants respectively, significantly inhibited the *S. aureus* biofilm formation. The contribution of the major components of each of the two herbs to that effect was then determined. The two major components of *Rosmarinus Officinalis:* α-pinene and camphor at different concentrations inhibited the biofilm formation of all the tested isolates. On the other hand, the two major components of *Origanum syriacum*: Thymol and Carvacrol at different concentrations did not affect the *S. aureus* biofilm formation. The results of this study revealed the previously undetermined ability of the tested plants to inhibit the *S. aureus* biofilm formation and demonstrated the significance of α-pinene and camphor as the effective inhibitors and suggested their use to prevent the serious infections that may be caused by the organism.

Keywords: Biofilm formation, *Rosmarinus Officinalis, Origanum syriacum, Staphylococcus aureus,* Prevention of biofilm

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List of Abbreviations

ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10

Agr: Accessory gene regulator

AIP: auto-inducing peptide

Atl: autolysin

BAP: biofilm associated surface proteins

bbp: gene encoding bone sialoprotein-binding protein

BHI: Brain Heart Infusion

C3b: Complement component 3b

C5a: Complement component 5a

C5aR: Complement component 5a receptor

CA-MRSA: community acquired methicillin resistant *Staphylococcus aureus*

CCI: chronic constriction injury

Ccr: cassette chromosome recombinase

clfA: gene encoding clumping factor A

clfB: gene encoding clumping factor B

cna: collagen adhesion-encoding gene

Coa: canonical coagulase

COX-2: cyclooxygenase 2

Dsg-1: Desmoglein 1

DVLO forces: Derjaguin, Landau, Verwey and Overbeek forces

ebps: gene encoding elastin binding protein

ECM: extracellular matrix

eno: gene encoding enolase

EPS: Exopolysaccharide

FnBPa: Fibronectin binding protein a

FnBPb: Fibronectin binding protein b

GMP: guanosine monophosphate

HAV: *Hepatitis A* virus

HIV-1: Human Immunodeficiency virus

HLA: Human Leukocyte Antigen

Hld: gene encoding delta hemolysin

Hsc70: heat shock cognate protein

HSV1: *Herpes simplex* virus 1

ICA: Intercellular adhesion protein

ica: Intercellular adhesion gene cluster

ICD: Irritant corneal dermatitis

Il: Interleukin

LAUMC-RH: Lebanese American University Medical Center Rizk Hospital

MCF7: breast cancer cell line

MHA: Muller-Hinton Agar

MHC II: major histocompatibility complex class II

MIC: minimum inhibitory concentration

MLS: macrolide, lincosamide and streptogramin resistance

MRSA: Methicillin resistant *Staphylococcus aureus*

MSA: Mannitol Salt Agar

MSCRAMM: microbial surface components recognizing adhesive matrix molecules

MSSA: Methicillin susceptible *Staphylococcus aureus*

NET: Neutrophil extracellular trap

NF-kB: Nuclear Factor kappa-light-chain enhancer of activated B cells

NIH: National Institute of Health

NO: nitric oxide

NOS: nitric oxide synthase

PBP-2A: penicillin binding proteim 2A

PFGE: Pulse Field Gel Electrophoresis

PIA: polysaccharide intercellular antigen

PMBC: peripheral blood mononuclear cells

PMN: polymorphonuclear neutrophils

PNAG: poly-β(1-6)-*N*-acetyl-glucosamine

PS/A: Capsular polysaccharide/ adhesion

PSM: phenol-soluble modulins

PVL toxin: Panten Valentine Leukocidin toxin

RNS: free reactive nitrogen species

SA: Super antigen

SAK: Staphylokinase

SasC: *S. aureus* surface protein C

SasG: *S. aureus* surface protein G

SCC*mec*: Staphylococcal chromosome cassette *mec*

SEB: Staphylococcal Enterotoxin B

sma1: Serratia marcescens restriction enzyme

sspA: gene encoding for Staphylococcal-derived staphopain A

SspB: Staphylococcal-derived staphopain B

SSSS: Staphylococcal Scalded Skin Syndrome

STAT-3: Signal transducer and activator of transcription 3

TAR: Trans activating region

Tat: Trans activator of transcription

TCR: T-cell restricted

TNF-α: Tumour Necrosis Factor alpha

TSA: Tryptic soy agar

TSST1: Toxic Shock Syndrome Toxin

UTI: urinary tract infection

UVB: ultraviolet B

VISA: vancomycin intermediate-resistant *Staphylococcus aureus*

VRSA: vancomycin resistant *S. aureus*

vWbp: von Willbrand factor binding proteins

Chapter I
Introduction

1.1. *Staphylococcus aureus*

1.1.1. *Staphylococcus aureus* characteristics

Staphylococcus aureus is a Gram-positive coccus that grows usually in grape-like clusters but can grow in pairs and less commonly in short chains (Foster, 1996). It has a relatively small colony size that appears light yellow to gold on Tryptic Soy Agar (TSA). It is differentiated from *Staphylococcus epidermidis* on Mannitol Salt Agar (MSA) where, unlike *Staphylococcus epidermidis*, it appears yellow as a result of acid production from fermentation of mannitol turning the phenol red indicator yellow. Similar to other Gram-positive bacteria, *S. aureus* possesses a thick cell wall composed of cross-linked peptidoglycan strands that create a complex mesh. This thick peptidoglycan allows retention of crystal violet of the Gram staining thechnique providing *S. aureus* and other similar bacteria with their classification as gram-positive bacteria. This thick peptidoglycan cell wall contains teichoic acid which is a polymer of sugar-alcohol and phosphate (Pommerville & Pommerville, 2014). Teichoic acid is essential for the viability of the bacterial cells as it is thought to protect the cells from autolytic enzymes and is considered as a virulence factor in the host (Pommerville & Pommerville, 2014). It gives positive catalase and coagulase tests. *S. aureus* can also be identified through testing for production of thermostable deoxy-ribonuclease through agglutination with Immunoglobulin G and Fibrinogen which bind *S. aureus* surface protein A (Foster, 1996). Staphylococci possess a 2.8 Mbps low G+C content genome. Their genome is relatively conserved, yet *Staphylococcus aureus* has in its genome highly variable sequences that are large in size (Baba et al., 2008). *S. aureus*

significant ability to acquire beneficial genes from other organisms through horizontal gene transfer possibly explains the presence of these variable regions in its genome, as well as, the complexity of this genome (Kuroda et al., 2001). These regions are distinguished as acquired prophages, pathogenicity islands or chromosomal cassettes (Baba et al., 2008). The combined effect of the presence of these variable regions draws the genetic profile for the pathogenicity of infectious *S. aureus* strains (Baba et al., 2002). It is a facultative anaerobe that produces ATP through aerobic respiration in the presence of oxygen or through fermentation in its absence (AO Research Institute et al., 2002). It has been considered a non-motile organism yet recently it was shown to possess "spreading motility" on soft agar in a passive motion to form outward spread circular colonies or radially extended lobes (Pollitt & Diggle, 2017). It is a non-spore forming organism (AO Research Institute et al., 2002). It requires a set of nutritional elements mainly nitrogen provided by a set of 5 to 12 amino acids such as valine and B vitamins (Kloos and Schleifer, 1986; Wilkinson, 1997). *Staphylococcus aureus* grows in complex media enriched with peptone which provides the bacterial cells with the necessary amino acids and peptides, beef extract which provides the cells with necessary minerals and vitamins for their growth and sodium chloride (NaCl) to provide it with sodium and chloride ions (Pommerville & Pommerville, 2014).

1.1.2. *Staphylococcus aureus* pathogenesis

S. aureus is usually a commensal organism that colonizes the skin, the mucosal surfaces (mainly the anterior nares), the axillae, the groin, and the gastrointestinal tract of individuals (Eriksen, et al., 1995; Gordon & Lowy, 2008; Taylor & Unakal, 2019). *S. aureus* does not infect the host once it exists on those mentioned sites of the host body, yet it can turn into a serious pathogen if it enters into the blood stream of the host or invades its internal tissues. This invasion of the host tissues is facilitated by the

ability of *S. aureus* to evade the host immune system through intracellular survival, avoiding the host antibodies, inhibition of chemotaxis between the cells of the immune system and biofilm formation (Taylor & Unakal, 2019). Few of the infections caused by *S. aureus* are skin infections (e.g., impetigo, scalded skin syndrome …), respiratory infections (e.g., pneumonia), urinary tract infections, toxic shock syndrome, meningitis, and gastroenteritis (Taylor & Unakal, 2019). *S. aureus* particularly the methicillin resistant strains have been characterized as nosocomial pathogens responsible for hospital outbreaks since more than three decades. In 1982, methicillin resistant *S. aureus* were responsible for outbreaks in more than 85 % of hospitals in the United States and were considered as endemic nosocomial pathogens (Thompson, Cabezudo & Wenzel, 1982). Hospitalized patients, who are usually more immunocompromised than healthy individuals, diabetic individuals or drug addicts, who use needles frequently, in addition to, health care workers have higher colonization rates of *S. aureus* (Taylor & Unakal, 2019). Health care workers are considered the major contributors to patient-to-patient spread of *S. aureus* because of transient hand carriage allowing the spread of nosocomial infections leading to hospital outbreaks (Thompson, Cabezudo, & Wenzel, 1982).

1.1.3. *Staphylococcus aureus* toxins and extracellular enzymes

Staphylococcus aureus damages the host cell by secreting different types of toxins including toxins that damage the host cell membrane or that interfere with the host receptors and secreted enzymes that damage the host cell.

1.1.3.1. Toxins targeting the host cell membrane

One of the toxins produced by *Staphylococcus aureus* is α-hemolysin (Hla) which is a pore forming toxin that targets the host innate and adaptive immune cells as well as the red blood cells (Berube & Bubeck Wardenburg, 2013; López de Armentia, Gauron

& Colombo, 2017; Otto, 2014). The α-toxin is a transmembrane β-barrel composed of 293 amino acid residues that form a single polypeptide chain where the carboxy terminus and amino terminus of the polypeptide are separate by a glycine rich sequence (Valeva et al., 1997; Walker et al., 1995). The attachement of the toxin to host cell membrane occurs by binding of the water-soluble monomer to the lipid bilayer. This monomer then aggregates to become an oligomer forming the pre-pore complex on the outer leaflet of the host cell membrane which is then converted into the completely assembled transmembrane pore constituting a hydrophilic channel through the cell membrane (Walker et al., 1995). This trans-membrane pore formation causes the release of intracellular ATP from the cell and influx of calcium into the host cell leading to degradation of host proteins to form prostaglandins and leukotrienes. In addition, pore formation leads to activation of transcription factors such as NF-kB and production of pro-inflammatory cytokines. All together these alterations activate the host inflammatory response leading eventually to necrotic tissue damage. The pore formation depends on the interaction of the toxin with the host cell receptor ADAM10 which is expressed on the surface of distinct cells including epithelial cells, primary endothelial cells and red blood cells (Berube & Bubeck Wardenburg, 2013). This interaction between the toxin and ADAM10 leads to degradation of a major substrate of ADAM10, which is E-cadherin, hence disrupting cell to cell junctions. This ADAM10-HLA interaction disrupt the focal adhesion of host cell to the basement membrane. These alterations explain the ability of the toxin to disrupt the host tissue barriers and the ability of *S. aureus* to infect the host lung and skin leading to diseases such as pulmonary edema or necrotic dermal injury as well as damage of the blood vessels and mucous membranes (Berube & Bubeck Wardenburg, 2013).

Anoher pore forming toxin is the Panten Valentine Leukocidin toxin which is encoded by the *PVL* gene and it is composed of two subunits LukS-PV and LukF-PV (Genestier et al., 2005). PVL toxin also interacts with the host complement receptors (C5aR and C5L2) (Spaan et al., 2013). This toxin induces the host immune system stimulating the release of histamine from basophils as well as activation of neutrophils and chemokines and production of oxygen radicals leading to host tissue damage and necrosis (Genestier et al., 2005).

1.1.3.2 Toxins interacting with host cell receptors

Staphylococcus aureus produces enterotoxins which belong to the family of super-antigens and that are related to food poisoning in the host (Argudín, Mendoza, & Rodicio, 2010). Super-antigens (SAs) allow the interaction between major histocompatibility complex class II (MHC II) on antigen presenting cells and the T cell receptors activating them and causing their proliferation. The outcome of this process is the production of pro-inflammatory cytokines leading to an exaggerated inflammatory response that causes toxic shock (Proft & Fraser, 2003). Nineteen distinct enterotoxins and enterotoxin-like toxins have been identified in *S. aureus* (Thomas et al, 2007). This classification is based on their emetic activity where super-antigens that cause emesis in primate models are referred to as staphylococcal enterotoxins while super-antigens lacking this emetic activity are referred to as enterotoxin-like (Argudín et al., 2010). One of the *S. aureus* enterotoxins is the 28 kDa enterotoxin B (SEB) which is resistant to proteolytic enzymes of the gastrointestinal tract. It is associated with non-menstrual toxic shock, dermatitis and asthma (Fries & Varshney, 2013).

Another well-known super-antigen produced by *S. aureus* is the Toxic Shock Syndrome Toxin (TSST1) which causes toxic shock characterized by massive

production of pro-inflammatory cytokines such as Interleukin-2 (Il-2) and Tumor Necrosis Factor-α (TNF)-α leading eventually to tissue damage (Otto, 2014). TSST1 was first discovered in 1979 and it was characterized, two years later, as the causative agent of menstrual Toxic Shock Syndrome (TSS) (Kulhankova, King, & Salgado-Pabón, 2014). TSST1 does not possess an emetic activity but causes symptoms that range from fever and rash to organ failure in the host (Argudín et al., 2010; Kulhankova et al., 2014; Lappin & Ferguson, 2009).

Furthermore, other exotoxins produced by *S. aureus* are epidermolytic toxins A and B which are also referred to as exfoliative toxins (Bukowski, Wladyka, & Dubin, 2010). These toxins are responsible for Staphylococcal Scalded Skin Syndrome (SSSS) whose severity ranges from scarce blisters on a certain region of the skin to wide spread exfoliation covering the entire surface of the body (Mishra, Yadav, & Mishra, 2016). The nomenclature of the syndrome came from the features of the blisters which resembled burned or scalded skin (Farroha et al, 2012). The proteolytic activity of *S. aureus* exfoliative toxins hydrolyses Desmoglein 1 (Dsg-1) which is a cadherin responsible for cell-to-cell adhesion causing disrupted skin integrity and leading to SSSS (Bukowski et al., 2010).

1.1.3.3. Proteolytic Enzymes of *Staphylococcus aureus*

Staphylococcus aureus enzymes are exo-proteases that degrade specific proteins of the host cell (Kolar et al., 2013). These enzymes include metalloprotease like aureolysin. Aureolysin is a zinc dependent metalloproteinase that is composed of 301 amino acids that assemble into two domain: a beta sheet rich and an alpha helix rich domains (Banbula et al., 1998). Aureolysin is an inhibitor of the complement system through preventing the binding of C3b to the bacterial cell membrane and the release of C5a (Laarman et al., 2011). In addition it inhibits phagocytosis of the bacteria and their

degradation by neutrophils (Laarman et al., 2011). Moreover, aureolysin is involved in *S. aureus* dependent bone destruction causing osteomyelitis (Cassat et al., 2013).

Another type of enzymes secreted by *S. aureus* are serine proteases such as the V8 protease which is encoded by the *sspA* gene (Rice et al., 2001). V8 protease is a glutamyl endopeptidase (Houmard & Drapeau, 1972). It plays a key role in infections caused by *S. aureus* in vivo through promoting its survival as well as significantly contributing to adhesion of *S. aureus* to host surfaces (Rice et al., 2001).

A third class of *S aureus* proteases are cysteine proteases such as staphopain B (SspB) (Kulig et al., 2007). SspB protects *S. aureus* from phagocytosis by host immune cells, neutrophils and monocytes (Smagur et al., 2009). In addition, SspB is capable of degrading proteins of the host extracellular matrix such as collagen and is involved in impairing blood clots and tissue damage during *S. aureus* infections (Ohbayashi et al., 2011).

Staphylococcus aureus releases coagulases which are the canonical coagulase (Coa) and von Willbrand factor binding proteins (vWbp). *Staphylococcus aureus* coagulases bind and convert prothrombin to thrombin which in its turn influences the conversion of fibrinogen into its insoluble counterpart, fibrin causing clotting in the host. Those coagulases play a role in the pathogenesis of *S. aureus* such as abscesses, sepsis, and endocarditis and contributes to biofilm formation in the host (McAdow, Missiakas, & Schneewind, 2012; Moreillon et al., 1995; Zapotoczna et al, 2015).

On the other hand¸ *Staphylococcus aureus* produces Staphylokinase (SAK) which promotes degradation of fibrin through activating plasminogen allowing bacterial dissemination through the host barriers (Peetermans et al., 2014). Additionally, the complex “plasminogen-staphylokinase” protects the bacteria from being lysed by the host bactericidal peptides, α-defensins (Bokarewa, Jin, & Tarkowski, 2006).

Staphylokinase producing *S. aureus* strains have been mainly isolated from skin or mucosal sites of infections and have been associated with implant associated infections (Bokarewa et al. 2006; Aubin et al., 2015)

1.1.4. *Staphylococcus aureus* drug resistant strains

In 1940, *Staphylococcus aureus* infections were treated with the newly introduced β-lactam, penicillin (Chambers & DeLeo, 2009). After two years of introducing penicillin, penicillin resistant *Staphylococcus aureus* strains emerged in hospitals and two decades later they became widespread in the community due to acquisition of β-lactamase enzymes by these strains allowing them to hydrolyse the β-lactam ring of penicillin (Miragaia, 2018). The major contributor to the development of β-lactams resistant staphylococci was exposure of these bacteria to β-lactams in the environment whether in the soil where staphylococci are in contact with fungi that produce penicillin or on animal farms where β-lactam antibiotics are extensively used as food additives (Miragaia, 2018). This widespread resistance to penicillin in *S. aureus* strains led to development of penicillinase-resistant penicillin such as methicillin (Miragaia, 2018). Yet, certain *Staphylococcus aureus* strains were capable of developing resistance against methicillin through acquisition of the *mecA* gene (Jevons, 1961). On the other hand, the *S. aureus* strains that remained susceptible to methicillin were termed Methicillin Susceptible *Staphylococcus aureus* (MSSA). Methicillin resistant *Staphylococcus aureus* (MRSA) were first reported in 1960 and they were referred to as "hetero-resistant" strains because they were resistant to all beta-lactam antibiotics (Barrett, McGehee & Finland, 1968). Infections of MRSA are associated with health care environments including catheter associated urinary tract infections, ventilator associated respiratory tract infections and prolonged hospitalization with extensive use of antibiotics especially quinolones (Barrett et al., 1968; Duarte et al., 2018). MRSA

remained nosocomial until the 1990s where community acquired MRSA (CA-MRSA) emerged causing soft tissue infections, head and neck infections, as well as, otolaryngologic infections (Duarte et al., 2018). Community acquired MRSA outbreaks occurred among prisoners where in 2002 inmates of Los Angeles Jail suffered from CA-MRSA infections ranging from skin infections to severe diseases such as endocarditis, bacteraemia and osteomyelitis (Centers for Disease Control and Prevention (CDC), 2003). Other CA-MRSA outbreaks were reported among military trainees where MRSA associated skin and soft tissue infections (SSTIs) occurred at 4-6% rate of U.S military trainees causing approximately 41,951 ambulatory visits for military members on duty and 1,054 admissions to the hospital (Landrum et al., 2017; Zinderman et al., 2004). In addition to that, CA-MRSA occurred among children in day care, players of contact sports like professional football players, intravenous drug users and naval ship crewmembers (Kazakova et al., 2005; Zinderman et al., 2004).

The *mecA* gene, which confers the *S. aureus* resistance to methicillin, encodes for the production of penicillin binding protein PBP 2A (Wielders et al., 2002). Penicillin binding proteins catalyse the transpeptidation between the peptidoglycans that form the cell wall of Gram positive bacteria, however PBP 2A exhibits unusual reduced affinity to β-lactams allowing normal cell wall synthesis even in the presence of lethal concentrations of β-lactam antibiotics (Lim & Strynadka, 2002). PBP 2A is composed of a transmembrane N-terminal domain containing Ser403 residue in the α-helix α2. Normally, the PBP interaction with the β-lactam inhibitor leads to formation of a Michaleis complex through noncovalent interaction, followed by a nucleophilic attack by the Serine residue in the active site on the β-lactam ring to form the intermediate acyl-PBP, however PBP 2A has its Ser403 within the active site in a position that is poor for nucleophilic attack leading to altered formation of the acyl-PBP intermediate,

hence rendering the strains producing PBP 2A resistant to β-lactams (Lim & Strynadka, 2002). The *mecA* gene is encoded on the 21-60 kb mobile genetic element, staphylococcal chromosome cassette *mec* (SCC*mec*) (Wielders et al., 2002).

In addition to the *mecA* gene, the SCC*mec* contains within the *mec* complex the *mecA* regulators (*mecI* and *mecR1) (*Katayama et al., 2000). Still another major element of the SCC*mec* is the cassette chromosome recombinase (*Ccr)* (*ccrAB* or *ccrC)* (Saber et al., 2017) responsible for the insertion and the excision from a site within the chromosomal open reading frame (*orfX)* which encodes ribosomal RNA methyltransferase (Foster & Geoghegan, 2015). Thirteen distinct types of SCC*mec* have been identified (Miragaia, 2018) where the hospital acquired MRSA are associated with the complex SCC*mec* types (I,II,III,VI, and VIII) while community acquired MRSA are associated with smaller SCC*mec* types (IV, V and VII) (Foster & Geoghegan, 2015). Types I, IV, V and VI encode resistance to β-lactam antibiotics only while types II and III confer resistance properties to multiple drugs because they encode plasmid pUB110 responsible for aminoglycoside resistance, pI258 responsible for resistance to heavy metals, and pT181, as well as, transposable element Tn554 which provides MLS (macrolide, lincosamide and streptogramin) resistance (Saber et al., 2017). Moreover, the majority of methicillin resistant *S. aureus* strains associated with community acquired infections including SSTIs and pneumonia encode in their genome the PVL toxin (Landrum et al., 2017).

Severe infections caused by MRSA strains are usually treated with vancomycin (McGuinness, Malachowa, & DeLeo, 2017). Vancomycin is a glycopeptide that inhibits the synthesis of bacterial cell wall, as well as, affecting the permeability of the cell membrane and the synthesis of DNA by the bacterial cells (Watanakunakorn, 1984). Intermediate resistance of *Staphylococcus aureus* to Vancomycin emerged in

isolates from Japan in 1997 and they were referred to as vancomycin intermediate-resistant *Staphylococcus aureus* (VISA) as they exhibited increased minimal inhibitory concentration to vancomycin ranging between 3–8 µg/ml (Gardete & Tomasz, 2014). Strains with complete resistance to vancomycin at MIC 128 µg/ml were first reported by the CDC in a patient in the United States in 2002 and those strains were termed vancomycin resistant *S. aureus* (VRSA) (CDC, 2002). Resistance to vancomycin is conferred by the *vanA* operon that was transferred on the transposable element Tn1546 from resistant enterococci to *S. aureus* (Perichon & Courvalin, 2009). Vancomycin binds with high affinity to D-alanyl-D-alanine peptide residue of Lipid II, a cell wall precursor, yet Tn1546 encodes the altered residue D-alanyl-D-lactate which exhibits a lower binding affinity to vancomycin (Gardete et al., 2014). A wide range of infections are associated with VRSA including wound infections, orthopaedic infections and was isolated from patients with diabetes, chronic skin ulcers, obesity or renal insufficiency (Antony, 2014; Sievert et al., 2008; Tosh et al., 2013).

1.2. Bacterial biofilm

1.2.1. Definition of a biofilm

Biofilms are bacterial cell populations embedded in a hydrated exopolysaccharide matrix that adhere to one another or to solid-liquid interfaces as well as biotic or abiotic surfaces (Costertonet al., 1995; Flemming & Wingender, 2010). Cell aggregates existing within the biofilm communicate between one another through physical and chemical interaction as well as acquiring new genetic properties through horizontal gene transfer, yet, competition exists between the coexisting bacterial cells within the biofilm (Costerton et al., 1995). Both Gram negative and Gram positive bacteria are

biofilm formers. Around 60% of nosocomial infections are associated with biofilms formed by Gram positive bacteria (O'Toole, Kaplan, & Kolter, 2000).

1.2.2. Stages of biofilm formation

Biofilm development passes through five distinct stages which are: reversible bacterial adhesion which is a reversible type of attachment of bacterial cells to a particular surface, irreversible bacterial adhesion established through production of extracellular polymers, initiation and maturation of a structured biofilm and finally detachment and dispersal of bacterial cells from the biofilm matrix (Stoodley et al., 2002).

1.2.2.1. Stage 1: Reversible Bacterial Adhesion

The first step of biofilm formation is the conditioning layer which is the scaffold of the biofilm composed of organic or inorganic elements. Those elements present within the fluid and by means of the gravitational force can deposit on a substrate to find this conditioning layer. In turn, this layer will allow the access of bacterial colonies to the substrate which will stabilize the bacterial colonies and provide the nutrients required for their enhanced growth. This stabilization is favored by the tensile forces and surface charges between the substrate and the conditioning layer (Garrett, Bhakoo, & Zhang, 2008).

Free-floating bacterial cells move from flowing fluids to the conditioned substrate through either their appendages or via the physical forces and adsorb to the surface. Those physical forces that mediate this reversible adhesion of the bacterial cells are known as DVLO forces sand include Van Der Waals forces in combination electrostatic interactions (Garrett, 2008). The DVLO theory was established in 1940s and explained by Derjaguin, Landau, Verwey and Overbeek (DVLO) and is used as a qualitative and quantitative measure to calculate the energy variations that affect adhesion of bacteria to surfaces, assuming that bacteria are colloidal particles and their

adhesion to a surface is based on the balance between additive Van Der Waals forces and electrostatic interactions of these particles (Adair, Suvaci, & Sindel, 2001; Hermansson, 1999; Ohshima, 2014).

Other factors that contribute to bacterial adhesion to a surface are its cells surface proteins and its capsular polysaccharides (Veerachamy, Yarlagadda, Manivasagam, & Yarlagadda, 2014). *Staphylococcus aureus* genome contains the *ica* locus which encodes for an intercellular adhesion (ICA) and a high molecular weight capsular adhesion (PS/A). Those two proteins contribute to the attachment of the bacteria to the substratum (O'Toole et al., 2000).

A shift from a reversible to an irreversible mode of bacterial adhesion occurs. The bacterial cells within a bacterial community that are capable of overcoming the repulsive physiochemical forces and forming a stable bond between their appendages and the conditioning surface through oxidative reactions are now more tightly attached to the biotic or abiotic surface (Garrett, Bhakoo, & Zhang, 2008).

1.2.2.3. Stages 3 and 4: Initiation and maturation of a structured biofilm

After they attach to the substratum, bacterial cells adapt to surviving within the biofilm by producing EPS and acquiring resistance to antibiotics (O'Toole et al., 2000).

On the adhesion surface, bacterial cells start to proliferate and adhere to one another via their polysaccharide intercellular antigen (PIA) forming micro-colonies which further proliferate and aggregate in layers of bacterial clusters to become macro-colonies (Veerachamy, et al., 2014). At this stage, bacterial macro-colonies are surrounded by a protective extracellular matrix. The EPS is composed of a large proportion of branched exopolysachharides, proteins and extracellular DNA from lysed bacterial cells. Exopolysaccharides include homopolysaccharides such as glucans and fructans that are responsible for the architecture of the biofilm (Flemming

& Wingender, 2010). Proteins of the matrix include extracellular enzymes that degrade long hydrophilic or hydrophobic biological polymers present within the biofilm (DNA, protein, cellulose…). They include, as well, matrix degrading enzymes which degrade the matrix and allow dispersal of the bacteria when the nutrients become scarce in the biofilm environment (Flemming & Wingender, 2010). Structural proteins are also part of the EPS and their role is to strengthen and maintain the structure of the extracellular matrix and they include the biofilm associated surface proteins (BAP) of *S. aureus* which promote biofilm formation by *S. aureus* (Flemming & Wingender, 2010). Those closely aggregated cells within the biofilm unlike planktonic cells communicate with one another through intercellular signals known as quorum sensing (Parsek & Greenberg, 2005). This communication influences a set of distinct bacterial functions including adhesion, virulence, and horizontal gene transfer of resistance and other genes (Valen & Scheie, 2018). Mature biofilms that now contain millions of closely packed colonies have the "pillar and mushroom" shape (Watnick & Kolter, 2000). The environment within the biofilm is heterogeneous varying in pH, amount of oxygen and availability of nutrtients (Uppuluri & Lopez-Ribot, 2016).

The EPS surrounding the aggregated bacterial cells has a set of significant functions. It promotes the aggregation and immobilization of bacterial cells within the biofilm (Deka et al., 2018; Kumar Singha, 2012). It additionally keeps the biofilm environment hydrated preventing bacterial death from desiccation and provides a nutrient rich environment for the bacteria through up-taking of the host's nutrients (Sutherland, 2001). The EPS protect the bacterial cells within the biofilm. It allows the bacterial cells to evade the host immune system by sterically hindering the access of phagocytes and other immune constituents such as complement components and antimicrobial

peptides into the biofilm as well as sequestering positively charged immune components via the eDNA of the matrix (Gunn, Bakaletz, & Wozniak, 2016).

1.2.2.4. Stage 5: Detachment and dispersal of bacterial cells from the biofilm matrix

Biofilm dispersal is determined by a set of cues including nutrient abundance, levels of oxygen and nitric oxide and density of cells within the biofilm (Guilhen, Forestier, & Balestrino, 2017; Uppuluri & Lopez-Ribot, 2016). In certain bacterial strains, the enrichment of the biofilm environment with nutrients induces the dispersal of the bacterial cells from the biofilm to colonize distinct site as in the case of *Pseudomonas aeruginosa* (Uppuluri & Lopez-Ribot, 2016). On the other hand, biofilm dispersal is induced by nutrient limitation or metabolic by-products accumulation in the biofilm environment (Solano, Echeverz, & Lasa, 2014). Similarly, low oxygen or nitric oxide levels within the biofilm trigger the detachment of bacterial colonies such as *S. aureus* and their dispersal (Uppuluri & Lopez-Ribot, 2016). Sensing those environmental cues is mediated by the bacterial quorum sensing systems such as the Agr system in *S. aureus* and second messengers such as cyclic di-GMP (Solano et al., 2014; Uppuluri & Lopez-Ribot, 2016). The intracellular level of cyclic di-GMP is critical for transition from planktonic to sessile growth state such that low cyclic di-GMP levels induce dispersal of bacteria from the biofilm while its elevated levels promote biofilm formation (Romling, Galperin, & Gomelsky, 2013).

1.2.3. Sites of biofilm formation: non-biological and biological surfaces

Bacterial biofilms form in nature and are associated with a variety of environmentally useful functions. Biofilms form in bioreactors and aid via the biofilm channel like structures formed in treatment of groundwater contaminated with petroleum (Massol-Deyá, Whallon, Hickey, & Tiedje, 1995). In addition to that, biofilms form in extreme environments such as under low pH conditions, at extremely high or low temperatures.

Bacterial biofilms can form in acid mine drainage such as those of *Leptospirillum* where they oxidize sulfur regulating the release of this toxic metal into the environment (Baker & Banfield, 2003; Ram et al., 2005). Additionally, bacteria such as actinobacteria or cyanobacteria can survive and form biofilms in hot springs such as those formed on the carbonate rocks of the Danube River (Borsodi et al., 2018). They can, on the other hand, form polar biofilm such as marine bacteria of Antarctica (Jeong et al., 2014). Clinically, bacterial biofilms are strongly associated with increased pathogenesis of the organism. Bacterial biofilms are able to form on teeth leading to dental plaques, on urinary catheters leading to urinary tract infections (UTI) and on prosthetic devices associated with chronic diseases such as osteomyelitis (Hall-Stoodley & Stoodley, 2009; Stickler, 2008; Wagner, Aytac, & Hänsch, 2011).

1.2.4. Mechanical and biological factors involved in *S. aureus* Biofilm formation

Surface attachment of *S. aureus* during biofilm formation involves non-covalent interactions with the surface, particularly, hydrophobic and acid-base interactions. *S. aureus* biofilm formation involves, as well, bacterial surface proteins that possess adhesive properties or that alter the physiochemical properties of the bacterial cell surface (Abdallah et al., 2014; Otto, 2013). *S. aureus* attachment to an abiotic surface such as a catheters or prosthetic devices involves non-covalently attached surface proteins and exopolymers produced by the bacteria (Jaglic et al., 2014). Autolysins, in particular the major autolysin AtlA are well-known *S. aureus* surface proteins that are bound to the bacterial cell surface by weak polar and non-polar interactions (Jaglic et al., 2014). They possess a dual role where they act as hydrolytic enzymes involved in degradation of bacterial cell wall during binary fission on one hand and as adhesive proteins critical for adhesion of *S. aureus* to abiotic surfaces such as polystyrene surfaces on the other hand (Porayath et al., 2018). AtlA is composed of 1257 amino

acids and exists in an inactive pro-protein form which is activated upon proteolytic cleavage. AtlA is cleaved into a signaling sequence, a pro-peptide sequence not well characterized, and two catalytic proteins which are an amidase and a glucosaminidase (Bose et al., 2012). In addition to mediating adhesion to abiotic surfaces, AtlA plays a role in binding to a variety of host cellular proteins allowing colonization and infection in the host such as fibronectin, vitronectin, thrombosondin 1, gelatin and heparin as well as uptake of *S. aureus* into non-professional phagocytic cells mediated by the heat shock cognate protein (Hsc70) (Jaglic et al., 2014; Porayath et al., 2018). Moreover, other exopolymers produced by *S. aureus* such as the extracellular DNA (eDNA) plays a significant role in adhesion of *S. aureus* to abiotic surfaces such as glass surfaces depending on the physical and chemical properties of the surface and the surrounding environment (Regina et al, 2014). The production of eDNA is thought to be mediated by autolysins such as AtlA mentioned previously and other autolysins such as cidA which promote the lysis of bacterial cells leading to the release of the bacterial DNA which is then used by the remaining cells for adhesion and biofilm formation (Qin et al., 2007; Rice et al., 2007). In addition, the negatively charged teichoic acid in the cell wall of *S. aureus* promotes its adhesion to abiotic surfaces mainly those that are positively charged yet, it still can, via the Van der Waals forces, attach to hydrophobic or low negatively charged surfaces (Gross et al., 2001).

On the other hand, adherence of *S. aureus* to biotic surfaces is mediated by a set of surface proteins that are termed: MSCRAMM which stands for microbial surface components recognizing adhesive matrix molecules. MSCRAMM are covalently linked to the bacterial cell wall peptidoglycan via their LPXTG sorting signal motif (Roche, 2003). Those proteins have the ability to bind to the host extracellular matrix proteins such as fibronectin, fibrinogen, elastin, heparin, collagen…etc. (Patti et al.,

1994). *S. aureus* fibronectin binding proteins are FnBPa and FnBPb. They play a role in adhesion of *S. aureus* to host endothelial and epithelial tissues as well as to other abiotic polystyrene surfaces (Foster et al., 1999; Houston et al., 2011; Patti et al, 1994). This adhesion promotes initiation of biofilm formation and development. Additionally, fibronectin binding proteins are critical for invasion of host tissues through allowing internalization of *S. aureus* into non-professional phagocytes such as fibroblasts through bridging the bacteria to the host $\alpha_5\beta_1$ integrin (Sinha et al., 1999). *S. aureus* Fibrinogen binding proteins, also known as clumping factors encoded by *clfA and clfB* genes, elastin binding proteins encoded by *ebps*, collagen adhesin encoded by *cna,* Enolase, which binds laminin, encoded by *eno* and bone sialoprotein (Bbp) encoded by *bbp,* function in binding to a particular component of the host extracellular matrix promoting adhesion of *S. aureus* to host tissue (Carneiro et al, 2004; Downer et al., 2002; Patti et al, 1994; Tung et al., 2000). In addition, other *S. aureus* surface proteins involved in adherence to host tissues and biofilm formation include *S. aureus* surface proteins such as SasG and SasC. SasG that allow adherence of *S. aureus* to host nasal epithelial tissues; while SasC also plays a role in cell aggregation and formation of biofilms (Roche, 2003; Schroeder et al., 2009).

Furthermore, *S. aureus* proteins that are critical in biofilm formation are the Biofilm Associated Proteins (Bap). Bap proteins are high molecular weight proteins composed of 2276 amino acid chain and possessing multiple domains similar to other Gram positive cell surface proteins (Lasa & Penadés, 2006). It was first identified in *S. aureus* strain V329 of bovine origin (Cucarella et al., 2001). Bap play an essential role in attachment and cell to cell adhesion on abiotic surfaces. However, it negatively regulates attachment to *S. aureus* to biotic surface such as host tissues during the primary stages of biofilm formation yet, it contributes to persistence of biofilm on host

tissues at later stages of biofilm formation (Cucarella et al., 2002; Cucarella et al., 2004; Lasa & Penadés, 2006).

During biofilm maturation, the primary mediator of cellular aggregation and cell-cell adhesion is the polysaccharide intercellular adhesion (PIA) (Otto, 2008). It is a linear glucosaminoglycan also termed poly-β(1-6)-*N*-acetyl-glucosamine knowns as PNAG (Otto, 2008). In addition to its role in cellular aggregation, PIA plays a role in the structure of the biofilm matrix, adhesion to abiotic surfaces and escaping the host immune system (Otto, 2008). PIA is encoded by the *ica* operon which is part of the accessory genome hence it isn't present in all *S. aureus* strains (Fluckiger et al., 2005; Otto, 2008). The *ica* locus carries a set of genes, *icaA, icaD, icaB, icaC and icaR* (Arciola, Campoccia, Ravaioli, & Montanaro, 2015). *icaA* encodes for an N-acetylglucosaminyltransferase which synthesizes the intercellular adhesion polysaccharide from UDP-N-acetylglucosamine. *icaD* optimises the function of the enzymatic activity of transferase. *icaC* is responsible for exporting the synthesized polysaccharide to the bacterial surface. *icaB* encodes for a protein deacetylase responsible for partially de-acetylating PIA allowing its fixation on the outer surface promoting the structuring of the biofilm polysaccharide matrix. *icaR* encodes for a negative transcriptional regulator of the *ica* operon (Cerca, Brooks, & Jefferson, 2008). PIA is a significant virulence factor of *S. aureus* where it is associated with catheter infections and infections associated with prosthetic devices (Arciola, Baldassarri, & Montanaro, 2001; Fluckiger et al., 2005).

On the other hand, the detachment and dispersal of the bacterial cells is mediated by a set of enzymatically active proteins capable of disrupting the matrix of the biofilm under certain environmental conditions and allowing bacterial cells to detach from the biofilm matrix. These proteins include, the previously mentioned, *S. aureus* proteases

such as the V8 serine protease (SspA), Aureolysin metalloprotease and staphopain cysteine protease (SspB) (Lister & Horswill, 2014). SspA and Aureolysin are both capable of degrading the biofilm associated protein (Bap) which is essential for biofilm formation (Martí et al., 2010). In addition to that, SspA is capable of degrading the fibronectin binding protein (FnBP) while Aureolysin is capable of cleaving the clumping factor ClfB both of which are major components of the biofilm extracellular matrix (Abraham & Jefferson, 2012; McGavin, Zahradka, Rice, & Scott, 1997). This proteolytic activity of the *S. aureus* enzymes plays a key role in biofilm disruption and matrix degradation promoting bacterial cell detachment from the biofilm. Moreover, *S. aureus* nucleases Nuc1 and Nuc2 were also shown to play a role in evasion of the immune system by altering the neutrophil extracellular traps (NETs) and were negatively regulated with accumulation of eDNA in the biofilm matrix (Lister & Horswill, 2014). This indicated a potential contribution of *S. aureus* nucleases to disruption of the matrix and dispersal in the host during infection. However, the role of *S. aureus* proteases and nucleases was only demonstrated in vitro (Otto, 2013). Moreover, significant effectors in *S. aureus* dispersal in vitro and in vivo were found to be the phenol-soluble modulins (PSMs). PSMs are short peptides rich in an amphipathic alpha-helix composed of both hydrophobic and hydrophilic amino acids providing them with surfactant properties (Le et al., 2014; Peschel & Otto, 2013). They are divided into PSMα, PSMβ and PSM δ-toxin. PSMα1 up to PSMα4 are the smaller peptides composed of around 20 amino acids encoded at the *psm*α locus. PSMβ1 and PSMβ2 are longer by 44 amino acids peptides encoded at the *psmβ* locus (Le et al., 2014). PSM δ-toxin is encoded by the gene *hld* within the RNAIII locus (Peschel & Otto, 2013). PSMs are exported outside the cell via specialized transporters termed Pmt which stands for PSM transporter. PSMs surfactant-like property enables their

aggregation to one another forming oligomers allowing them to spread on surfaces or form biofilms via their physiochemical properties (Peschel & Otto, 2013). On the other hand, they contribute significantly to biofilm detachment and dispersal through altering the non-covalent interactions including the hydrophobic and polar interactions between the molecules forming the matrix (Otto, 2013). In addition to their role in biofilm structuring and dispersal, PSMs activate the host pro-inflammatory response, in particular PSM α and PSM δ-toxin and alter phagocytosis after engulfment of *S. aureus* in vitro through killing of neutrophils (Wang et al., 2007). PSMs are under the direct and strict regulation of the *S. aureus* quorum sensing system, Agr which induces PSM production at high cell concentration in the biofilm (Peschel & Otto, 2013).

The Agr system is the *S. aureus* quorum sensing system and it stands for accessory gene regulator system. It is encoded by the *agrBDCA* operon within the core genome of *S. aureus* (Kavanaugh & Horswill, 2016). The agr locus consists of two promoters P2 which directs transcription of RNAII and P3 which directs transcription of RNAIII (Le & Otto, 2015). The genes *agrA, agrB, agrC* and *agrD* are present within the RNAII locus. *agrD* encodes the precursor of AIP which is the auto-inducing peptide that is further processed by the endopeptidase and chaperone protein AgrB allowing the maturation of AIP and its export outside the bacterial cell (Tan, Li, Jiang, Hu, & Li, 2018). Once the extracellular concentration of AIP exceeds a particular threshold of around 10 μM, the *agr* system is activated (Kavanaugh & Horswill, 2016; Tan et al., 2018). The *agrA* and *agrC* loci encode a two component signal transduction system where AgrC is a histidine phosphokinase which auto-phosphorylates and transfers the phosphate group to AgrA which is a response regulator (Le & Otto, 2015; Novick et al., 1993; Tan et al., 2018). Then AgrA in its turn bind and activates P2 directing the expression of *agrBDCA* operon and P3 promoter expressing RNAIII which is a

transcriptional regulator of several *S. aureus* virulence factors (Tan et al., 2018). Agr quorum sensing systems regulates around 15 distinct virulence factors of *S. aureus* including exoenzymes like proteases and lipases as well as coagulases and haemolysins (Abdelnour et al., 1993; Kavanaugh & Horswill, 2016). At high cell density, Agr quorum sensing system upregulates the expression of *S. aureus* exoenzymes and downregulates proteins involved in adhesion and surface attachment (Cheung, Wang, Khan, Sturdevant, & Otto, 2011).

During the initial stages of biofilm development and at low cell density attachment to the host surface is crucial for biofilm initiation so expression of adhesive proteins is induced. On the other hand, at later stages of biofilm lifecycle where the cell density has increased and the infection is established quorum sensing signals are detected by the Agr system to upregulate *S. aureus* lytic enzymes. At this stage, bacterial cells prefer to leave the biofilm environment where nutrients are becoming scarce and disperse to acquire nutrients from the host tissues and express molecules necessary to evade the host immune response (Cheung et al., 2011).

1.2.5. Contribution of biofilms to pathogenesis

The National Institute of Health (NIH) indicated that the 65% to 80% of infections caused by microbes are associated with biofilm development (Jamal et al., 2018).

Bacteria within the biofilm are significantly more resistant to antibiotics as a result of reduced nutrient supply within the biofilm leading to decreased growth rate, production of various polymers to form the biofilm matrix capable of modifying the antibiotic or affecting its diffusion into the bacterial cell and increase in the rate of horizontal gene transfer of resistance genes between the cells within the biofilm as a result of increased cell density (Figueiredo, et al., 2017; Gilbert, Das, & Foley, 1997).

In addition to that, biofilms are associated with medical devices related infections. Biofilms can develop on joint prosthetic devices, breast implants, mechanical heart valves, ventricular catheters, urinary catheters, defibrillators and pacemakers (Jamal et al., 2018). In the case of mechanical heart valves, these can damage the tissue surrounding the site of implantation causing platelets and fibrin clots to form at that region, thus allowing bacteria to colonize the surrounding tissue of the attached valve leading to native valve Endocarditis (Donlan, 2001; Donlan & Costerton, 2002).

Moreover, urinary catheters are well known to be colonized by *S. aureus.* Infections associated with urinary catheters are known to be affected by the period of catheterization, where a period longer than 4 weeks will inevitably allow establishment of biofilms in the patient's urinary bladder (Jones et al., 2006). The urinary catheters are attractive sites for biofilm formation because of the irregular area surrounding the eye hole of the catheter which promotes aggregation of the bacterial cells, as well as, the texture of the catheter which is made up of silicon or latex that also promote biofilm adhesion initiating biofilm formation and spreading causing catheter blockage and UTIs (Jones et al., 2006). Furthermore, biofilm formation was associated with ophthalmic diseases such as Keratitis, where bacterial adhesion and biofilm formation on the storage cases of contact lenses were related to microbial eye, Keratitis (McLaughlin-Borlace et al., 1998).

On the other hand, there are non-device related bacterial biofilms. Such types of biofilms are associated with airway infections ranging from acute or chronic sinusitis (rhinosinusitis) in the upper airway of the host to cystic fibrosis in the lower airway of the host in addition to osteomyelitis, periodontitis, chronic otitis media and wound infections (Del Pozo, 2018).

S. aureus is the primary agent responsible for osteomyelitis which is a bone infection that can be either a haematogenous infection or caused by contiguous spread of the infectious agent, through a surgical wound for example (Calhoun, Manring, & Shirtliff, 2009). *S. aureus* has the ability to adhere to the bone matrix or to bone prosthetic implants in the host via its fibrinogen binding protein (FnBp) which is capable of binding fibronectin of the host extracellular matrix as well as other components of the extracellular matrix (ECM) (Ciampolini, 2000). This interaction will facilitate colonization and biofilm formation on the orthopaedic device which in turn protects the bacteria within the biofilm from host immune response, cause the bacteria to enter into a dormant state and reduce phagocytosis by the host PMNs due to the presence of the implant. The above combined factors will eventually lead to *S. aureus* mediated osteomyelitis.

In addition, the prevalence of *S. aureus* in the oral cavity of a host is around 13% to 15% and it is associated with peri-implantitis which is an infection of the soft tissue around the tooth implant and less frequently with periodontitis which is an inflammation of the supporting bones of the tooth (Archer et al., 2011).

1.3. *Origanum syriacum*

1.3.1. Taxonomy and distribution of *Origanum syriacum*

Origanum syriacum is a plant that belongs to the genus *Origanum* which belongs to the family *Lamiaceae*. This genus includes 70 different species and subspecies that mainly grow in the wild in the Mediterranean region (Torres et al., 2012). It is commonly and widely spread though-out Lebanon in many regions including the coastal areas surrounding the Beirut river as well as in areas of higher altitudes such as Arz Al-Chouf and Baskinta. It is commonly known as Zaatar (Tohme & Tohme, 2007).

1.3.2. Description of *Origanum syriacum* morphology

Origanum syriacum is a woody base plant that can grow between 30 cm and up to 80 cm in height. Its leaves are obtuse and the petiole which attaches the stem of the plant with the leaf blade is covered with tiny hairs. The petiole length ranges between 1 cm and 3 cm in length. Its flowers are white in color. It has a fasciculate terminal and axillary spikes and its calyx is 2 mm indented (Tohme & Tohme, 2007).

1.3.3. *Origanum syriacum* chemical components

1.3.3.1 The *Origanum syriacum* essential oils

Based on the literature, its content of essential oils, in specific, vary between one season and the other and between one area and the other. However, analysis of the major essential oils constituting *Origanum syriacum* revealed that these were thymol and carvacrol (Zein et al., 2010). Analysis of the components of the Lebanese *Origanum syriacum,* conducted at the Lebanese University, showed that these essential oils made up thirty-six compounds (Loizzo et al., 2009). These compounds mainly included γ-terpinene (12.6%), p-cymene (8.7%), 2-isopropyl-1-methoxy-4-methylbenzene (7.9%) and α-terpinene (2.5%) (Loizzo et al., 2009) while the two main components of the essential oil were thymol (24.7 %) and carvacrol (17.6 %).

1.3.3.2. The *Origanum syriacum* fatty acids

Analysis of the fatty acids present in both wild and cultivated *Oriagnum syriacum* in Lebanon revealed that it contained predominantly myristic acid present in 61.50 % and 79.07 % in wild and cultivated leaves, respectively, which allows for their use to synthesize cholesterol by the liver (Zein et al., 2010). In addition, *Oriagnum syriacum* contained two essential fatty acids that could not be biologically synthesized by the human body: α-linolenic acid present in 51.73 % in the wild and in 49.34 % in the cultivated *Oriagnum syriacum* and Lenoleic acid present in about 10% in *Oriagnum*

syriacum (Zein et al., 2010). These two essential fatty acids are known to be involved in a variety of functions in the human body. Moreover, palmitic acid was also an essential acid present in around 15% in *Oriagnum syriacum.* This renders *Oriagnum syriacum* a major source of fatty acids necessary for the human body.

1.3.3.3. The *Origanum syriacum* minerals

It was shown that a population of *Origanum syriacum* contained a variety of minerals that varied between flowering and non-flowering plants but was found to be higher in the cultivated as compared to the wild plants. Some of the minerals present included Potassium (K) 65 mg, calcium (Ca) 40 mg, manganese (Mn) 1.25 mg and iron (Fe) 5 mg (Zein et al., 2010).

1.3.4. Pharmacological significance of *Origanum syriacum*

1.3.4.1. Antimicrobial effect of *Origanum syriacum*

Antibacterial effect of Origanum syriacum

Origanum syriacum was shown to possess an antimicrobial effect against a variety of microorganisms. Methanolic extract of *Origanum syriacum* had a minimum inhibitory concentration (MIC) of 1 mg/ml against Gram positive bacteria such as *Staphylococcus aureus* and an MIC of 2 mg-ml against *Pseudomonas aeruginosa* (Assaf et al., 2016). It was shown as well that the antibacterial effect of *Origanum syriacum* extended against additional Gram-negative bacteria including *Proteus* spp., *Klebsiella pneumoniae* and *Yersenia enterocolitica.* The MIC_{50} of *Origanum syriacum* against *Proteus* spp. and *Klebsiella pneumonia* was 1.5 µl/ml while MIC_{50} against *Yersenia enterocolitica* was 6.25 µl/ml (Al-Mariri & Safi, 2014). Moreover, *Oregano.* Spp. were shown to possess a bactericidal effect on *Helicobacter pylori,* which is the primary cause of gastric ulcers in humans (O’Mahony, 2005).

Antifungal effect of Origanum syriacum

Origanum syriacum was shown to possess an antifungal effect against the pathogenic fungus *Candida albicans* with an MIC of 1 mg/ml (Assaf et al., 2016). In addition, the antifungal activity of *Origanum syriacum* was demonstrated against three other species of fungi, namely: *Penicillium* spp., *Fusarium oxysporum* and *Aspergillus niger* with a MIC of 0.1 µl/ml (Daouk, Dagher, & Sattout, 1995). Moreover, the essential oils of *Origanum syriacum* were also shown to possess an inhibitory effect against the plant fungal pathogen *Sclerotinia sclerotiorum,* where treatment of soil with its essential oils remarkably inhibited the growth of the fungus and was able to increase the growth of tomato seeds by 69.8% (Jiang, Fu, Guoqing, & Ghabrial, 2013; Soylu, Yigitbas, Soylu, & Kurt, 2007)

Antiviral effect of Origanum syriacum

It was shown that *Origanum syriacum* major constituents, which are thymol, and carvacrol possessed an antiviral effect against the activity of *Herpes Simplex* Virus 1 (HSV-1) such that the IC_{50} was reached at 7 µM of the essential oils, with a 90% inhibition of the viral activity (Lai et al., 2012). In addition, it was shown that thymol was capable of reducing the infection ability of Hepatitis A virus (HAV), while rosmarinic acid, another component in *Origanum* spp., possessed antiviral activity against norovirus: a virus which infects the human gastrointestinal tract leading to nausea, vomiting and diarrhea but can persist for long periods of time in immunocompromised patients (Sánchez & Aznar, 2015). Moreover, rosmarinic acid was also shown to have an anti-viral effect against feline calcivirus, a virus that infects the upper respiratory tract of cats (Caswell & Williams, 2016; Niendorf et al., 2016; Sánchez & Aznar, 2015).

1.3.4.2. Anti-inflammatory effect of *Origanum syriacum*

Origanum syriacum was shown to possess an anti-inflammatory effect, for it was shown to inhibit the pro-inflammatory cytokine Interleukin 6 (IL-6) and to reduce the release of anti-inflammatory cytokine IL-10 (Assaf et al., 2016). In addition, it was shown that Rosmarinic acid, a major phenolic compound in Oregano, possessed an anti-inflammatory effect on murine mice models with a pulmonary inflammation (Sanbongi et al., 2004). Rosmarinic acid was also shown to reduce the increase of eosinophils in the alveolar fluids of the murine models and reduced the expression of the pro-inflammatory cytokines Il-4 and IL-5 in the lungs (Sanbongi et al., 2004). Moreover, it was shown that rosmarinic acid was capable of inhibiting the T-cell restricted (TCR) signaling, preventing the proliferation of T cells (Won et al., 2003), and was shown also to promote apoptosis of T cells in patients with rheumatoid arthritis, via the mitochondrial pathway (Hur et al., 2007).

1.3.4.3. Anti-oxidant role of *Origanum syriacum*

It was shown that *Origano* possessed high level of antioxidants ranging between 75 and 138 mmol/100 g (Dragland et al., 2003). In addition, *Origano* was shown to possess the ability to suppress the production of the free radical nitric oxide (NO) by the lipopolysaccharide-activated macrophages, in vitro, through inhibiting the enzymatic activity of nitric oxide synthase (NOS) (Tsai et al., 2007). Nitric oxide is produced by the enzyme Nitric oxide synthase induced by the inflammatory response of macrophages, hepatocytes, fibroblasts and other host defense cells and is directed towards pathogens invading the host. Excessive NO production will increase the free reactive nitrogen species (RNS) leading to host tissue damage (Tsai et al., 2007). In the absence of an *in vivo,* enzymatic defense mechanism to reduce the damage of RNS,

Origano was shown to possess an anti-inflammatory response to protect against host tissue damage.

1.3.4.4. Anti-tumor effect of *Origanum syriacum*

Origanum syriacum showed an antipoleferative effect on the MCF7 breast cancer cell line at an IC_{50} of 6.4 μg/mL (Al-Kalaldeh, Abu-Dahab, & Afifi, 2010). *Origanum syriacum* also was shown to possess a cytotoxic effect on THP-1 human leukemia cells. It significantly reduced the viability of the THP-1 cells in a concentration dependent manner and IC_{50} was achieved at a concentration of 2.126 mg/ml (Ayesh, Abed, & Faris, 2014). Moreover, *Origanum syriacum* possessed a more potent effect on peripheral blood mononuclear cells (PMBC), where the IC_{50} was reached at 0.4247 mg/ml of the *Origanum syriacum* ethanolic extract (Ayesh, Abed & Faris, 2014). This concentration was remarkably lower than the IC_{50} of *Origanum syriacum* ethanolic extract against THP-1 cancer cell line.

1.3.4.5. Other pharmacological benefits of *Origanum syriacum*

It was demonstrated that *Origanum syriacum* possessed an amoebicidal effect against pathogenic amoeba such as *Acanthamoeba castellanii* which causes keratitis, a sight threatening disease (Degerli et al., 2012; Hurt, Proy, Niederkorn, & Alizadeh, 2003). The methanolic extract of *Origanum syriacum* possessed the ambeobicidal effect against the trophozites and cysts of *Acanthamoeba castellanii* at a concentration of 32 mg/ml of extract (Degerli, et al., 2012). In addition, it was reported that rosmarinic acid, a component of *Origanum syriacum,* demonstrated an anti-depressive effect that was revealed in the forced-swimming test of murine mice models (Takeda et al., 2002).

1.4. *Rosmarinus officinalis*

1.4.1. Taxonomy and distribution of *Rosmarinus officinalis*

Rosmarinus officinalis which is commonly known as rosemary belongs to the *Lamiaceae* family of herbs (Begum et al., 2013; de Oliveira, Camargo, & de Oliveira, 2019). It grows mainly in the Mediterranean region but is also present in different regions around the world. *Rosmarinus officinalis* is a perennial shrub that remains green all year long (González-Trujano et al., 2007).

1.4.2. Description of *Rosmarinus officinalis* morphology

Rosmarinus officinalis is an aromatic plant. Its shrub can grow between 0.8 m to 2 m. Its dark green leaves are narrow in width and rolled into the inner side (González-Trujano et al., 2007). It is melliferous and has a bi-labiate calyx that is white in color and bi-labiate corolla violet in color (Tohme & Tohme, 2007).

1.4.3. *Rosmarinus officinalis* chemical components

Rosmarinus officinalis is composed of two major classes of chemical constituents which are essential oils and phenolic compounds. The abundance of these chemical constituents vary between one season and the other and one region and the other (Özcan & Chalchat, 2008).

1.4.3.1 *Rosmarinus officinalis* essential oils

Rosmarinus officinalis contains several distinct essential oils. The two major essential oils of *Rosmarinus officinalis* are 1,8 cineole which is also known as Eucalyptol and α-pinene (Imad, Israa, & Hawraa, 2015; Fadil et al., 2018). The percentage of 1,8-cineole in *Rosmarinus officinalis* ranges between 19.59 % to 27.23 % while that of α-pinene ranges between 14.2% to 19.43 % (Celiktas et al., 2007, Özcan & Chalchat, 2008; Wang, Wu, Zu, & Fu, 2008). Another essential oil of *Rosmarinus officinalis* is camphor which exists at a percentage ranging from 9.9 % to 18.35 % (Celiktas et al., 2007; Özcan & Chalchat, 2008; Wang, et al., 2008). *Rosmarinus officinalis* contains additional essential oils that possess lower abundance in the plant including β-pinene

(6.08% to 10.27%) and Camphene, Isoborneol, Linalool, Mycrene, Terpinen-4-ol, γ-Terpinene and eugenol that exist in concentrations ranging from 0.54 % to 3.34 % (Miladi et al., 2013; Özcan & Chalchat, 2008; Wang, et al., 2008).

1.4.3.2. *Rosmarinus officinalis* phenolic and flavonoid compounds

Rosmarinus officinalis is composed of three significant phenolic compounds which are rosmarinic acid, carnosic acid and carnosol. Additionally, *Rosmarinus officinalis* contains around 15 distinct flavonoids among which are luteolin, kaemoferol, ladanein, 6''-O-(E)-feruloylnepitrin (2) and 6''-O-(E)-p-coumaroylnepitrin (Bai et al., 2010).

1.4.4. Pharmacological significance of *Rosmarinus officinalis*

Rosmarinus officinalis possesses a variety of functions. It is used as a food additive as well as a food preservative instead of synthetic preservatives (Miladi et al., 2013). *Rosmarinus officinalis* is also a major constituent of folk medicine because of its various medicinal roles. It is used as an antimicrobial, anti-inflammatory, anti-diabetic and antinociceptive agent.

1.4.4.1. Anti-inflammatory effect of Rosmarinus officinalis

Rosmarinus officinalis displayed an anti-inflammatory effect in murine models. The essential oils of *Rosmarinus officinalis,* mainly camphor, possessed an anti-inflammatory effect in rats (Borges et al., 2018). In that experiment, oedema, which is the accumulation of fluids in the tissues as a result of inflammation (*Collins Dictionary of Medicine*, 2004) was induced in the rat paws using the inflammatory agent, carrageenan (Borges et al., 2018). In that model, treatment with *Rosmarinus officinalis* significantly reduced inflammation in the mouse model at ED_{50}= 261 mg/kg. In addition, *Rosmarinus officinalis* was reported to protect against irritant contact dermatitis (ICD) in humans (Fuchs, Schliemann-Willers, Fischer, & Elsner, 2005).

Moreover, *Rosmarinus officinalis* anti-inflammatory effect was revealed in its ability to reduce sunburn caused inflammation through its component, carnosol (Yeo et al., 2018). Mice with induced skin inflammation through exposure to Ultraviolet B (UVB) radiation and treated topically with carnosol displayed a wide range of anti-inflammatory responses compared to untreated mice. This anti-inflammatory effect of carnosol was revealed through reduced levels of pro-inflammatory cytokines including tumor necrosis factor α (TNFα) and Interleukin-1β (IL-1β), reduced expression of protein markers activated during an inflammatory response such as iNOS and COX-2 and reduced activation of the transcription factor STAT-3 that is responsible for activating the pro-inflammatory response in the host. Furthermore, *Rosmarinus officinalis* was shown to possess an anti-cancer activity where its diterpenes carnosic acid, carnosol and rosmanol were shown to possess the ability to modulate altered signaling pathways that are involved in various tumors (Petiwala & Johnson, 2015).

1.4.4.2. Antimicrobial effect of *Rosmarinus officinalis*

Antibacterial effect of Rosmarinus officinalis

Rosmarinus officinalis was reported to possess a bacteriostatic property against Gram positive bacteria like *Staphylococcus aureus* and *Staphylococcus enteritidis* at a MIC of 15 μL/mL and *Listeria monocytogenes* at a MIC 5 μL/mL (Ait-Ouazzou et al., 2011). In addition to that, *Rosmarinus officinalis* was shown to possess antibacterial effect against Gram negative bacteria like the opportunistic pathogen *Pseudomonas aeruginosa* at a MIC higher than 2 mg/ml (Assaf, et al., 2016). Moreover, *Rosmarinus officinalis* was reported to also have an antibacterial effect against the foodborne pathogen *Aeromonas hydrophila* (Alves de Azerêdo, Stamford, Queiroz de Figueiredo, & Leite de Souza, 2012). Treatment of *Aeromonas hydrophila* bacterial culture with 20 μL/ml *Rosmarinus officinalis* essential oils led to a drastic decrease in

bacterial count, as well as, morphological disruption within 3h of exposure to the *Rosmarinus officinalis* essential oils and to a loss of attachment between bacterial cell wall and cell membrane and thus disruption of the bacterial outer membrane, leading to release of the cytoplasmic components. *Aeromonas hydrophila* which is present in water habitats and colonizes fish and raw meat, has the potential to become a foodborne pathogen associated with a wide range of infections including septicemia, wound infection and gastrointestinal disturbances (Daskalov, 2006). So, the bactericidal effect *Rosmarinus officinalis* on this microorganism suggested that as a food additive, it can insure food safety against *Aeromonas hydrophila.* Another foodborne pathogen on which *Rosmarinus officinalis* was effective were *Salmonella typhimirium,* at a MIC of 2 % (v/v) (Fadil et al., 2018). In addition to that, combining *Rosmarinus officinalis* with *Thymus vulgaris* and *Myrtus communis* in a mixture increased susceptibility of *Salmonella typhimirium* lowering the MIC (Fadil et al., 2018).

Antifungal effect of Rosmarinus officinalis

Rosmarinus officinalis was reported to possess an antifungal effect against the pathogenic microorganism *Candida albicans* at a MIC higher than 2 mg/mL (Assaf, et al., 2016). In another experiment, *Rosmarinus offiicinalis* displayed anti-fungal acitivty against *Candida albicans* isolated from bovine animals having mastitis and *Rosmarinus officinalis* possessed a MIC between 23.99 mg/mL and 31.08 mg/mL (Ksouri et al., 2017). In addition, *Rosmarinus officinalis* was shown to display an antifungal effect against a wide range of fungi that infected vegetables and certain types of mushrooms such as *Botrytis cinerea, Sclerotinia sclerotiorum, Fusarium oxysporum*, *Phytophthora parasitica, Cladobotryum mycophilum* and *Trichoderma aggressivum* using the disk diffusion technique (Diánez et al., 2018). Moreover,

Rosmarinus officinalis displayed a mild yet present antifungal effect against *Malassezia* species which cause atopic dermatitis in dogs (Khosravi, Shokri, & Fahimirad, 2016). *Rosmarinus officinalis* essential oils had a MIC ranging between 100 and 850 mg/mL.

Antiviral effect of Rosmarinus officinalis

An antiviral effect against Human Immunodeficiency virus (HIV-1) was reported for *Rosmarinus officinalis.* Its essential oils interfered with the HIV-1 Trans activator of transcription (Tat) proteins responsible for viral transcription and significantly reduced its interaction with the trans activating region (TAR) RNA, a process which normally increases the number of viral transcripts in the host (Feriotto et al., 2018). In addition, *Rosmarinus officinalis* showed an inhibitory effect against Human respiratory syncytial virus which infects the lower region of the human respiratory tract through inhibiting the expression of viral proteins in infected cells, displaying 66 folds decrease in production of viral particles (Shin et al., 2013). Moreover, *Rosmarinus officinalis* displayed an inhibitory effect against *Herpes simplex* virus 1 (HSV-1) and HSV-2 through targeting the free form of the virus prior to adsorption. These results suggested that *Rosmarinus officinalis* can act as a potential topical treatment for Herpes infections (Nolkemper, Reichling, Stintzing, Carle, & Schnitzler, 2006).

1.4.4.3. Antinociceptive effect of Rosmarinus officinalis

Rosmarinus officinalis was reported to possess the ability to act as a pain reliever. It was shown that treatment of three pain induced murine models with *Rosmarinus officinalis* relieved their pain with a potential similar to pain killers (González-Trujano et al., 2007). The first murine model was a mouse injected with acetic acid which induced writhing in the mouse. Treatment with *Rosmarinus officinalis* ethanolic extracts displayed a dose dependent reduction in writhing movement of the mouse

similar to post injection of acetic acid with an ED_{50}= 108.84 mg/kg. The second murine model, subjected to formalin test, displayed after treatment with *Rosmarinus officinalis,* a decrease in both neurogenic pain and inflammatory pain behavior characterized by licking and trembling in early and late stages of the test, similar to what was observed upon treating the mice with Tramadol. Similarly, in the third murine model which was a pain-induced functional impairment model in the rate (PIFIR), treatment with *Rosmarinus officinalis* extract displayed a dose dependent antinociceptive effect in the rat similar to that of Tramadol and Acetylsalicylic acid at an ED_{50}=222.78 mg/kg. These experiments revealed the characteristics of *Rosmarinus officinalis* as a pain reliever. Furthermore, in a different experiment, mice model that had induced neuropathy due to chronic constriction injury (CCI) of sciatic nerved were treated with *Rosmarinus officinalis.* This treatment was successful in reducing neuropathic pain in mice with CCI compared to untreated mice (Ghasemzadeh et al., 2016).

1.4.4.4. Other pharmaceutical uses of *Rosmarinus officinalis*

Rosmarinus officinalis was also shown to have an anti-hypotensive effect on patients that were suffering from low blood pressure (Fernández, Palomino, & Frutos, 2014). Treatment of the patients with *Rosmarinus officianlis* essential oils, revealed a significant increase in blood pressure during the period of treatment as compared to prior treatment and this was reflected as well on improved physical and psychological well-being related to improved blood pressure. In addition, *Rosmarinus officinalis* was found to possess a protective function on the heart. It was shown that *Rosmarinus officinalis* extract reduced ischemic myocardial infarction resulting from increased oxidative stress in the patient through reducing oxidant levels and regulating vasoconstrictors and vasodilators level (Cuevas-Durán et al., 2017). Moreover,

rosmarinic acid which is a component of *Rosmarinus officinalis* was shown to possess a cardioprotective role against acute myocardial infarction and arrhythmia (Javidanpour, et al., 2017). Furthermore, *Rosmarinus officinalis* was shown to be a potential agent to protect from cases of toxicity caused by certain drugs such as the antibiotic gentamicin (Hegazy et al., 2018). It was shown that co-administration of *Rosmarinus officinalis* aqueous extract into gentamicin treated rats displayed a hypolipidemic effect characterized by decreases in lipid parameters and pancreatic lipases as well as protection against gentamicin associated liver toxicity indicated by reduced secretion of liver enzymes and levels of bilirubin (Hegazy et al., 2018).

1.5. Pulse Field Gel Electrophoresis (PFGE)

1.5.1. Definition of Pulse Field Gel Electrophoresis

Pulse Field Gel Electrophoresis (PFGE) is a molecularly based technique that is used mainly for typing microorganisms, which means determining the relatedness of different isolates of a certain species of a microorganism, and for mapping genes based on analysis of the genomic DNA isolated from that microorganism, digested by restriction enzymes and separated on agarose gel under the impact of an electric field alternating in direction (Simner, Khare, & Wengenack, 2015; Stubbs, 2001).

1.5.2. History of PFGE

PFGE was first described in 1980 in the time when gene mapping and localization of disease-causing genes was evolving (Simner, Khare, & Wengenack, 2015; Stubbs, 2001). During that time, research efforts were aiming at finding genetic markers, allocating their position and studying their linkage to genes that caused inherited diseases. Yet, the distance between the marker and the gene was poorly determined by chromosome walking and other primitive cloning techniques. Gel Electrophoresis was the commonly used technique to measure the size of DNA fragments such that DNA

fragments cut by restriction enzymes migrated on an agarose gel in a unidirectional electric field in a size dependent manner. Yet, gel electrophoresis succeeded in separating DNA fragments with sizes that did not exceed 30-40 kilobases long. However, in 1982, David Schwartz, Charles Cantor and their colleagues introduced Pulse Field Gel Electrophoresis in which the electric field was made to alternate periodically in opposite directions throughout the run (Stubbs, 2001). PFGE was directed by the electric field generated by two focused electrodes placed at each side of the gel generating an electric current which intersected at 45° angle. This electric field alternated directions such that each set of electrodes alternated with the other set of electrodes while generating the current for a specific duration and at specific time intervals allowing large DNA fragments to reorient themselves and separate from one another according to their size while migrating forward away from the well (Simner, Khare, & Wengenack, 2015; Stubbs, 2001).

1.5.3. PFGE Methodology

PFGE is based on extraction of chromosomal DNA of the bacteria through lysis of the cell wall within plugs of agarose. Chromosomal DNA is digested with restriction endonucleases into 12 or more high molecular weight DNA fragments that can reach up to 2 Megabases in size and that migrate on the agarose gel through the alternating electric field (Golding et al., 2015; Reed, Stemper, & Shukla, 2007; Sharma-Kuinkel, Rude, & Fowler, 2016). The result of fragmentation pattern of each isolate is termed a pulsotype and all the pulsotypes together are displayed in what is called a dendrogram. The bands of each isolate are compared with other bands of other isolates in order to identify the relatedness between these isolates. Isolates that share a common pattern and have the same bands in common indicate the pattern of the outbreak strain which is termed Type A (Tenover et al., 1995). The remaining isolates are then classified

based on how related they are to the outbreak pattern such that those that differ in only 2 to 3 bands from the outbreak pattern are considered closely related to the outbreak while those that differ in 4 to 6 bands are considered possibly related to the outbreak pattern (Tenover et al., 1995). Closely and possibly related patterns are classified as subtypes of A such as A1, A2…etc. Patterns that have more than 7 bands difference from the outbreak pattern are considered different and classified as a distinct type such as type B, type C… etc. Concerning the epidemiological interpretation of the PFGE patterns, indistinguishable patterns are part of the outbreak, closely or possibly related patterns are closely or possibly part of the outbreak, respectively, while different patterns are not part of the outbreak (Tenover et al., 1995).

1.5.4. Advantages of PFGE

PFGE is often considered as a gold standard due to its discriminatory power, reproducibility, and ease of execution, data interpretation, cost, and availability (Bannerman et al., 1995). The PFGE equipment is available and suits moderate sized laboratories (Reed, Stemper, & Shukla, 2007). This method allows to determine the genetic relatedness of certain bacterial isolates and identify those that have generated from an outbreak, those that are similar and supposed to have originated from a common parent and those that are totally distinct isolates (Tenover et al., 1995). Based on the genetic relatedness determined by PFGE and the epidemiological relatedness of the isolates they are classified as the same or as distinct strains and determined whether they are outbreak strains or endemic strains (Tenover et al., 1995). Computer based software is then used to analyze the results and compare them to information in databases allowing strain identification.

1.5.5. Characterization of *Staphylococcus aureus* through PFGE

Since 1990, PFGE was considered of the most sensitive techniques to genetically characterize MRSA (Reed, Stemper, & Shukla, 2007). The restriction endonuclease *SmaI* is the enzyme mainly used to fragment *S. aureus* chromosomal DNA (Golding et al., 2015). PFGE with its genotypic typing of strains was found to be more effective in typing *Staphylococcus aureus* compared to other typing techniques such as phage typing, capsular polysaccharride serotyping, zymotyping, ribotyping and plasmid profile typing which are less reproducible (Schlichting et al., 1993). In addition to that, PFGE is capable of detecting minor changes such as point mutations in the bacterial genome as well as recent genetic evolution in the bacterial genome providing more sensitivity in determining phylogenetic and epidemiological classification of the isolates (Struelens et al., 1992).

Chapter II
Materials and methods

2.1. *Staphylococcus aureus* isolates collection

The *Staphylococcus aureus* isolates included in this study were obtained from Lebanese American University Medical Center-Rizk Hospital (LAUMC-RH). Each bacterial sample was then streaked on Tryptic Soy Agar (TSA) for definitive identification.

2.2. Definitive identification of the *Staphylococcus aureus* isolates

2.2.1. *Staphylococcus aureus* growth on Mannitol Salt Agar (MSA)

Isolated *Staphylococcus aureus* colonies from the initial culture plates were used to streak Mannitol Salt Agar (MSA) plates (biolab®) and were incubated overnight at 35° C. The colonies that appeared were yellow in color as expected for *Staphylococcus aureus.*

2.2.2. *Staphylococcus aureus* identification by the coagulase test

Definitive identification of *Staphylococcus aureus* was done by the coagulase test using rabbit plasma. The rabbit plasma powder was dissolved in sterile deionized water as recommended by the manufacturer and transferred into sterile tubes. Then, a loopful of each *Staphylococcus aureus* isolate was transferred into one of the tubes which were incubated for 4 hours at 35° C. The tubes were then examined for clot formation. Isolates that formed a clot of plasma were confirmed to be *Staphylococcus aureus.* Those that did not form a clot after four hours were left overnight and examined on the following day for clot formation.

2.3. Differentiation of isolates into methicillin susceptible and methicillin resistant *Staphylococcus aureus*

As per the standard procedure outlined by the "Clinical and Laboratory Standard Institute 2015" guidelines (CLSI guide, 2015), isolated colonies of each *Staphylococcus aureus* isolate were inoculated using a loop or a sterile swab in 0.85 % saline solution to achieve a turbidity equivalent to 0.5 McFarland standard. The inoculum would contain around 1.5 x 10^{8} CFU/ml.

The Muller-Hinton Agar (MHA) (acumedia ®) was prepared based on the manufacturer's instructions and 25 ml were poured into each sterile petri dish. A sterile cotton swab was then dipped into each adjusted suspension tube, rotated several times on the inner walls of the tube above the fluid level and pressed against the wall of the tube to remove excess fluid. The MHA plates were inoculated by passing the swab over the entire surface of the plate (CLSI guide, 2015). This process was repeated two additional times after rotating the plate 60° every time. Finally, the swab was passed around the rim of the plate. The plates were then left for few minutes before the drug-impregnated disk was added.

The 30 µg Cefoxitin disk was then added at the center of the agar plate. Cefoxitin is used to determine *mecA* mediated oxacillin resistance (CLSI guide, 2015). The plate were then incubated at 35° C for 16-18 hours after which the diameter of the inhibition zone was measured. As per the standard procedure, a diameter ≤ 21 mm indicated a methicillin-resistant *Staphylococcus aureus* (MRSA) strain while a diameter ≥ 22 mm indicated a methicillin-susceptible *Staphylococcus aureus* (MSSA) strain.

2.4. *Staphylococcus aureus* typing using Pulse field Gel Electrophoresis (PFGE)

2.4.1 Materials used in PFGE

1. Brain Heart Infusion (BHI) agar (biolab®)

   -Prepared per the manufacturer instructions

2. Tryptic soy broth (biolab®)

   -Prepared per the manufacturer instructions

3. NaOH solution (10N)

   -NaOH pellets: 40 g

   -deionized water: 60 ml

   They were dissolved by stirring and heating at 45˚ C. Once the pellets completely dissolved, deionized water was then added to 100 ml final volume.

4. Tris-EDTA-NaCl (TEN) buffer (500 ml)

   -Trisma Base: 6 g (0.1 M)

   -NaCl: 4.35 g (0.15 M)

   -Di-Na-EDTA: 3.72 g (0.1 M)

   The pH was adjusted to 7.5

   Deionized water was then added to reach 500 ml final volume. The buffer was then autoclaved for 20 minutes.

5. EC buffer (Modified)

   -Tris-HCl: 948 mg (6 mM)

   -NaCl: 58 g

   -Di-Na-EDTA: 37 g

   -N-Lauroyl sarcosine: 5 g (0.5%)

-NaOH: 5 g

-deionized water 900 ml

The pH of the buffer was adjusted to 7.5 using 10N NaOH.

Deionized water was then added to reach 1000 ml final volume

The buffer was then autoclaved for 20 minutes

6. TE buffer (Modified)

-Tris-HCl stock: 50 ml (10 mM)

-Di-Na EDTA stock: 200 ml (20 mM)

-deionized water 4 L

Deionized water was then added to reach 5 L final volume

The buffer was then autoclaved for 20 minutes

7. 2% SeaKem agarose gel

-Seakem agarose: 0.6 g

-TE buffer: 30 ml

The agarose was dissolved in TE buffer by heating in a microwave for 1 minute and then heating at intervals of 30 sec until the agarose completely dissolved

8. Lysostaphin (1 mg/ml)

4 mg of Lysosaphin (BioChemika, 62965) were completely dissolved in 4 ml of sterile deionized water

9. ES buffer

-Di-NA-EDTA: 186 g (0.5 M)

-N-Lauroyl Sarcosine: 10 g (1%)

-NaOH: 20 g

-deionized water: 800 ml

The pH of the buffer was adjusted to 9.0 with 10 N NaOH.

Deionized water was added to reach 1000 ml final volume

The buffer was then autoclaved for 20 minutes

10. Proteinase K (20 mg/ml)

11. 1.0 M Tris-HCl Stock

-Tris-HCl: 15.76 g (1 M)

-deionized H_2O: 80 ml

The pH of the buffer was adjusted to 7.6 with 10 N NaOH.

Deionized was then added to reach 100 ml final volume.

The buffer was then autoclaved for 20 minutes in a liquid cycle

12. 0.5 M EDTA

-Di-Na EDTA: 93 g (0.5 M)

-deionized water: 400 ml

The pH was adjusted to 8 with 10 N NaOH.

Deionized water was then added to reach 500 ml final volume

The EDTA solution was then autoclaved for 20 minutes

13. 0.5X TBE buffer

2360 ml of 0.5X TBE buffer was prepared from 10X TBE buffer stock solution

-10X TBE buffer: 118 ml

-Deionized water: 2242 ml

14. 1.0 % SeaKem agarose running gel

-SeaKem agarose: 1.5 g

-0.5X TBE buffer: 150 ml

The mixture was heated in a microwave for 1 minute and then heated at separate intervals of 30 seconds until the agarose completely dissolved.

15. *SmaI* restriction endonuclease (ThermoScientific)

2.4.2. PFGE procedure

2.4.2.1. Preparation of plugs

1. Each bacterial isolate was streaked on fresh BHI agar plate and incubated overnight at 37˚C
2. Isolated colonies from each plate were inoculated in 10 ml of fresh TSB to read an initial optical density of 0.1 at 540 nm. The TSB conical was then incubated at 37˚C until achieving an optical density of 1.0.
3. The bacterial cells were then harvested through centrifuging the inoculated TSB tubes at 5000 rpm for 25 minutes at 4 ˚C
4. The supernatant was discarded and the pellet was resuspended in 5 ml TEN buffer
5. The cell suspension was then centrifuged at 5000 rpm for 25 minutes at 4 ˚C
6. The supernatant was discarded and the pellet was resuspended in 2 ml of modified EC buffer
7. Chef plug molds (BioRad, 1703713) were used to prepare the plugs
8. 1 ml of the each bacterial cell suspension was mixed with 1 ml of 2% SeaKem agarose gel
9. 100 µL of 1 mg/ml Lysostaphin was added into each mixture (in step 8)
10. Very rapidly, 100 µL of each mixture was pipetted into the plug mold
11. The plugs were left to solidify for 30 minutes at 4 ˚C

12. After the plugs had completely solidified, they were ejected from the plug mold into 20 ml EC buffer.
13. The plugs were then incubated overnight at 37˚C with gentle shaking (50 rpm)
14. The following day, EC buffer was removed and 20 ml of ES buffer were added
15. 50 µL of 20 mg/ml Proteinase K were added to each conical as well
16. The conicals were incubated for 24 hours at 50 ˚C with gentle shaking (50 rpm)
17. After 24 hours, ES buffer was removed and 20 ml of TE buffer were added to each conical
18. The conicals were kept gently shaking (50 rpm) at room temperature for two hours.
19. Step 17 and 18 were repeated two more times.
20. The plugs can be then directly subjected to restriction by the restriction endonuclease or they can be stored in fresh TE buffer at 4 ˚C for up to two months.

2.4.2.2. Running the PFGE

1. One-third of each plug was cut using a blade and gently placed in a 1.5 ml micro-centrifuge tube
2. The restriction enzyme master mix was prepared and added to each micro-centrifuge tube:

   - 172 µL sterile deionized water

   - 25 µL of 10X Tango buffer

   - 4 µL *SmaI* restriction endonuclease

The plugs were then incubated overnight (16 hours) at 27 ˚C

3. After 16 hours, the restriction master mix was removed and 500 µL of 0.5X TBE were added to the micro-centrifuge tubes and incubated at room temperature for 30 minutes
4. While the plugs were equilibrating, the agarose for the running gel was then poured into the gel casting apparatus. It was left for 45 minutes to solidify
5. The plugs were then added each separately into the wells of the gel and the wells were then closed using agarose
6. 2200 ml of 0.5X TBE buffer were added in the pulse field chamber
7. The pump was turned on allowing the buffer to circulate
8. The cooling module was then turned on and allowed to cool to 14 ˚C
9. The gel was then placed in the pulse field chamber
10. The parameters were set as follows:

    - Initial Switch Time: 5 seconds
    - Final Switch Time: 40 seconds
    - Run Time: 20 hours
11. After the run was finished, the staining buffer was prepared

    - Ethidium Bromide (10 mg/ml): 40 µL
    - Deionized water: 400 ml
12. The gel was placed in the staining buffer and left shaking gently (50 rpm) for 45 minutes
13. The gel was then visualized under UV light using UV transilluminator as the source of light and a Polaroid Camera for capturing the gel image
14. The results were then analyzed using BioNumerics software version 4.0 (Applied Maths, Belgium) following the manual's instructions

2.5. Detection and quantification of *Staphylococcus aureus* biofilms

2.5.1 Preparation of the *S. aureus* strains

Based on the protocol used by Zmantar and his collogues (2010), biofilm production of *S. aureus* was determined using a semi-quantitative assay in a 96 well microtiter plate (costar®). The *S. aureus* isolates were streaked on TSA plates and incubated overnight at 35 °C. After incubation, a loopful of each *S. aureus* strain was inoculated in tryptic soy broth (TSB) (biolab®) and incubated again overnight. The overnight culture of TSB was then diluted 1:100 in TSB with 2% (w/v) glucose and 200 µL of the cell suspension was transferred into the 96 well microtiter plate. Each isolate was tested in eight wells and for each isolate eight wells that contained 200 µL of sterile TSB only were considered as a negative control. The 96 well microtiter plates were incubated aerobically overnight at 35 °C.

2.5.2. Staining of the 96 well microtiter plates containing *S. aureus*

The following day, the culture plates were removed from the incubator. The cell suspension was discarded from the wells and the wells were washed with 200 µL phosphate-buffered saline (PBS) (BioWhittaker ®) to remove the non-adherent cells. The plates were then inverted to remove the PBS. The plates were washed two more times with 200 µL PBS. 200 µL of 95% ethanol were added into each well to fix the biofilms. The ethanol was then removed and the wells were then stained with 100 µL of 1% (w/v) crystal violet (Bio Basic Canada INC., 548-62-9) for 5 minutes. Unbound crystal violet was then removed, and the wells were washed for three times with 300 µL of sterile distilled water.

2.5.3. Quantification of *S. aureus* biofilm

The optical density of each well was read on ELISA (Thermo Scientific, Varioskan flash) at 570 nm wavelength. Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Zmantar et al., 2010).

2.6. Processing of the tested plants

2.6.1. Collection of the plant Samples

Samples of *Origanum syriacum* commonly known as "Zaatar" was collected from the wilderness in Gharifeh located in Al-Chouf region in Mount Lebanon while the samples of *Rosmarinus officinalis* commonly known as "Rosemary" were collected from a domestically planted shrub in the same region. The plant samples were harvested in the month of April. The samples were washed thoroughly with tap water and dried at room temperature for one day. They were then placed in polystyrene bags and carried to the Microbiology Laboratory at the Lebanese American University, where they were kept at room temperature for processing.

2.6.2. Plant samples' preparation

The wooden stem of both *Origanum syriacum* and *Rosmarinus officinalis* was removed. Then, 25 g of each plant was weighed using a digital scale.

2.6.3. Preparation of the plants' extracts

2.6.3.1. Preparation of the methanolic extract *Origanum syriacum*

A concentration of 0.1 g/ml of *Origanum syriacum* methanolic extract was prepared. Twenty-five grams of *Origanum syriacum* were added to 250 ml of 80 % methanol. The mixture was then blended together in a blender until all the plant sample was heavily grinded. The obtained mixture was then poured through a sterile funnel into a

sterile Erlenmeyer flask. The Erlenmeyer flask was then well sealed and placed in shaking incubator at 42° C and 80 rpm for one week.

After one week, the mixture was removed from the shaking incubator and filtered to remove the remaining plant debris and was collected in a sterile container. The extract was then stored at 4° C for further use.

2.6.3.2. Preparation of the methanolic extract of *Rosmarinus officinalis*

Similarly, a concentration of 0.1 g/ml of *Rosmarinus officinalis* methanolic extract was prepared. Twenty-five grams of *Rosmarinus officinalis* were added to 250 ml of 80 % methanol. The mixture was then blended together in a blender until all the plant sample was heavily grinded. The obtained mixtue was then poured into a sterile Erlenmeyer flask. The Erlenmeyer flask was then well sealed and placed in a shaking incubator at 42° C and 80 rpm for one week.

After one week, the mixture was removed from the shaking incubator and filtered to remove the remaining plant debris and collected in a sterile container. The extract was then stored at 4° C for further use.

2.6.4. Studying the effect of *Origanum syriacum* and *Rosmarinus officinalis* methanolic extracts on the growth of *Staphylococcus aureus*

2.6.4.1. Preparation of the *Staphylococcus aureus* inoculum and test plates

As per the standard procedure of the "Clinical and Laboratory Standard Institute 2015" guidelines, isolated colonies of each *Staphylococcus aureus* isolate were inoculated using a loop or a sterile swab in 0.85 % saline solution to achieve a turbidity equivalent to 0.5 McFarland standard (CLSI guide, 2015). This suspension contained around 1.5 x 10^{8} CFU/ml. The Muller-Hinton Agar (MHA) (acumedia®) was prepared as per the manufacturer's instructions and 25 ml of MHA were poured into each sterile petri dish (CLSI guide, 2015)

2.6.4.2. Inoculation of the plates with *Staphylococcus aureus*

As per the standard procedure of the “Clinical and Laboratory Standard Institute 2015” guidelines, a sterile cotton swab was then dipped into each adjusted suspension tube, rotated several times on the inner walls of the tube above the fluid level and pressed against the wall of the tube to remove excess fluid (CLSI guide, 2015). The MHA plates were inoculated by streaking the entire surface of the plate with the swab. This process was repeated two additional times after rotating the plate 60° every time. Finally, the swab was passed around the rim of the plate.

2.6.4.3. Addition of the *Origanum syriacum* and *Rosmarinus officinalis* methanolic extracts to the plates

To each of the inoculated plates, a well was introduced in the center of each of the plates using a cork borer. In each of these wells, either *Origanum syriacum* or *Rosmarinus officinalis* methanolic extract was added. For each, *Origanum syriacum* or *Rosmarinus officinalis* methanolic extract different volumes were tested on the bacterial strains: 50 μL, 100 μL, 150 μL, 200 μL and 300 μL. The plates were then incubated at 35° C for 16-18 hours. The diameter of the zone of inhibition of growth was then measured, and the anti-bacterial effect of the extracts was determined for each tested volume.

2.6.5. Studying the effect of *Origanum syriacum* and *Rosmarinus officinalis* methanolic extracts as inhibitors of *Staphylococcus aureus* biofilm formation

A protocol similar to the one used by Zmantar and his collogues (2010), was used to study the inhibitory effect of *Origanum syriacum* and *Rosmarinus officinalis* on *S. aureus* biofilm formation.

2.6.5.1. Addition of the extract to the 96 well microtiter plates

The effect of the methanolic extract of *Origanum syriacum* or *Rosmarinus officinalis* on inhibition of *S. aureus* biofilm was tested at a concentration of 0.02 g/ml which is five times lower than the concentration of the initially prepared extract (0.1 g/ml) and at a concentration of 0.01 g/ml which is ten times lower than the concentration of the initially prepared extract (0.1 g/ml). Each of the indicated concentrations (0.02 g/ml and 0.01 g/) were tested at volumes where no inhibition zone was detected (based on the results of section 2.6.4.3) in order to determine the inhibitory effect of the extracts on *S. aureus* biofilm formation. The volumes used were 50 μL and 100 μL for *Origanum syriacum* and 100 μL and 150 μL for *Rosmarinus officinalis.*

A 96 well microtiter plate was used to determine the inhibitory effect of the extracts on *S. aureus* biofilm formation. For *Origanum syriacum*, each of the previously assigned volumes for each concentration (0.02 g/ml and 0.01 g/ml) were added, separately, to the well of the 96 well microtiter plate. Each volume of each concentration was repeated in 6 wells. Six additional wells for that same volume at that same concentration served as negative control. The plates were then incubated at 35 ° C until the wells containing the extract completely dried. The same procedure was carried with *Rosmarinus officinalis* methanolic extract.

2.6.5.2. Preparation of the *S. aureus* strains

The *S. aureus* isolates were streaked on TSA plates and incubated overnight at 35 °C. After incubation, a loopful of *S. aureus* was inoculated in tryptic soy broth (TSB) and incubated overnight.

2.6.5.3. Addition of *S. aureus* to the 96 well plates containing the extract

The overnight culture of TSB was then diluted 1:100 in TSB with 2% (w/v) glucose and 200 μL of bacterial cell suspension was transferred into the well that contained the

dried extract. This step was carried in the 6 wells that contained the dried plant extract. The remaining 6 wells that contained the dried extract served as controls to measure the optical density of the extract alone. Also, 200 μL of bacterial cell suspension were then added into an empty well to compare the difference in the optical density in the well containing *S. aureus* alone to that containing *S. aureus* with the plant extract reflecting inhibition of biofilm formation of *S. aureus* in the presence of the extract. This step was repeated in 6 wells.

2.6.5.4. Staining of the 96 well microtiter plates containing *S. aureus* with either *Origanum syriacum* or *Rosmarinus officinalis* methanolic extracts

The same procedure mentioned in section 2.5.2. was used in order to stain the 96 well microtiter plate.

2.6.6. Studying the effect of the methanolic solution of major chemical components of *Origanum syriacum* and *Rosmarinus officinalis* as inhibitors of *Staphylococcus aureus* biofilm formation

2.6.6.1. The major chemical components of the tested plants

For each plant whether *Origanum syriacum* or *Rosmarinus officinalis* the major chemical components were determined based on data from the literature. The major chemical components of *Origanum syriacum* were thymol existing in 24.7 % and carvacrol existing in 17.6 % (Loizzo et al., 2009). The major chemical components of *Rosmarinus officinalis* were 1,8-cineole existing in 27.23 %, α-pinene existing in 19.43 % and camphor existing in 14.26% (Wang et al., 2008).

The chemical components utilized in the experiments were Thymol (Sigma-Aldrich, 16254-100G), Carvacrol (Aldrich), Camphor (Aldrich, 148075-100G), α-pinene (MERCK-Schuchardt, 232-067-8) and Eucalyptol (1,8-cineole) (Aldrich, C80601-100ml).

2.6.6.2. Preparation of the solutions of the major chemical components of each of the tested plants in the appropriate concentrations

Based on the literature, a solution of each of the previously mentioned chemical components reflecting their concentration in the plant extract dissolved in 80% methanol was prepared. The chemical components were also tested at concentrations five times and ten times higher than their concentration in the extract.

For Thymol, the concentrations tested were 0.0247 g/ml which is equivalent to its concentration in the plant extract, 0.124 g/ml which is five times higher than its concentration in the plant extract and 0.247 g/ml which is ten times higher than its concentration in the plant extract.

For Carvacrol, the concentrations tested were 0.0176 g/ml which is equivalent to its concentration in the plant extract, 0.088 g/ml which is five times higher than its concentration in the plant extract and 0.176 g/ml which is ten times higher than its concentration in the plant extract.

For α-pinene, the concentrations tested were 0.0194 g/ml which is equivalent to its concentration in the plant extract, 0.097 g/ml which is five times higher than its concentration in the plant extract and 0.194 g/ml which is ten times higher than its concentration in the plant extract.

For Camphor, the concentrations tested were 0.0143 g/ml which is equivalent to its concentration in the plant extract, 0.072 g/ml which is five times higher than its concentration in the plant extract and 0.143 g/ml which is ten times higher than its concentration in the plant extract.

For 1,8-cineole (Eucalyptol), the concentrations tested were 0.0273 g/ml which is equivalent to its concentration in the plant extract, 0.135 g/ml which is five times

higher than its concentration in the plant extract and 0.273 g/ml which is ten times higher than its concentration in the plant extract.

2.6.6.3. Addition of the chemical components of *Origanum syriacum* or *Rosmarinus officinalis* to the 96 well microtiter plates

The effect of each of these chemical components on inhibition of *S. aureus* biofilm formation was tested separately. The chemical components were tested at the three concentrations: equivalent to their concentrations in the extract, a concentration five times than their concentration in the extract and ten times higher than their concentration in the extract.

The volume of each chemical component added to the wells at each of the indicated concentrations was equivalent to the previously assigned volumes in section 2.6.5.1.

Similar to the procedure described in section 2.6.5.1., each volume of each component at each indicated concentration was transferred into a well of the 96 well microtiter plate. This step was carried out in 6 wells and six additional wells for that same volume at that same concentration of the solution of the chemical component served as negative controls. The plates were then incubated at 35 ° C until the solution completely dried.

2.6.6.4. Preparation of the *S. aureus* isolates

The *S. aureus* strains were prepared as mentioned in section 2.6.5.2.

2.6.6.5. Addition of *S. aureus* strains to the 96 well plates containing the chemical components of *Origanum syriacum* or *Rosmarinus officinalis*

Similar to the procedure mentioned in section 2.6.5.3., the overnight culture of TSB was then diluted 1:100 in TSB with 2% (w/v) glucose and 200 μL of bacterial cell suspension was transferred into the well that contained the chemical component. This step was carried out in 6 wells that contained the dried chemical component. The

remaining 6 wells that contained the dried chemical component served as controls to measure the optical density of the chemical component alone. Also, 200 µL of bacterial cell suspension were added to an empty well to compare the difference in the optical density in the well containing *S. aureus* alone to that containing *S. aureus* with the chemical component, reflecting inhibition of biofilm formation by *S. aureus* in the presence of the chemical component. This step was also carried out in 6 wells.

2.6.6.6. Staining of the 96 well microtiter plates containing *S. aureus* with each of the major chemical components of either *Origanum syriacum* or *Rosmarinus officinalis*

The same procedure mentioned in section 2.5.2. was used in order to stain the 96 well microtiter plate.

2.7. Data analysis of the effect of the extracts and their major components on inhibition of *S. aureus* biofilm formation

The percentage of inhibition of biofilm formation of the extracts or their major components was determined according to the following formula:

% inhibition =

$$\frac{O.D_{570nm} \text{ of isolate alone} - O.D_{570nm} \text{ of isolate in the experimental well}}{O.D_{570nm} \text{ of isolate alone}} \times 100$$

In order to determine the significance of this variation between the control well where the *S. aureus* isolate was present alone and the experimental well where the *S. aureus* isolate was placed with either the methanolic extract of *Origanum syriacum,* the methanolic extract of *Rosmarinus officinalis* or the solution of one of the major components of either *Origanum syriacum* or *Rosmarinus officinalis*, a 95% confidence interval was considered and the p value was calculated. A p value less than or equal to 0.05 but greater than 0.01 indicated a slightly significant variation between the experimental condition and the control (*), a p value less than or equal to 0.01 but

greater than 0.001 indicated a moderately significant variation between the experimental condition and the control (**) and a p value less than or equal to 0.001 indicated a highly significant variation between the experimental condition and the control (***).

Chapter III
Results

3.1. *Staphylococcus aureus* isolates identification

3.1.1. *Staphylococcus aureus* identification through the coagulase test

After incubation for four hours or overnight, all the plasma tubes with isolated *Staphylococcus aureus* colonies revealed a clot validating the identity of the isolates as *S. aureus.*

3.1.2. *Staphylococcus aureus* growth on Mannitol Salt Agar (MSA)

After incubation, all *S. aureus* isolates grew yellow colonies showing a typical mannitol fermentation while isolate number 7496 did not ferment mannitol which is expected for some *S. aureus* strains.

3.2. Differentiation of isolates into methicillin susceptible and methicillin resistant *Staphylococcus aureus*

Based on the diameter of the zone of inhibition of growth surrounding the cefoxitin disk (CLSI guide, 2015), six *Staphylococcus aureus* isolates were noted to be as resistant to methicillin (MRSA) and 15 were noted to be methicillin susceptible (MSSA) as shown in Table 1.

Table 1. Resistance of Staphylococcus aureus isolates to methicillin

	Isolate Number	Average Diameter width (mm)	Identity
1	12610	25.0 mm	MSSA
2	13073	26.3 mm	MSSA
3	12989	24.3 mm	MSSA
4	12634	25.3 mm	MSSA
5	2483	24.7 mm	MSSA
6	2484	26.3 mm	MSSA
7	2564	24.3 mm	MSSA
8	2553	26.0 mm	MSSA
9	7353	28.3 mm	MSSA
10	6281	24.0 mm	MSSA
11	45139	24.3 mm	MSSA
12	48865	26.3 mm	MSSA
13	10762	26.0 mm	MSSA
14	5862	27.0 mm	MSSA
15	14102	25.0 mm	MSSA
16	13074	17.7 cm	MRSA
17	12631	17.0 cm	MRSA
18	7496	17.0 cm	MRSA
19	4826	18.3 cm	MRSA
20	4588	15.0 mm	MRSA
21	43271	19.3 mm	MRSA

3.3. *Staphylococcus aureus* typing using Pulse field Gel Electrophoresis (PFGE)

The pulsotypes allowed to segregate the isolates into one major clade which was subdivided into two subclades where each contained a set of more closely related isolates (Figure 1). The rest of the isolates were each individually related to the other.

Notably, methicillin susceptible *Staphylococcus aureus* strains were more closely related to one another compared to methicillin resistant *Staphylococcus aureus* strains which were either grouped in pairs or individually. Interestingly, few methicillin susceptible isolates obtained from LAUMC-RH were more closely related to isolates obtained from another health center in Lebanon than to other methicillin susceptible isolates from the same hospital environment (Figure 1).

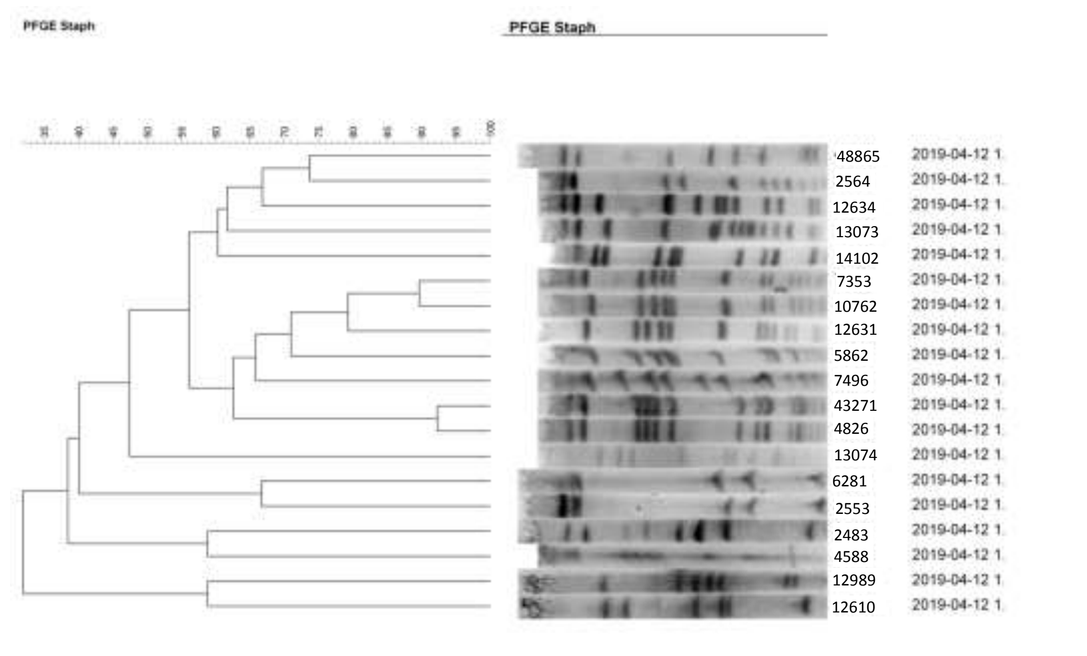


Figure 1. The dendrogram showing the relatedness between the tested Staphylococcus aureus isolates as indicated by pulse field gel electrophoresis (PFGE)

3.4. Detection and quantification of *Staphylococcus aureus* biofilms

The ability of the tested strains to form biofilms, was determined using the assay described by Zmantar et al., 2010. Based on their level of absorbance, the *Staphylococcus aureus* isolates were categorized into strong biofilm formers ($OD_{570\ nm} > 1$), low positive biofilm formers ($0.1 \leq OD_{570\ nm} \leq 1$) or non-biofilm formers ($OD_{570\ nm} < 0.1$). As indicated in Table 2.a absorbance of the stained 96 well microtiter plate measured at 570 nm, revealed that all the *Staphylococcus aureus* isolates were

biofilm formers. The majority of the isolates were low positive biofilm formers while only one isolate was a strong biofilm former (Table 2.b).

Table 2. *Staphylococcus aureus* biofilm formation

a. Biofilm formation of methicillin susceptible and methicillin resistant *S. aureus* strains measured at 570 nm

MRSA	TSB only	Isolate
13074	0.1352	0.6244
12631	0.1282	0.8979
7496	0.1362	0.3636
4826	0.1393	0.5948
4588	0.1352	0.5013
43271	0.1382	0.2504

MSSA	TSB only	Isolate
12610	0.1332	0.7159
13073	0.1238	0.4645
12989	0.1339	0.5054
12634	0.1305	0.4476
2483	0.1272	0.4184
2484	0.1334	0.4198
2564	0.1227	0.5777
2553	0.1369	0.3829
7353	0.1277	0.9077
6281	0.1244	0.3651
45139	0.1368	0.9343
48865	0.1032	0.4006
10762	0.1069	1.3069
5862	0.1774	0.3297
14102	0.1227	0.2844

b. The *Staphylococcus aureus* strains classification based on their biofilm forming abilities

Isolate Number	Identity	Δ (Bact., TSB)	Biofilm production
13074	MRSA	0.4892	+
12631	MRSA	0.7696	+
7496	MRSA	0.2274	+
4826	MRSA	0.4555	+
4588	MRSA	0.3661	+
43271	MRSA	0.1123	+
12610	MSSA	0.5828	+
13073	MSSA	0.3408	+
12989	MSSA	0.3715	+
12634	MSSA	0.3171	+
2483	MSSA	0.2912	+
2484	MSSA	0.2864	+
2564	MSSA	0.4551	+
2553	MSSA	0.2460	+
7353	MSSA	0.7800	+
6281	MSSA	0.2407	+
45139	MSSA	0.7975	+
48865	MSSA	0.2974	+
10762	MSSA	1.2000	+++
5862	MSSA	0.1523	+
14102	MSSA	0.1617	+

-: absence of biofilm formation

+: Low positive biofilm formation

+++: Strong biofilm formation

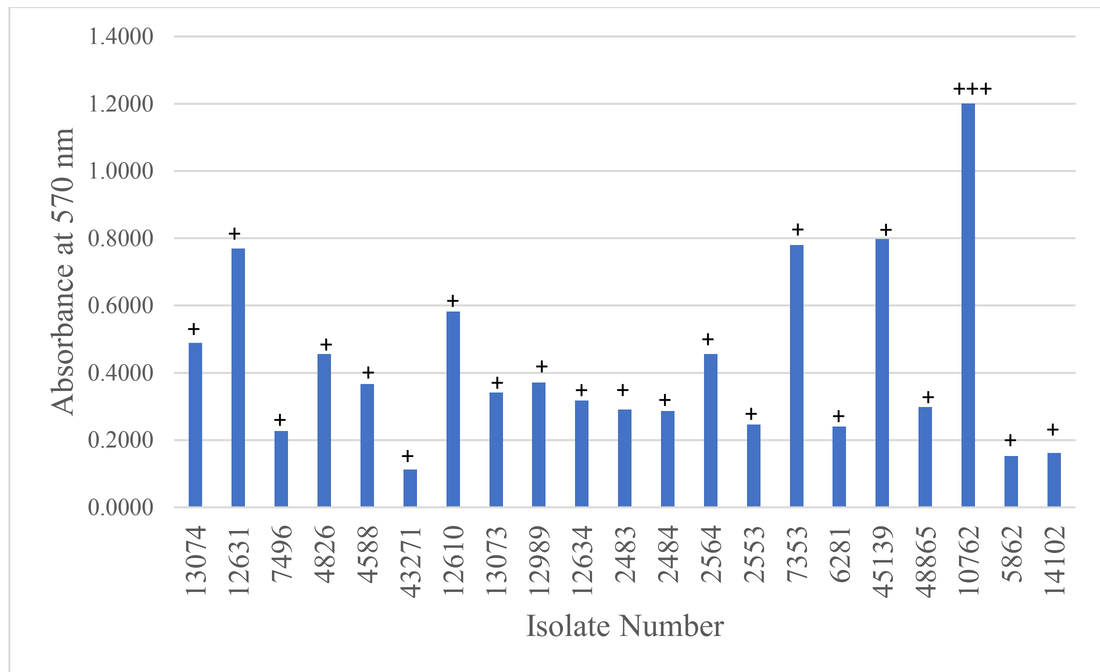


Figure 2. The *Staphylococcus aureus* biofilm forming abilitiy as demonstrated by variation of their optical density (at 570 nm) ((+): low positive biofilm formation; (+++): strong biofilm formation)

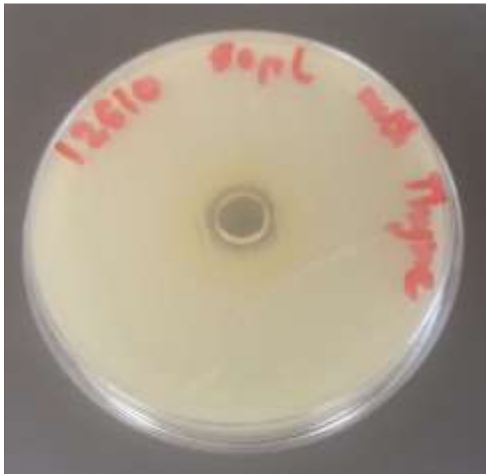
3.5. The effect of *Origanum syriacum* and *Rosmarinus officinalis* methanolic extracts on the growth of *Staphylococcus aureus*

3.5.1. The effect of *Origanum syriacum* methanolic extract on inhibition of *Staphylococcus aureus* growth

The *Origanum syriacum* methanolic extract was tested on the five isolates of *Staphylococcus aureus* numbered 12610, 12634, 12989, 13073 and 13074. The results revealed that *Origanum syriacum* methanolic extract of concentration 0.1 g/ml lacked a significant effect on the inhibition of *Staphylococcus aureus* growth at low volumes of 50 µL and 100 µL of the extract containing 0.005 g and 0.01 g of *Origanum syriacum* plant (Figure 3. (a, b), Figure 4. (a, b), Figure 5. (a, b), Figure 6. (a, b) and Figure 7. (a, b)). However, higher volumes of 150 µL, 200 µL and 300 µL of *Origanum syriacum* methanolic extract having a higher amount of 0.015 g, 0.02 g and 0.03 g of

Origanum syriacum plant, respectively, possessed a significantly increased inhibitory effect on the growth of the tested *Staphylococcus aureus* isolates indicated by the remarkably larger zone of inhibition of growth of the tested *Staphylococcus aureus* isolates treated with higher volumes of the *Origanum syriacum* methanolic extract (Figure 3. (c, d, e), Figure 4. (c, d, e), Figure 5. (c, d, e), Figure 6. (c, d, e) and Figure 7. (c, d, e)).

a.



b.

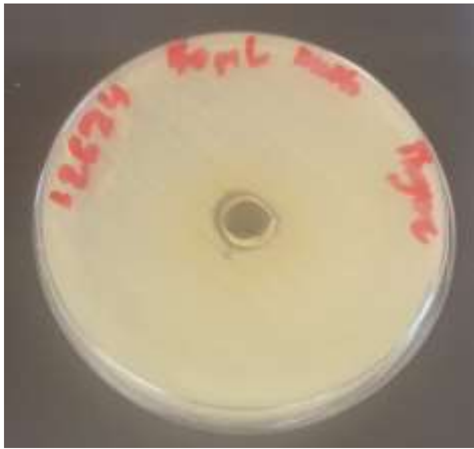
c.

d.

e.

Figure 3. Effect of different volumes of *Origanum syriacum* methanolic extract on growth of *Staphylococcus aureus* isolate 12610 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.



b.

c.

d.

e.

Figure 4. Effect of different volumes of *Origanum syriacum* methanolic extract on growth of *Staphylococcus aureus* isolate 12634 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.

b.

c.

d.

e.

Figure 5. Effect of different volumes of *Origanum syriacum* methanolic extract on growth of *Staphylococcus aureus* isolate 12989 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.

b.

c.

d.

e.

Figure 6. Effect of different volumes of *Origanum syriacum* methanolic extract on growth of *Staphylococcus aureus* isolate 13073 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.

b.

c.

d.

e.

Figure 7. Effect of different volumes of *Origanum syriacum* methanolic extract on growth of *Staphylococcus aureus* isolate 13074 a. 50 µL b. 100 µL c. 150 µL d. 200 µL e. 300 µL

3.5.2. The effect of *Rosmarinus officinalis* methanolic extract on the growth of *Staphylococcus aureus*

Rosmarinus officinalis methanolic extract was tested on the five isolates of *Staphylococcus aureus* numbered 12610, 12634, 12989, 13073 and 13074. The results revealed that *Rosmarinus officinalis* methanolic extract of concentration 0.1 g/ml lacked a significant effect on the inhibition of *Staphylococcus aureus* growth at the low volumes of 50 µL, 100 µL and 150 µL of the extract containing 0.005 g, 0.01 g and 0.015 g of *Rosmarinus officinalis* plant (Figure 8. (a, b, c), Figure 9. (a, b, c), Figure 10. (a, b, c), Figure 11. (a, b, c) and Figure 12. (a, b, c)). However, higher volumes of 200 µL and 300 µL of *Rosmarinus officinalis* methanolic extract having a higher amount of 0.02 g and 0.03 g of *Rosmarinus officinalis* plant, respectively, possessed an increased inhibitory effect on the growth of the tested *Staphylococcus aureus* isolates (Figure 8. (d, e), Figure 9. (d, e), Figure 10. (d, e), Figure 11. (d, e) and Figure 12. (d, e)).

a.

b.

c.

d.

e.

Figure 8. Effect of different volumes of *Rosmarinus officinalis* methanolic extract of concentration 0.1 g/ml on growth of *Staphylococcus aureus* isolate 12610. a. 50 µL b. 100 µL c. 150 µL d. 200 µL e. 300 µL

a.

b.

c.

d.

e.

Figure 9. Effect of different volumes of *Rosmarinus officinalis* methanolic extract on growth of *Staphylococcus aureus* isolate 12634. a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.

b.

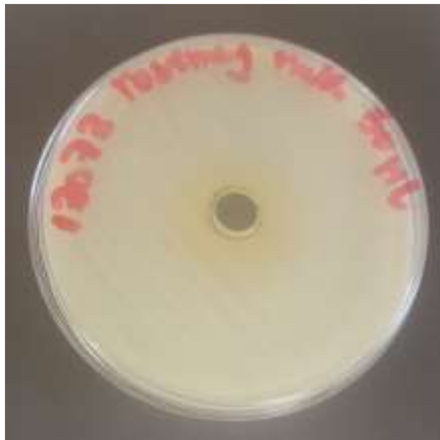
c.

d.

e.

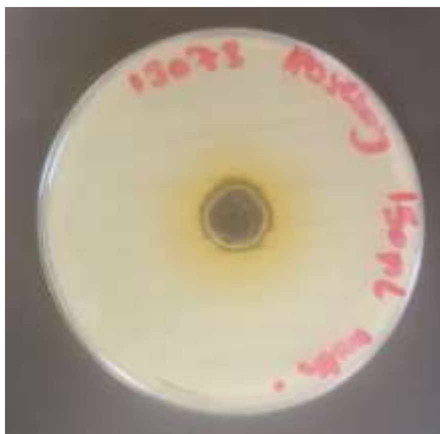
Figure 10. Effect of different volumes of *Rosmarinus officinalis* methanolic extract on growth of *Staphylococcus aureus* isolate 12989 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.



b.

c.



d.

e.

Figure 11. Effect of different volumes of *Rosmarinus officinalis* methanolic extract on growth of *Staphylococcus aureus* isolate 13073 a. 50 μL b. 100 μL c. 150 μL d. 200 μL e. 300 μL

a.

b.

c.

d.

e.

Figure 12. Effect of different volumes of Rosmarinus officinalis methanolic extract on growth of *Staphylococcus aureus* isolate 13074 a. 50 µL b. 100 µL c. 150 µL d. 200 µL e. 300 µL

3.6. The effect of *Origanum syriacum* and *Rosmarinus officinalis* methanolic extracts on inhibition of *Staphylococcus aureus* biofilm formation

3.6.1. The effect of *Origanum syriacum* methanolic extract on inhibition of *Staphylococcus aureus* biofilm formation

3.6.1.1. Effect of *Origanum syriacum* methanolic extract of concentration 0.02 g/ml reflecting a concentration five times less than that of the initially prepared *Origanum syriacum* methanolic extract (0.1 g/ml) on inhibition of *Staphylococcus aureus* biofilm formation

Effect of 50 µL volume of Origanum syriacum methanolic extract of concentration 0.02 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 50 µL of *Origanum syriacum* methanol extract of concentration 0.02 g/ml which contained 0.001 g of the active components of the extract, there was a significant inhibition in biofilm formation of the majority of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 3) and the significance of this inhibition in Figure 13. The percentage of inhibition of biofilm formation ranged from 27.82 % inhibition with isolate 12610 which was considered a significant decrease in biofilm formation and up to 83.11 % decrease in biofilm formation as with isolate 7353 which was considered a highly significant reduction in biofilm formation. However, it is important to note that 50 µL of 0.02 g/ml *Origanum syriacum* extract did not have an effect on two MSSA strains numbered 12634 and 2553. On the other hand, 50 µL of 0.02 g/ml *Origanum syriacum* extract inhibited the biofilm formation ability of four methicillin resistant *S. aureus* strains and this inhibition ranged from a slight insignificant reduction in biofilm formation with isolate 4826 which exhibited

only 2.24 % inhibition of biofilm formation to a highly significant inhibition of biofilm formation reaching 77.55 % with isolate 12631 (Table 3.b; Figure 14). However, the biofilm formation of two methicillin resistant *S.aureus* isolates which are 7496 and 43271, was not affected by exposure to 50 µL of 0.02

Table 3. Effect of 50 µL volume of the *Origanum syriacum* methanolic extract of concentration 0.02 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.02 g/ml *Origanum syriacum* extract	$O.D_{570\ nm}$ of 50 µL of 0.02 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.1798	0.1113	0.1297	27.82
2	13073	0.6461	0.1064	0.4058	37.19
3	12989	1.0567	0.1077	0.6136	41.93
4	12634	0.3653	0.1174	0.6289	
5	2483	0.3102	0.1031	0.1203	61.22
6	2484	0.2525	0.1190	0.1489	41.03
7	2564	0.7013	0.0924	0.5307	24.33
8	2553	0.3343	0.1012	0.3897	
9	7353	0.9077	0.1320	0.1533	83.11
10	6281	0.4467	0.1528	0.1587	64.47
11	45139	2.4291	0.1183	1.9285	20.61
12	48865	0.5390	0.0989	0.1548	71.28
13	10762	0.4261	0.1262	0.1224	71.28
14	5862	0.4589	0.1212	0.1426	68.93
15	14102	0.1696	0.0894	0.0981	42.16

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 50 µL of 0.02 g/ml *Origanum syriacum* extract	O.D570 nm 50 µL of 0.02g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.2332	0.1049	0.1903	18.40
2	12631	0.9804	0.1215	0.2201	77.55
3	7496	0.3070	0.0913	0.6808	
4	4826	0.8217	0.1068	0.8033	2.24
5	4588	0.5045	0.1297	0.1240	75.43
6	43271	0.1388	0.1038	0.1858	

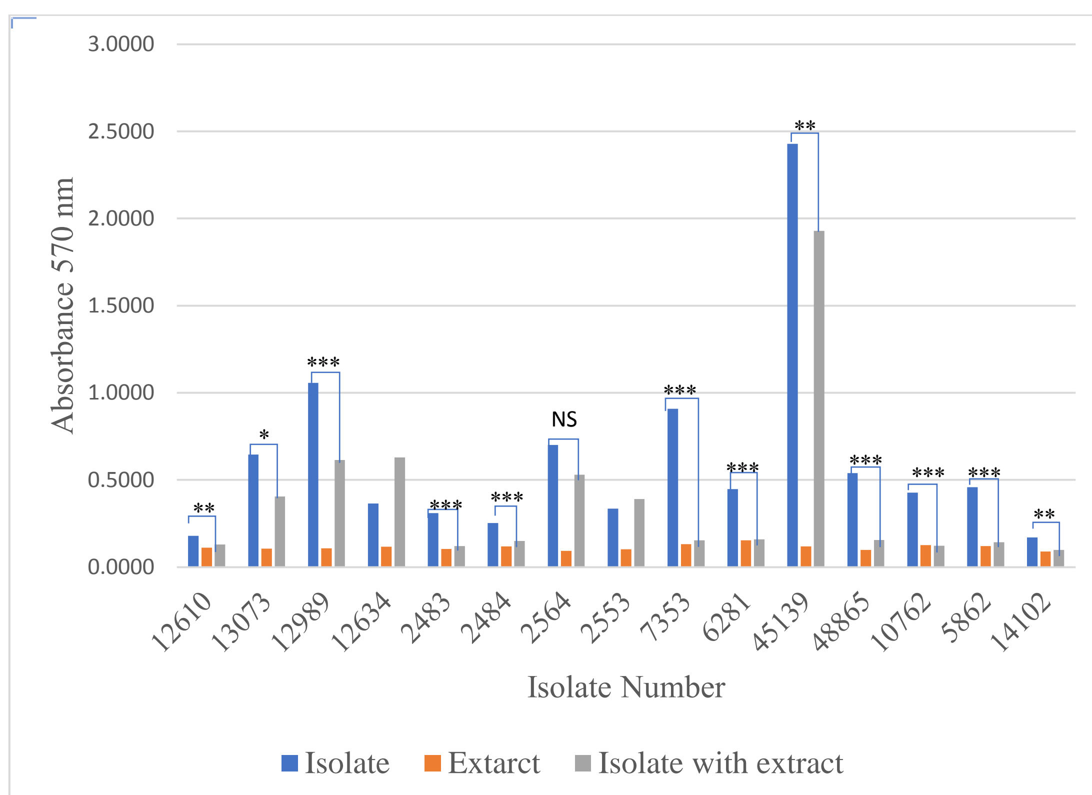


Figure 13. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.02 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

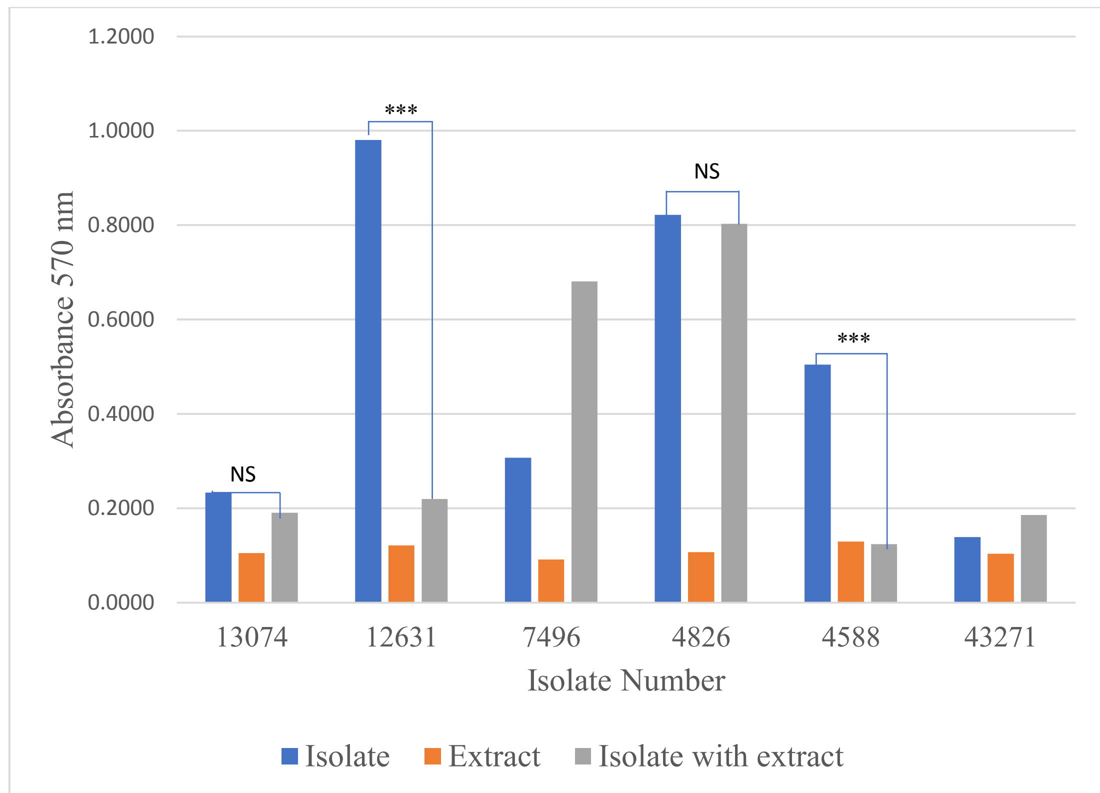


Figure 14. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 µL of 0.02 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

Effect of 100 µL volume of Origanum syriacum methanolic extract of concentration 0.02 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 100 µL of *Origanum syriacum* methanol extract of concentration 0.02 g/ml which contained 0.002 g of the active components of the extract, there was a significant inhibition in biofilm formation of the majority of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 4.a) and the significance of this inhibition (Figure 15). The percentage of inhibition of biofilm formation ranged from 26.25 % inhibition with isolate 7353 which was considered a significant decrease in biofilm formation and up to 75.29 % decrease in biofilm formation as with isolate 5862 which is considered a highly significant reduction in biofilm formation.

However, it is important to note that 100 µL of 0.02 g/ml *Origanum syriacum* extract did not have an effect on MSSA strain numbered 12634 which is the same strain whose biofilm formation was not affected previously by 50 µL of 0.02 g/ml of *Origanum syriacum* extract. Yet, isolate 2553 whose biofilm formation was not affected by 50 µL of 0.02 g/ml of *Origanum syriacum* extract showed a significant 36.96 % inhibition of biofilm formation with 100 µL of 0.02 g/ml *Origanum syriacum* extract. On the other hand, 100 µL of 0.02 g/ml *Origanum syriacum* extract significantly inhibited biofilm formation of all methicillin resistant *S. aureus* strains except isolate number 43271 and this inhibition ranged from 26.28 % with isolate 4588 to a highly significant inhibition of biofilm formation reaching 82.46 % with isolate 12631 (Table 4.b; Figure 16) which is the highest inhibition among the MRSA strains, similar to what it shown with 50 µL of 0.02 g/ml of *Origanum syriacum* extract. However, biofilm formation of *S. aureus* isolates 7496 which was not affected by exposure to 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract showed a significant 55.34 % decrease in biofilm formation with 100 µL of 0.02 g/ml *Origanum syriacum* extract while the methicillin resistant isolate 43271 whose biofilm formation was not inhibited with 50 µL of 0.02 g/ml *Origanum syriacum* still showed no effect when the volume of the extract used was increased to 100 µL.

Table 4. Effect of 100 μL volume of the *Origanum syriacum* methanolic extract of concentration 0.02 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.02 g/ml *Origanum syriacum* extract	$O.D_{570\ nm}$ of 100 μL of 0.02 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.2223	0.1747	0.1377	38.08
2	13073	0.7498	0.1173	0.2277	69.64
3	12989	1.5271	0.1079	0.5557	63.61
4	12634	0.4477	0.1622	0.5842	
5	2483	0.5267	0.1098	0.2098	60.17
6	2484	0.3120	0.1360	0.1727	44.65
7	2564	0.8186	0.1117	1.1919	
8	2553	0.3348	0.1057	0.2111	36.96
9	7353	0.6611	0.1222	0.4876	26.25
10	6281	0.4981	0.1244	0.1583	68.21
11	45139	2.5779	0.1263	0.8245	68.02
12	48865	0.4647	0.1170	0.1722	62.95
13	10762	0.3929	0.1535	0.1254	68.08
14	5862	0.6040	0.1709	0.1493	75.29
15	14102	0.1617	0.0912	0.1209	25.23

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.02 g/ml *Origanum syriacum* extract	$O.D_{570\ nm}$ of 100 µL of 0.02 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.8498	0.1174	0.2871	66.22
2	12631	1.2471	0.1185	0.2187	82.46
3	7496	0.5174	0.1088	0.2311	55.34
4	4826	0.9448	0.1471	0.3108	67.10
5	4588	0.5466	0.1354	0.4030	26.28
6	43271	0.1725	0.1540	0.2624	

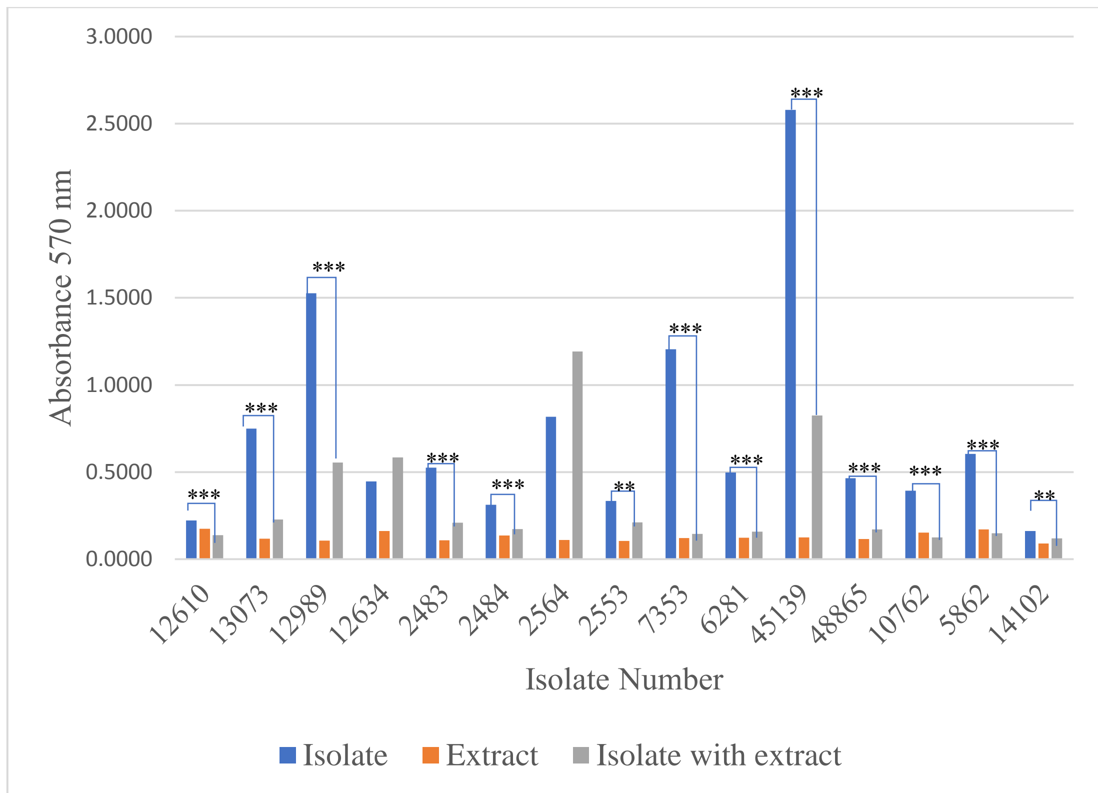


Figure 15. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.02 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

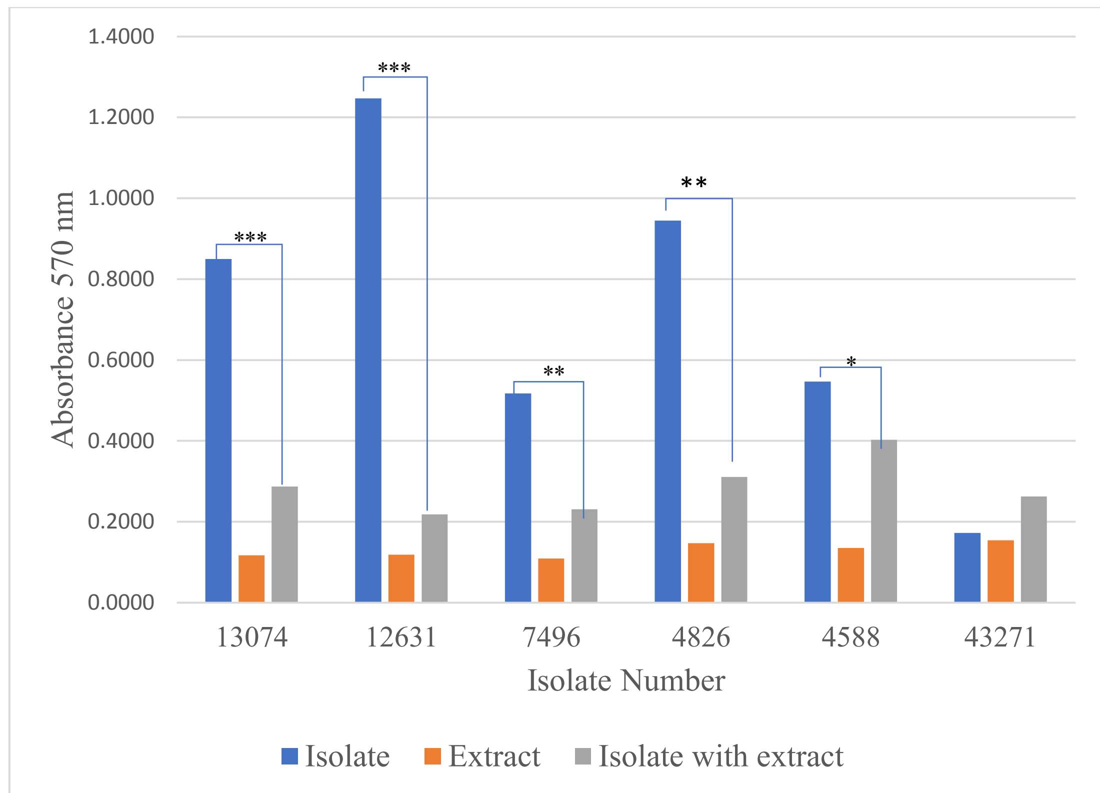


Figure 16. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.02 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.6.1.2. Effect of *Origanum syriacum* methanolic extract of concentration 0.01 g/ml reflecting a concentration ten times less than that of the initial *Origanum syriacum* methanolic extract (0.1 g/ml) on inhibition of *Staphylococcus aureus* biofilm formation

Effect of 50 µL volume of Origanum syriacum methanolic extract of concentration 0.01 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 50 µL of *Origanum syriacum* methanol extract of concentration 0.01 g/ml which contained 5×10^{-4} g of the active components of the extract, there was still a significant inhibition in biofilm formation of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 5.a) and the significance of this inhibition in

Figure 17, yet this inhibition in biofilm formation was observed in fewer isolates compared to the results observed with the higher concentration of 0.02 g/ml of the *Origanum syriacum* methanol extract at this same volume of 50 µL. The percentage of inhibition of biofilm formation was observed in 10 MSSA isolates and this inhibition ranged from insignificant 8.39 % with isolate 12989 inhibition and up to a highly significant decrease of 75.51 % in biofilm formation with isolate 7353 which also exhibited the highest inhibition in biofilm formation at the higher concentration 0.02 g/ml of *Origanum syriacum* methanol extract and remained the most affected by the *Origanum syriacum* methanol extract even at the lower 0.01 g/ml concentration. However, it is important to note that 0.01 g/ml of *Origanum syriacum* extract did not have an effect on isolates 13073, 12989 and 45139 whose biofilm formation was significantly inhibited at a higher concentration of 0.02 g/ml of *Origanum syriacum* extract at the same volume of 50 µL. On the other hand, 50 µL of 0.01 g/ml *Origanum syriacum* extract inhibited biofilm formation of only two methicillin resistant *S. aureus* strain which are isolates 12631 and 4588 by approximately 50 % (Table 5.b; Figure 18). The biofilm formation of these two isolates: 12631 and 4588 was also significantly inhibited by 0.02 g/ml *Origanum syriacum* extract at the same volume of 50 µL of the extract, but the percentage inhibition for both isolates was higher to more than 70% once they were exposed to the higher concentration of 0.02 g/ml of the extract. However, biofilm formation of the rest of the MRSA isolates was not affected by exposure to 50 µL of 0.01 g/ml *Origanum syriacum* methanol extract including isolates 4826 and 13074 whose biofilm formation was inhibited by 0.02 g/ml *Origanum syriacum* methanol extract.

Table 5. Effect of 50 μL volume of the *Origanum syriacum* methanolic extract of concentration 0.01 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 50 μL of 0.01 g/ml *Origanum syriacum* extract	O.D$_{570\ nm}$ of 50 μL of 0.01 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.1969	0.1093	0.1343	31.79
2	13073	0.6715	0.1104	2.3356	
3	12989	1.1411	0.1114	1.0454	8.39
4	12634	0.3978	0.1258	1.6126	
5	2483	0.4063	0.1147	0.1313	67.67
6	2484	0.2823	0.1344	0.1698	39.87
7	2564	0.5677	0.1010	2.3591	
8	2553	0.4161	0.1002	0.4417	
9	7353	1.0729	0.1455	0.2627	75.51
10	6281	0.4601	0.1097	0.1945	57.73
11	45139	2.6732	0.1216	2.7253	
12	48865	0.5593	0.1110	0.2116	62.16
13	10762	0.4037	0.1247	0.1511	62.57
14	5862	0.5309	0.1242	0.1479	72.13
15	14102	0.1592	0.1005	0.1292	18.84

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 50 µL of 0.01 g/ml *Origanum syriacum* extract	O.D570 nm of 50 µL of 0.01 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.3345	0.1145	0.9405	
2	12631	1.0458	0.1402	0.4412	57.81
3	7496	0.3267	0.0920	0.7783	
4	4826	1.0391	0.1033	2.4499	
5	4588	0.4413	0.1279	0.2089	52.66
6	43271	0.1348	0.1044	0.2204	

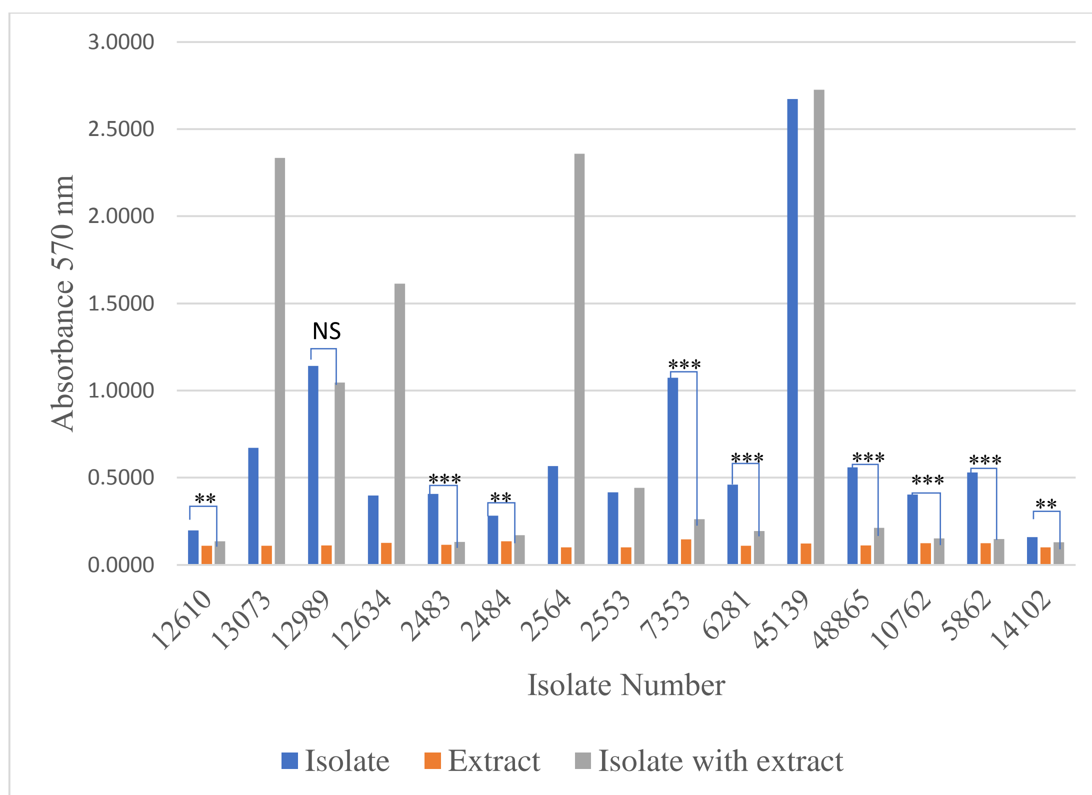


Figure 17. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.01 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

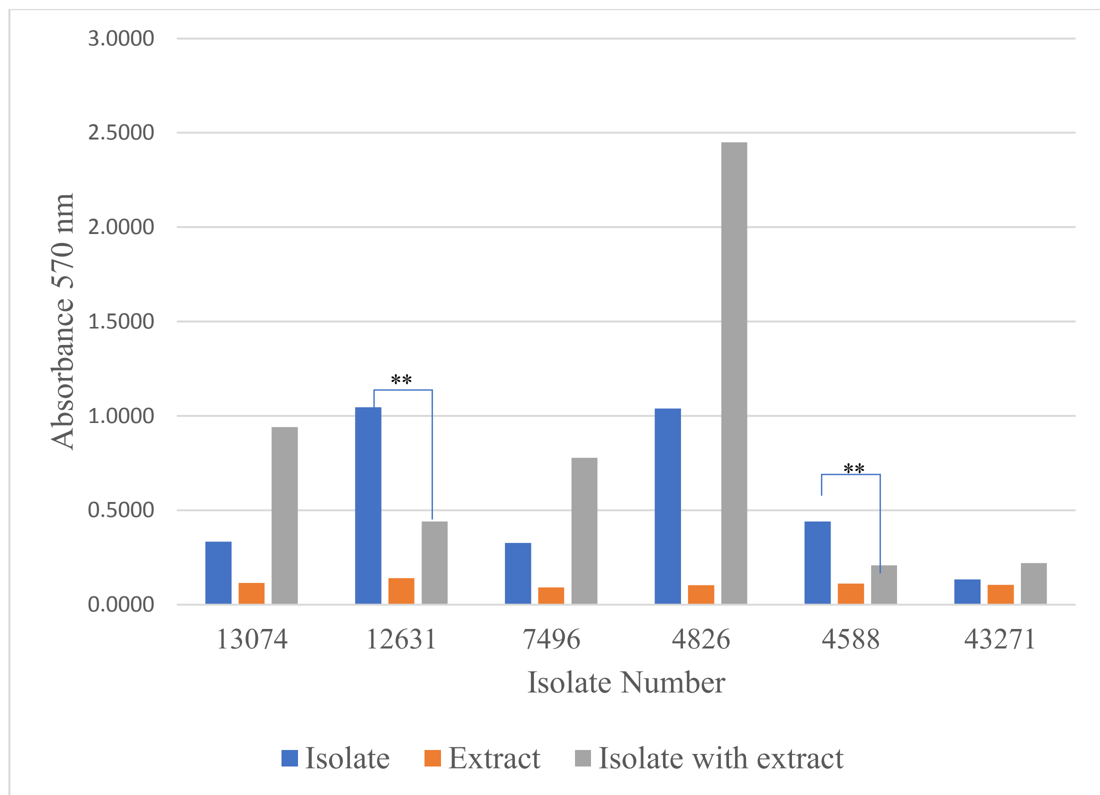


Figure 18. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 µL of 0.01 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

Effect of 100 µL volume of Origanum syriacum methanolic extract of concentration 0.01 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results with 100 µL of the lower concentration of 0.01 g/ml of the *Origanum syriacum* methanol extract which contained 0.001 g of the active components of the extract were nearly identical to the results shown with 50 µL of the 0.02 g/ml *Origanum syriacum* methanol extract which contained an equal amount of the active components of *Origanum syriacum* methanol extract. Under the indicated conditions, there was a significant inhibition in biofilm formation of the majority of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 6.a) and the significance of this inhibition (Figure 19). The percentage of inhibition of biofilm formation ranged from 20.07%

with isolate 12610, which is close to the 27.82 % inhibition observed with 12610 at 50 µL of the 0.02 g/ml *Origanum syriacum* methanol extract. The percentage of inhibition of biofilm formation was 83.75% with isolate 7353, which is the same percentage of inhibition observed previously with the same isolate at 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract (83.11 %). However, it is important to note that 100 µL of *Origanum syriacum* extract did not have an effect on isolate 12634, a result similar to what was observed with 50 µL of 0.02 g/ml *Origanum syriacum* extract. On the other hand, 100 µL of 0.01 g/ml *Origanum syriacum* extract inhibited biofilm formation of three methicillin resistant *S. aureus* strains (Table 6.b; Figure 20). This inhibition of biofilm formation reached 70% with isolate 12631 similar to the inhibition observed with 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract where inhibition of biofilm formation of isolate 12631 was 77.55 % (Table 3.b; Figure 14). The biofilm formation of the three *S. aureus* isolates: 13074, 4826 and 43271, was not affected by exposure to 100 µL of 0.01 g/ml *Origanum syriacum* methanol extract. The biofilm formation of isolates 4826 and 43271 was previously shown not to be inhibited with 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract, which coincided with the results observed here with 100 µL of 0.01 g/ml *Origanum syriacum* methanol extract. Eventhough isolate 7496 displayed a slight inhibition in biofilm formation, this inhibition was not significant, a result that coincides with the lack of inhibition of biofilm formation of isolate 7496 previously observed with 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract. These coinciding results between 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract and 100 µL of 0.01 g/ml *Origanum syriacum* methanol extract, that have equal amounts of the active components of the extract, confirmed the inhibitory effect on biofilm formation by *Origanum syriacum* methanol extract.

Table 6. Effect of 100 μL volume of the *Origanum syriacum* methanolic extract of concentration 0.01 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.01 g/ml *Origanum syriacum* extract	$O.D_{570\ nm}$ of 100 μL of 0.01 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.1934	0.1171	0.1546	20.07
2	13073	0.6794	0.1326	1.0663	
3	12989	1.2750	0.1123	0.8732	31.51
4	12634	0.3630	0.1141	1.0569	
5	2483	0.5170	0.1220	0.2632	49.08
6	2484	0.2528	0.1247	0.2017	20.22
7	2564	0.8102	0.0975	0.3678	54.61
8	2553	0.6261	0.1004	0.3372	46.15
9	7353	1.1629	0.1141	0.1890	83.75
10	6281	0.5304	0.1346	0.1852	65.08
11	45139	2.6569	0.1182	2.2455	15.49
12	48865	0.5089	0.1081	0.1818	64.28
13	10762	0.3134	0.1178	0.1681	46.38
14	5862	0.5060	0.1491	0.1494	70.48
15	14102	0.1676	0.0973	0.1077	35.74

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Origanum syriacum* extract	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Origanum syriacum* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.9128	0.1117	0.9535	
2	12631	0.9939	0.1225	0.2993	69.89
3	7496	0.9976	0.1124	0.7797	21.84
4	4826	0.9243	0.1183	1.0857	
5	4588	0.5032	0.1292	0.2406	52.19
6	43271	0.1480	0.1234	0.3332	

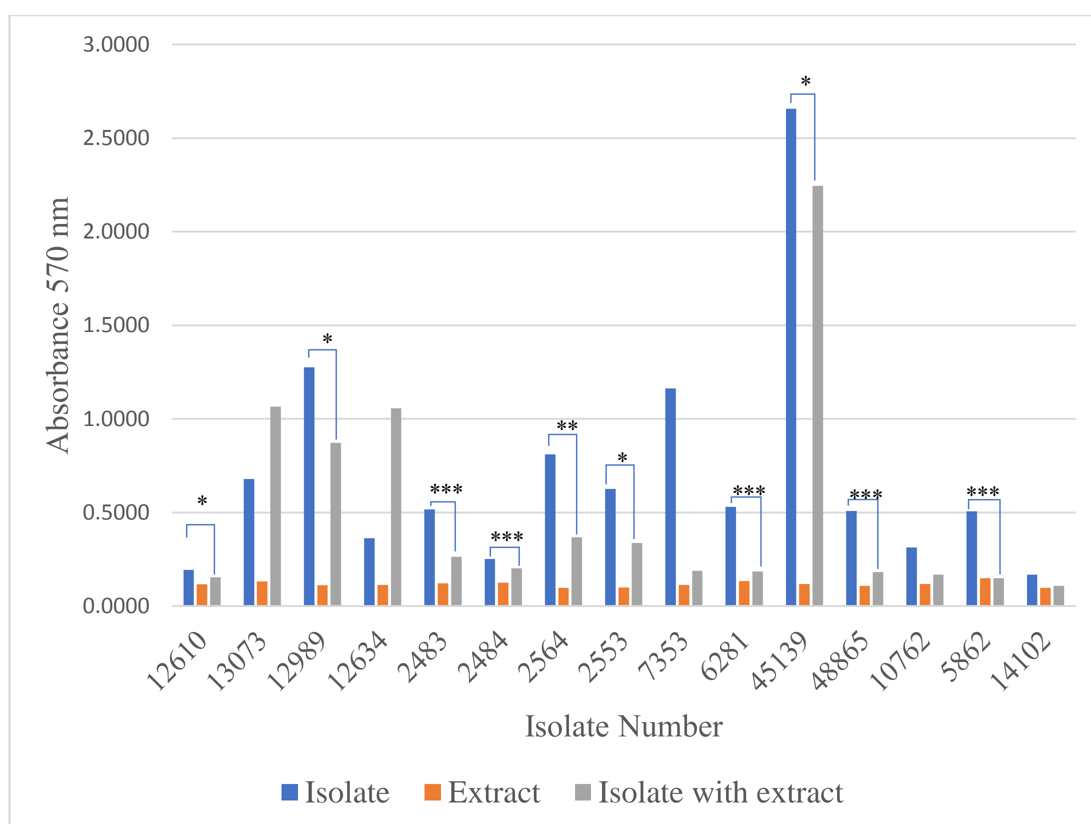


Figure 19. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.01 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

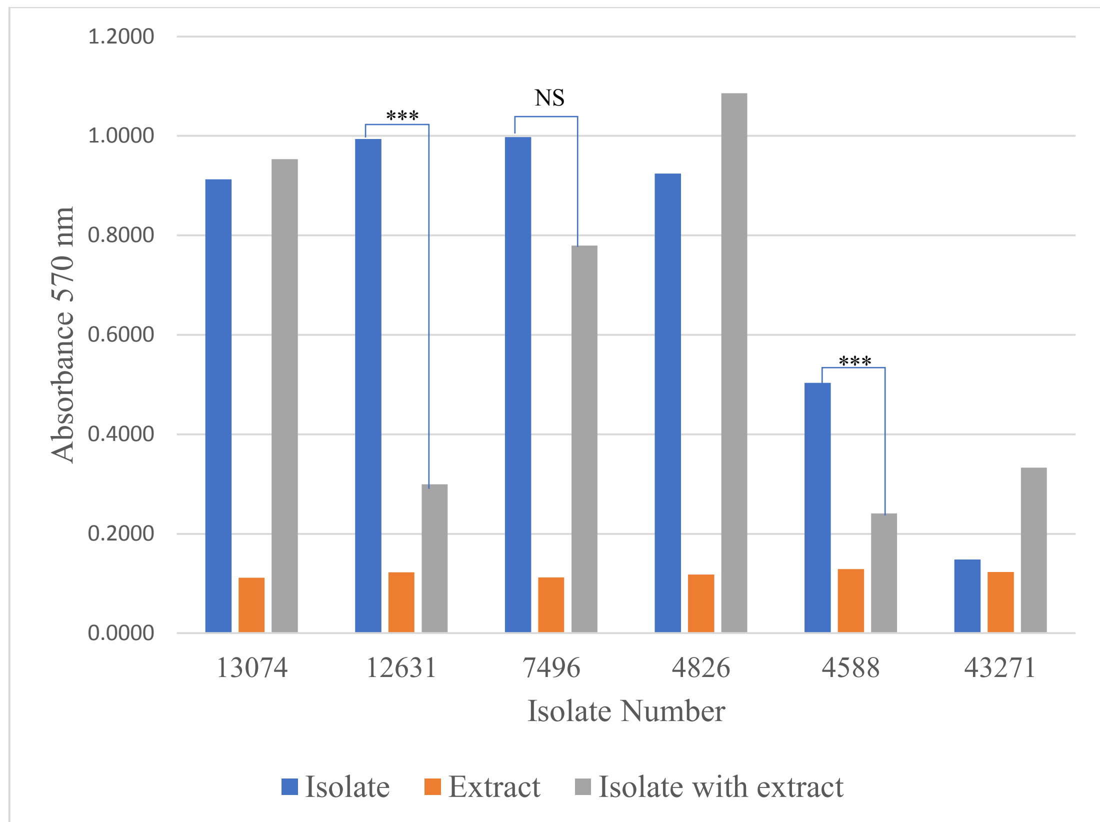


Figure 20. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.01 g/ml *Origanum syriacum* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.6.2. The effect of *Rosmarinus officinalis* methanolic extract on inhibition of *Staphylococcus aureus* biofilm formation

3.6.2.1. Effect of *Rosmarinus officinalis* methanolic extract of concentration 0.02 g/ml reflecting a concentration five times less than that of the initial *Rosmarinus officinalis* methanolic extract (0.1 g/ml) on inhibition of *Staphylococcus aureus* biofilm formation

Effect of 100 µL volume of Rosmarinus officinalis methanolic extract of concentration 0.02 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 100 µL of *Rosmarinus officinalis* methanol extract of concentration 0.02 g/ml which contained 0.002 g of the active components of the

extract, there was a significant inhibition in biofilm formation of the majority of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 7.a) and the significance of this inhibition (Figure 21). The percentage of inhibition of biofilm formation ranged from 43.06 % inhibition with isolate 2484 which was considered a moderately significant decrease in biofilm formation and up to 89.99 % decrease in biofilm formation as with isolate 45139, which is considered a highly significant effect for 100 µL of *Rosmarinus officinalis* methanol extract. Even though the decrease was not significant with isolates 14102 and 2553, there was an inhibition in biofilm formation by 6.22 % and 13.53 %, respectively. However, 100 µL of 0.02 g/ml *Rosmarinus officinalis* methanol extract did not have an effect on only two MSSA strains numbered 12610 and 2564. On the other hand, 100 µL of 0.02 g/ml *Rosmarinus officinalis* extract significantly inhibited biofilm formation of four methicillin resistant *S. aureus* strains and this inhibition ranged from a moderately significant reduction in biofilm formation with isolate 4588, which exhibited 48.07 % inhibition of biofilm formation to a highly significant inhibition of biofilm formation, reaching 82.19 % with isolate 4826 (Table 7.b; Figure 22). However, biofilm formation of two *S. aureus* isolates: 7496 and 43271 was not affected by exposure to 100 µL of 0.02 g/ml *Rosmarinus officinalis* methanol extract similar to the result we observed with 50 µL of 0.02 g/ml *Origanum syriacum* methanol extract on the same isolates (Table 3.b and Figure 14).

Table 7. Effect of 100 µL volume of the *Rosmarinus officinalis* methanolic extract of concentration 0.02 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 100 µL of 0.02 g/ml *Rosmarinus officinalis* extract	O.D$_{570\ nm}$ of 100 µL of 0.02 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.2249	0.1188	0.2833	
2	13073	0.6868	0.0852	0.1661	75.81
3	12989	1.0320	0.0943	0.1261	87.78
4	12634	0.4327	0.1121	0.2188	49.43
5	2483	0.4288	0.1207	0.1154	73.08
6	2484	0.3366	0.0970	0.1916	43.06
7	2564	0.5495	0.1097	0.7208	
8	2553	0.3595	0.1103	0.3108	13.53
9	7353	1.3627	0.1066	0.2117	84.46
10	6281	0.4277	0.1331	0.1913	55.27
11	45139	2.1312	0.1185	0.2133	89.99
12	48865	0.5270	0.1307	0.2029	61.50
13	10762	0.6276	0.1181	0.1909	69.58
14	5862	0.6253	0.1442	0.2877	53.99
15	14102	0.1335	0.1116	0.1252	6.22

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 100 µL 0.02 g/ml *Rosmarinus officinalis* extract	O.D$_{570\ nm}$ of 100 µL of 0.02 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	13074	1.0956	0.1079	0.3741	65.86
2	12631	1.1103	0.1278	0.2930	73.61
3	7496	0.3636	0.1101	0.4115	
4	4826	1.1475	0.1067	0.2044	82.19
5	4588	0.4268	0.1130	0.2216	48.07
6	43271	0.1524	0.1093	0.1747	

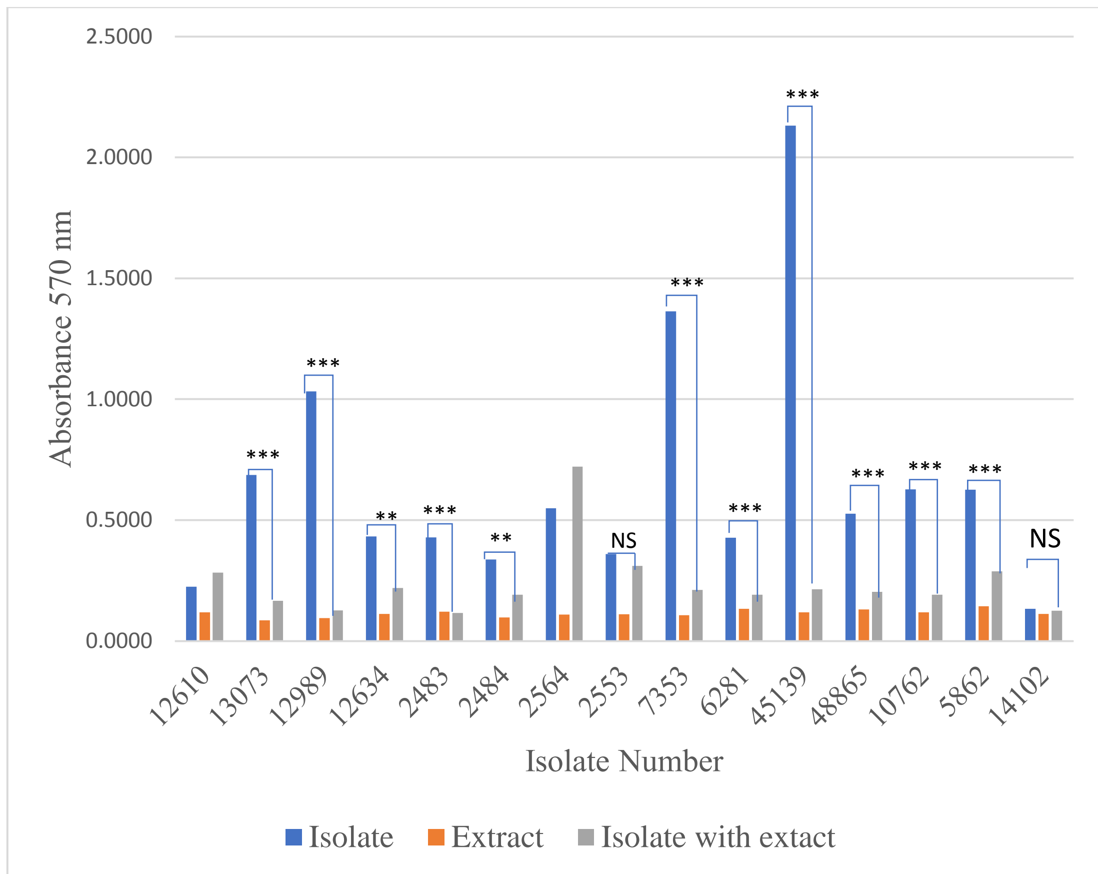


Figure 21. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.02 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

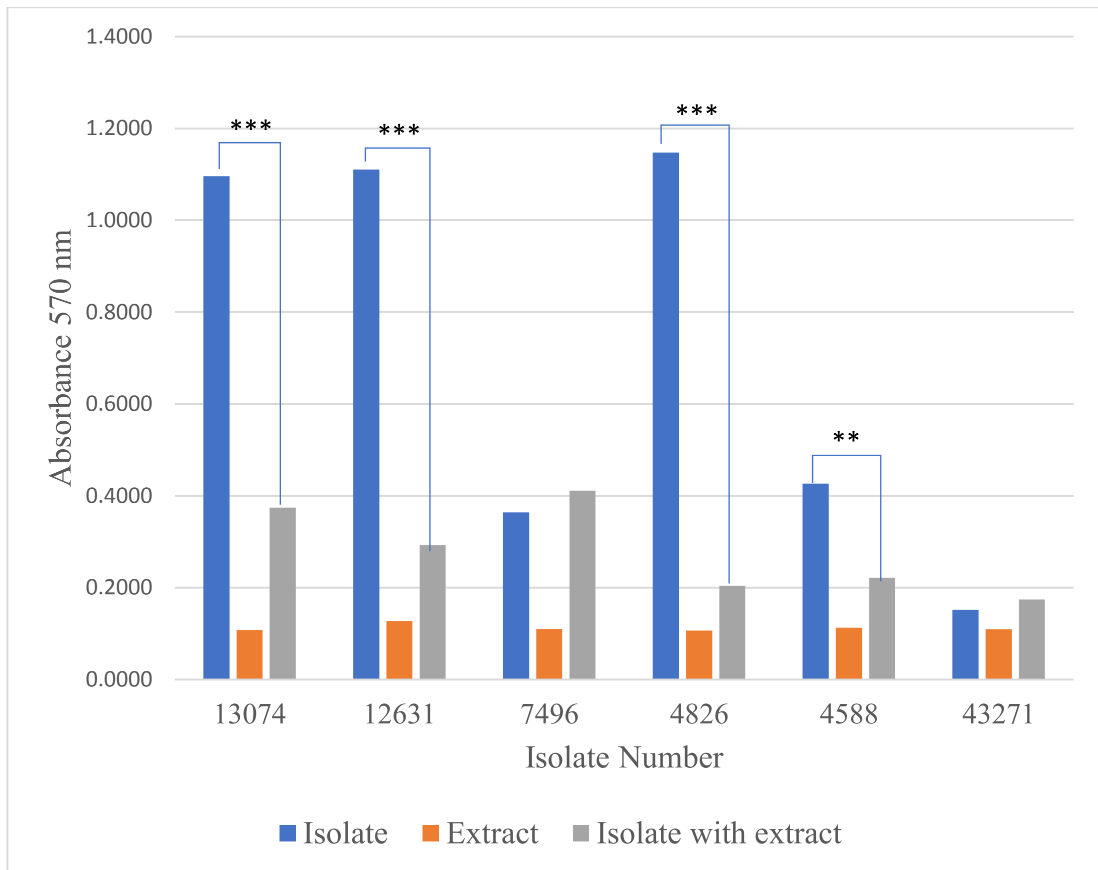


Figure 22. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.02 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

Effect of 150 µL volume of Rosmarinus officinalis methanolic extract of concentration 0.02 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 150 µL of *Rosmarinus officinalis* methanol extract of concentration 0.02 g/ml which contained 0.003 g of the active components of the extract, there was a significant inhibition in biofilm formation of the majority of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage of inhibition of biofilm formation (Table 8.a) and the significance of this inhibition (Figure 23). The percentage of inhibition of biofilm formation ranged from 39.09 % inhibition with isolate 12634, which was considered a significant decrease in biofilm formation, and up to 85.02 % decrease in biofilm formation as with isolate

45139, which was considered a highly significant effect on reduction in biofilm formation. However, it is important to note that 150 µL of 0.02 g/ml *Rosmarinus officinalis* extract did not have any effect on two MSSA strains numbered 12610 and 2484. The biofilm formation of isolate 12610 which was not affected previously by 100 µL of 0.02 g/ml of *Rosmarinus officinalis* was still not affected by 0.02 g/ml of *Rosmarinus officinalis* at a higher volume of 150 µL extract. Yet, isolate 2564 whose biofilm formation was not affected by 100 µL of 0.02 g/ml of *Rosmarinus officinalis* extract showed a significant 58.98 % inhibition of biofilm formation with 150 µL of 0.02 g/ml *Rosmarinus officinalis* extract. On the other hand, 150 µL of 0.02 g/ml *Rosmarinus officinalis* extract significantly inhibited biofilm formation of all methicillin resistant *S. aureus* strains except isolate number 7496 and this inhibition ranged from 16.09 % with isolate 43271, to a highly significant inhibition of biofilm formation reaching 85.05 % with isolate 4826 (Table 8.b; Figure 24) which is the highest inhibition among the MRSA strains, similar to its result with 100 µL of 0.02 g/ml of *Rosmarinus officinalis* extract. However, biofilm formation of *S. aureus* isolate 43271 which was not affected by exposure to 100 µL of 0.02 g/ml *Rosmarinus officinalis* methanol extract, showed a significant 16.09 % decrease in biofilm formation with 150 µL of 0.02 g/ml *Rosmarinus officinalis*.

Table 8. Effect of 150 μL volume of the *Rosmarinus officinalis* methanolic extract of concentration 0.02 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.02 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 150 μL of 0.02 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.1807	0.1249	0.3256	
2	13073	0.7969	0.1116	0.3586	55.00
3	12989	1.3292	0.1189	0.3299	75.18
4	12634	0.3094	0.1129	0.1885	39.09
5	2483	0.4058	0.1127	0.1818	55.19
6	2484	0.2756	0.1446	0.3234	
7	2564	0.7877	0.1431	0.3232	58.98
8	2553	1.0207	0.1299	0.4561	55.31
9	7353	1.2888	0.1163	0.2236	82.65
10	6281	0.3571	0.1393	0.1751	50.98
11	45139	2.2913	0.1430	0.3431	85.02
12	48865	0.4446	0.1230	0.1638	63.15
13	10762	0.3887	0.1107	0.1920	50.62
14	5862	0.5483	0.1501	0.4197	23.44
15	14102	0.1360	0.1306	0.1145	15.81

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.02 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 150 µL of 0.02 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.5299	0.1435	0.3784	28.59
2	12631	0.6868	0.1277	0.1291	81.20
3	7496	0.5376	0.1329	0.7724	
4	4826	0.9464	0.1112	0.1415	85.05
5	4588	0.4418	0.1221	0.1865	57.79
6	43271	0.1557	0.1217	0.1307	16.09

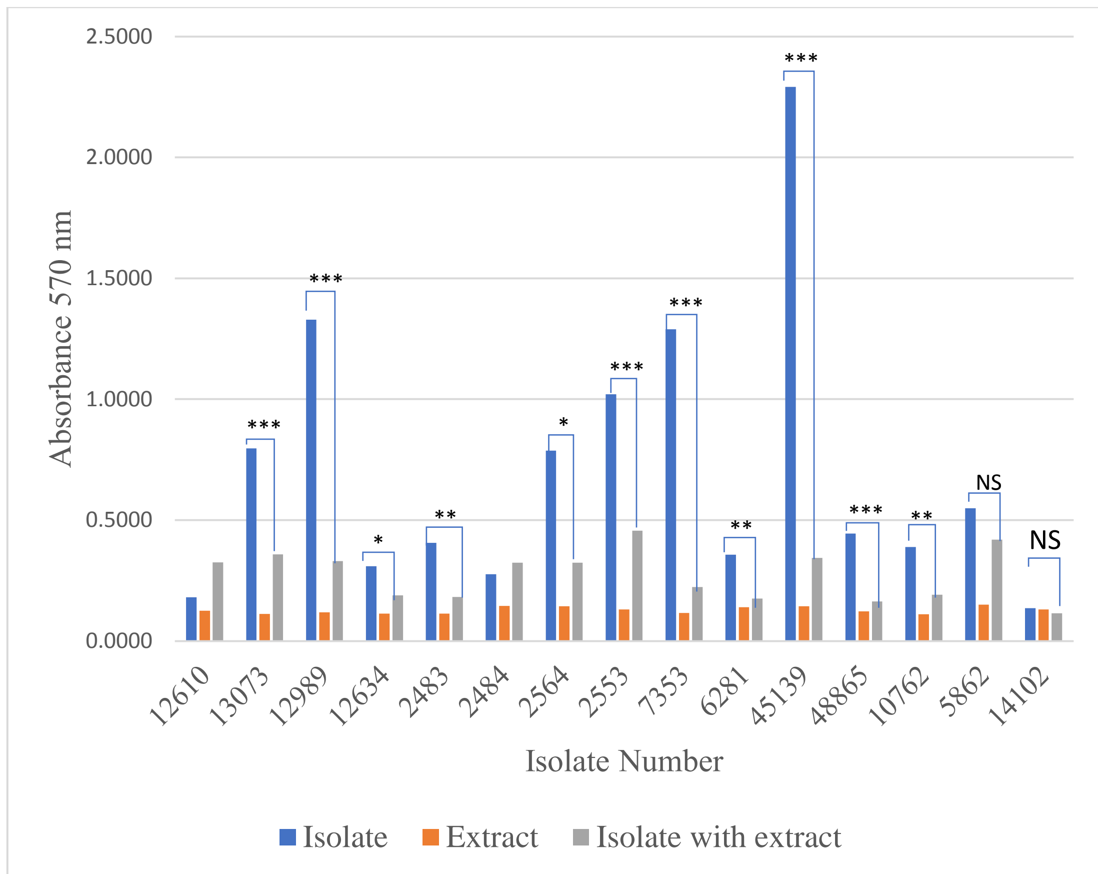


Figure 23. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.02 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

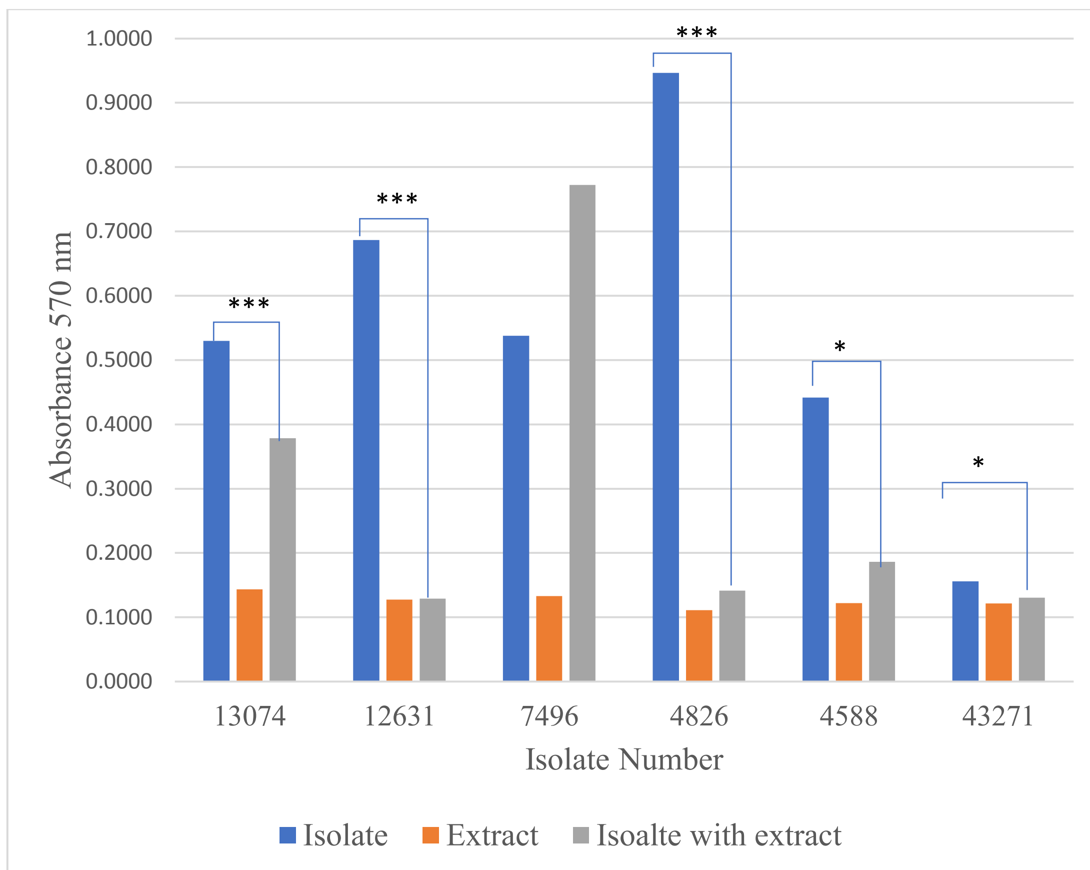


Figure 24. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.02 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.6.2.2. Effect of *Rosmarinus officinalis* methanolic extract of concentration 0.01 g/ml reflecting a concentration ten times less than that of the initial *Rosmarinus officinalis* methanolic extract (0.1 g/ml) on inhibition of *Staphylococcus aureus* biofilm formation

Effect of 100 µL volume of Rosmarinus officinalis *methanolic extract of concentration 0.01 g/ml on inhibition of* Staphylococcus aureus *biofilm formation*

The results showed that with 100 µL of *Rosmarinus officinalis* methanol extract of concentration 0.01 g/ml which contained 0.001 g of the active components of the extract, there was still a significant inhibition in biofilm formation of the methicillin susceptible *Staphylococcus aureus* isolates as indicated by the calculated percentage

of inhibition of biofilm formation (Table 9.a) and the significance of this inhibition in Figure 25. This inhibition was observed in all except one MSSA strain: isolate 2553 (Table 9.a and Figure 25). Although the inhibition in biofilm formation included the two isolates numbered 12610 and 2564, whose biofilm formation was not inhibited at 100 µL of the higher concentration of 0.02 g/ml of *Rosmarinus officinalis* methanol extract, this inhibition of biofilm formation of these two isolates was not significant. This indicated a consistency with the results observed with 0.02 g/ml of *Rosmarinus officinalis* methanol extract. The percentage of inhibition ranged from 45.12 % with isolate 2484 and up to a highly significant inhibition of 75.51 % in biofilm formation with isolate 7353. Isolate 7353 exhibited one of the highest inhibition in biofilm formation with 84 % at the higher concentration of 0.02 g/ml of *Rosmarinus officinalis* methanol extract and its biofilm formation was still highly affected by the *Rosmarinus officinalis* methanol extract even at the lower 0.01 g/ml concentration. On the other hand, 100 µL of 0.01 g/ml *Rosmarinus officinalis* extract inhibited biofilm formation of four methicillin resistant *S. aureus* strains and this inhibition ranged from 32.54 % with isolate 13074 to 72.68 % with isolate 12631 (Table 9.b and Figure 26). However, the biofilm formation of MRSA strains numbered 7496 and 43271 was not affected by 100 µL of 0.01 g/ml *Rosmarinus officinalis,* which is a consistent effect with the higher concentration of 0.02 g/ml of *Rosmarinus officinalis* methanol extract at the same volume. The results revealed that *Rosmarinus officinalis* methanol extract still exhibited an inhibitory effect on both methicillin susceptible and methicillin resistant *S. aureus* strains at a concentration five times lower than 0.02 g/ml.

Table 9. Effect of 100 µL volume of the *Rosmarinus officinalis* methanolic extract of concentration 0.01 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.2117	0.1474	0.1745	17.58
2	13073	0.7619	0.0998	0.2384	68.71
3	12989	1.3306	0.1160	0.4820	63.78
4	12634	0.3545	0.1153	0.1886	46.80
5	2483	0.3835	0.0969	0.1219	68.20
6	2484	0.2822	0.1065	0.1549	45.12
7	2564	0.9614	0.1038	0.8865	7.79
8	2553	0.6112	0.1199	0.7318	
9	7353	1.1257	0.1258	0.2115	81.21
10	6281	0.4335	0.1595	0.1993	54.03
11	45139	2.3826	0.1218	0.9886	58.51
12	48865	0.5114	0.1089	0.2376	53.55
13	10762	0.3685	0.1093	0.1813	50.81
14	5862	0.4667	0.1333	0.2207	52.70
15	14102	0.1339	0.1199	0.1152	13.97

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 100 µL of 0.01 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.9770	0.1230	0.6005	38.54
2	12631	1.0837	0.1376	0.2960	72.68
3	7496	0.6482	0.0909	0.7146	
4	4826	0.9030	0.1171	0.5370	40.53
5	4588	0.4507	0.0983	0.1499	66.74
6	43271	0.1467	0.0991	0.3379	

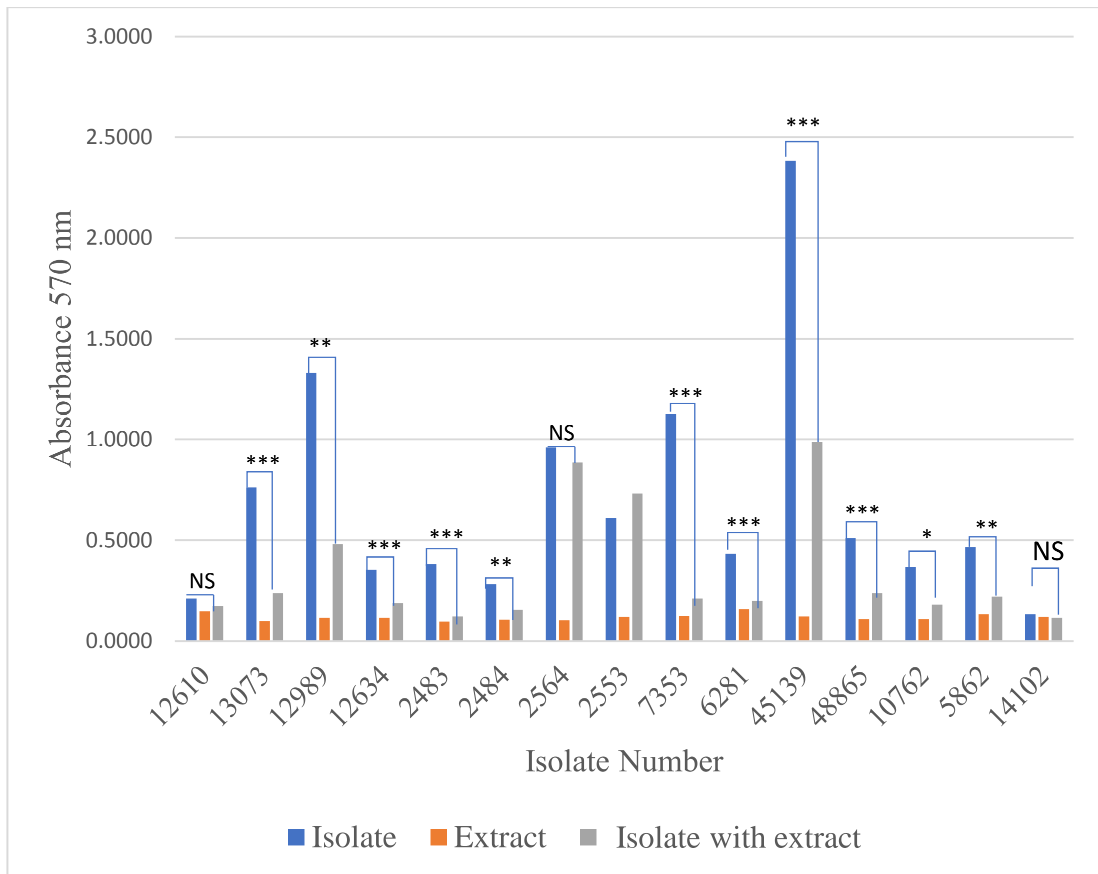


Figure 25. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.01 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

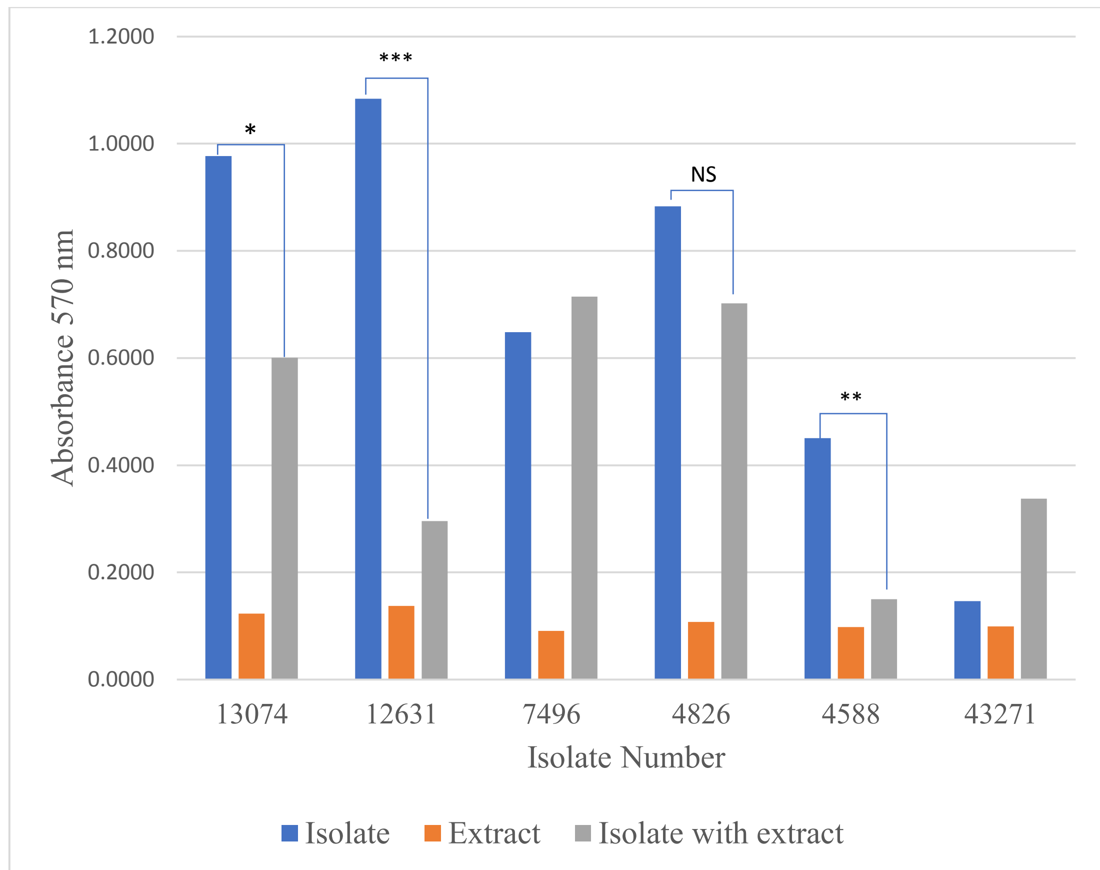


Figure 26. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.01 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

Effect of 150 µL volume of Rosmarinus officinalis methanolic extract of concentration 0.01 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed a comparable yet higher percentage of inhibition with 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract which contained 0.0015 g of active components as compared to 100 µL *Rosmarinus officinalis* methanol extract (Table 10.a and Figure 27). Isolate 2564 showed a significantly higher inhibition in biofilm formation with 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract. However, the biofilm of isolates 2484 and 6281, whose biofilm was previously inhibited by 100 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract, was not altered with 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract; while isolate

2553 whose biofilm formation was not affected by 100 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract showed a significant reduction in biofilm formation with 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract. On the other hand, methicillin resistant *S. aureus* isolates showed a similar inhibition in biofilm formation with 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract to the inhibition observed with 100 µL of 0.01 g/ml *Rosmarinus officinalis* methanol extract (Table 10.b and Figure 28).

Table 10. Effect of 150 µL volume of the *Rosmarinus officinalis* methanolic extract of concentration 0.01 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.01 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 150 µL of 0.01 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	12610	0.1804	0.1201	0.1761	2.42
2	13073	0.8081	0.1084	0.2217	72.56
3	12989	1.5556	0.1130	0.2734	82.43
4	12634	0.3068	0.1144	0.2143	30.17
5	2483	0.4620	0.0996	0.1406	69.57
6	2484	0.3007	0.1289	0.4030	
7	2564	0.6245	0.1107	0.4444	28.84
8	2553	1.0207	0.1299	0.4561	55.31
9	7353	1.2139	0.1148	0.1942	84.00
10	6281	0.3438	0.1339	0.4911	
11	45139	2.3166	0.1236	0.8066	65.18
12	48865	0.4810	0.1093	0.2266	52.89
13	10762	0.4671	0.1197	0.1983	57.54
14	5862	0.5030	0.1354	0.2564	49.02
15	14102	0.1365	0.0991	0.1009	26.08

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.01 g/ml *Rosmarinus officinalis* extract	$O.D_{570\ nm}$ of 150 µL of 0.01 g/ml *Rosmarinus officinalis* extract with bacterium	% Inhibition of biofilm formation
1	13074	0.6053	0.1004	0.5177	14.47
2	12631	1.0751	0.1157	0.2830	73.68
3	7496	0.6388	0.1113	1.1576	
4	4826	0.7731	0.1091	0.6528	15.56
5	4588	0.5043	0.1275	0.2118	58.00
6	43271	0.1314	0.1041	0.1719	

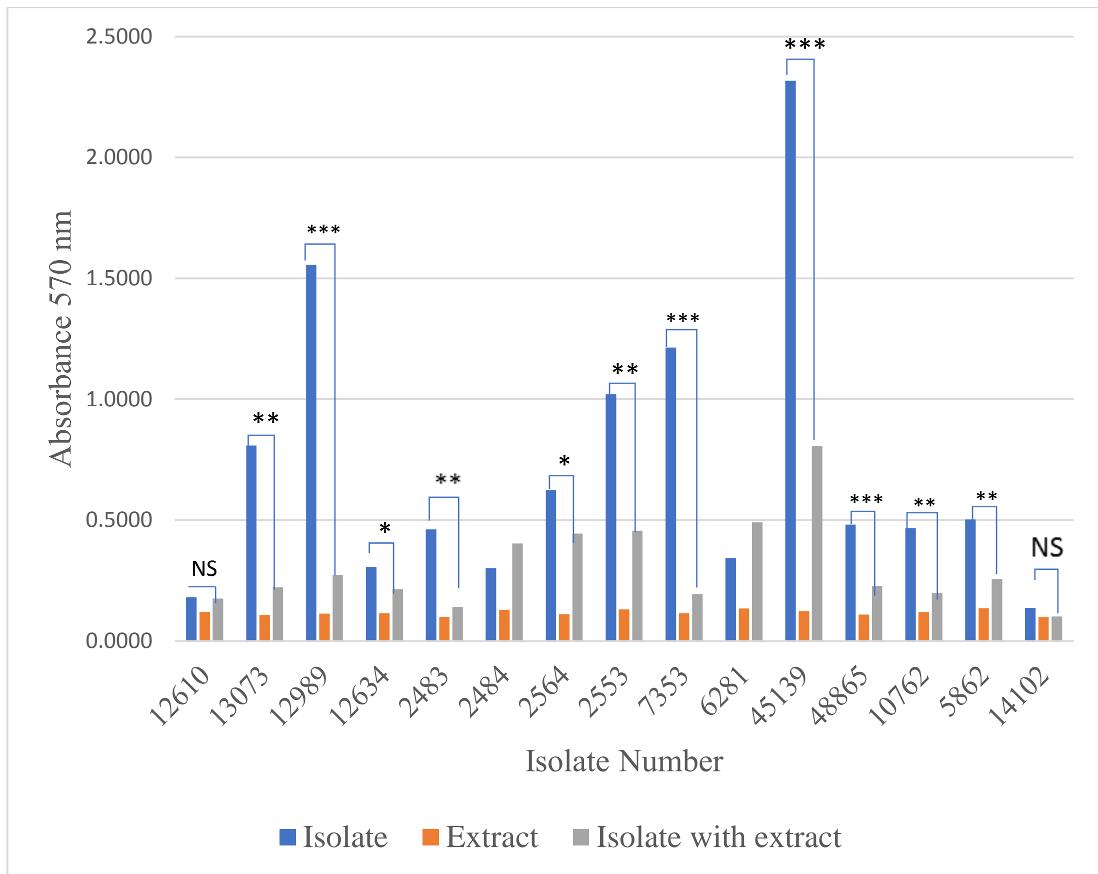


Figure 27. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

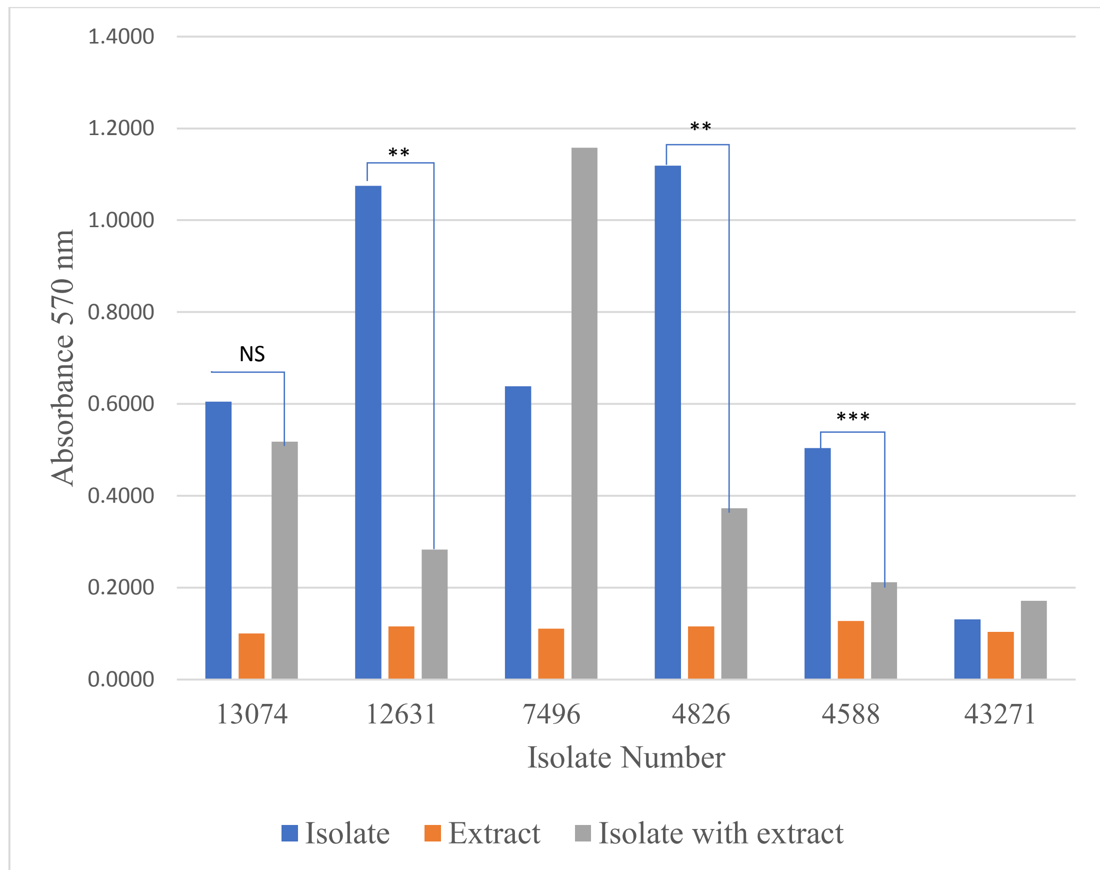


Figure 28. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.01 g/ml *Rosmarinus officinalis* methanolic extract ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.7. The effect of two major components of *Origanum syriacum* on inhibition of *Staphylococcus aureus* biofilm formation

3.7.1. The effect of carvacrol on inhibition of *Staphylococcus aureus* biofilm formation

In order to determine the effect of carvacrol which is one of the major components of *Origanum syriacum* on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated with different volumes of carvacrol at different concentrations

3.7.1.1. The effect of carvacrol solution of concentration 0.0176 g/ml, reflecting a concentration equivalent to the concentration of carvacrol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 μL of 0.0176 g/ml carvacrol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 50 μL of 0.0176 g/ml carvacrol solution, which contained 88 x 10^{-5} g of carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 11, Figure 29 and Figure 30).

Table 11. Effect of 50 μL volume of carvacrol solution of concentration 0.0176 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 μL of 0.0176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 50 μL of 0.0176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1347	0.2905	0.3263	
2	13073	0.2051	0.2283	0.2579	
3	12989	0.1378	0.2082	0.3383	
4	12634	0.1305	0.2668	0.2182	
5	2483	0.1819	0.2418	0.3610	
6	2484	0.2687	0.2315	0.3073	
7	2564	0.1765	0.2445	0.3015	
8	2553	0.1349	0.2356	0.2863	
9	7353	0.1145	0.2601	0.3157	
10	6281	0.1781	0.2599	0.2946	
11	45139	0.2741	0.2163	0.2447	
12	48865	0.1822	0.2199	0.3283	
13	10762	0.1482	0.2211	0.2679	
14	5862	0.1883	0.1656	0.2697	
15	14102	0.1651	0.6231	0.5032	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.0176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 50 µL of 0.0176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1064	0.1929	0.2240	
2	12631	0.1885	0.2019	0.3166	
3	7496	0.1219	0.2593	0.3119	
4	4826	0.1616	0.2541	0.2665	
5	4588	0.1771	0.3679	0.4304	
6	43271	0.2359	0.1897	0.2859	

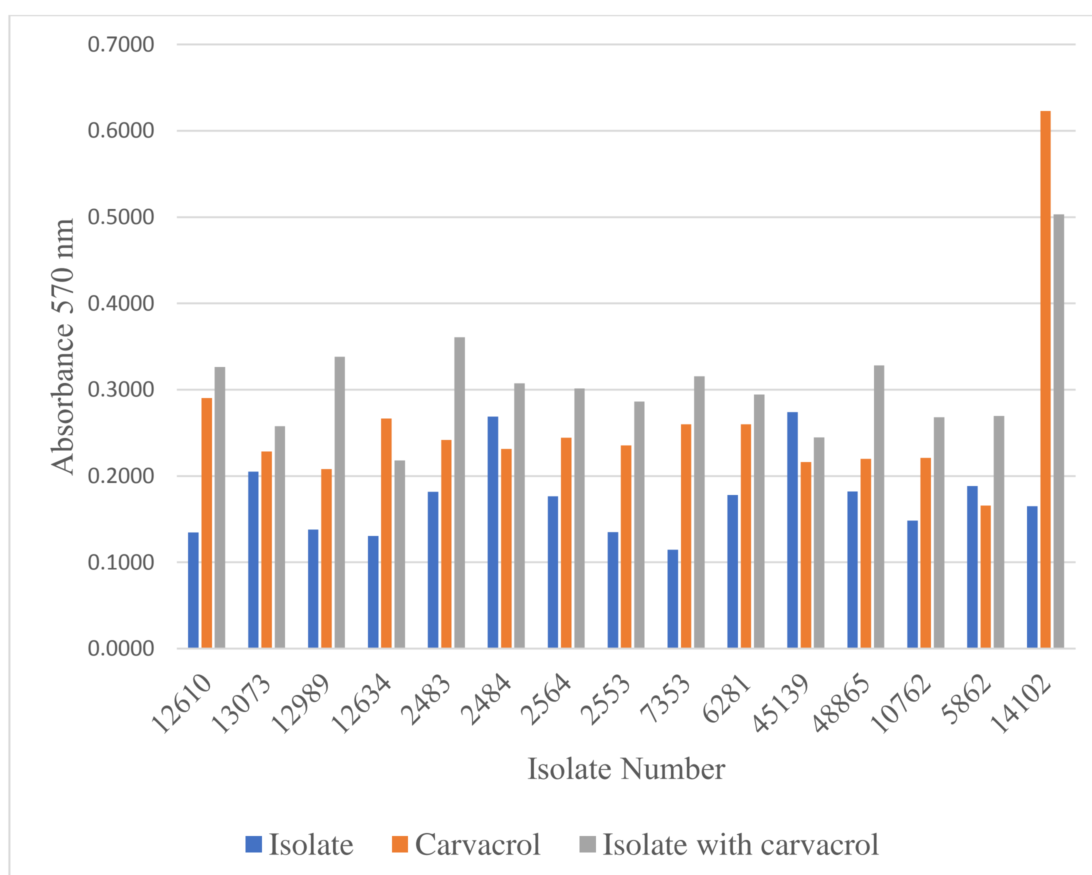


Figure 29. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.0176 g/ml carvacrol solution

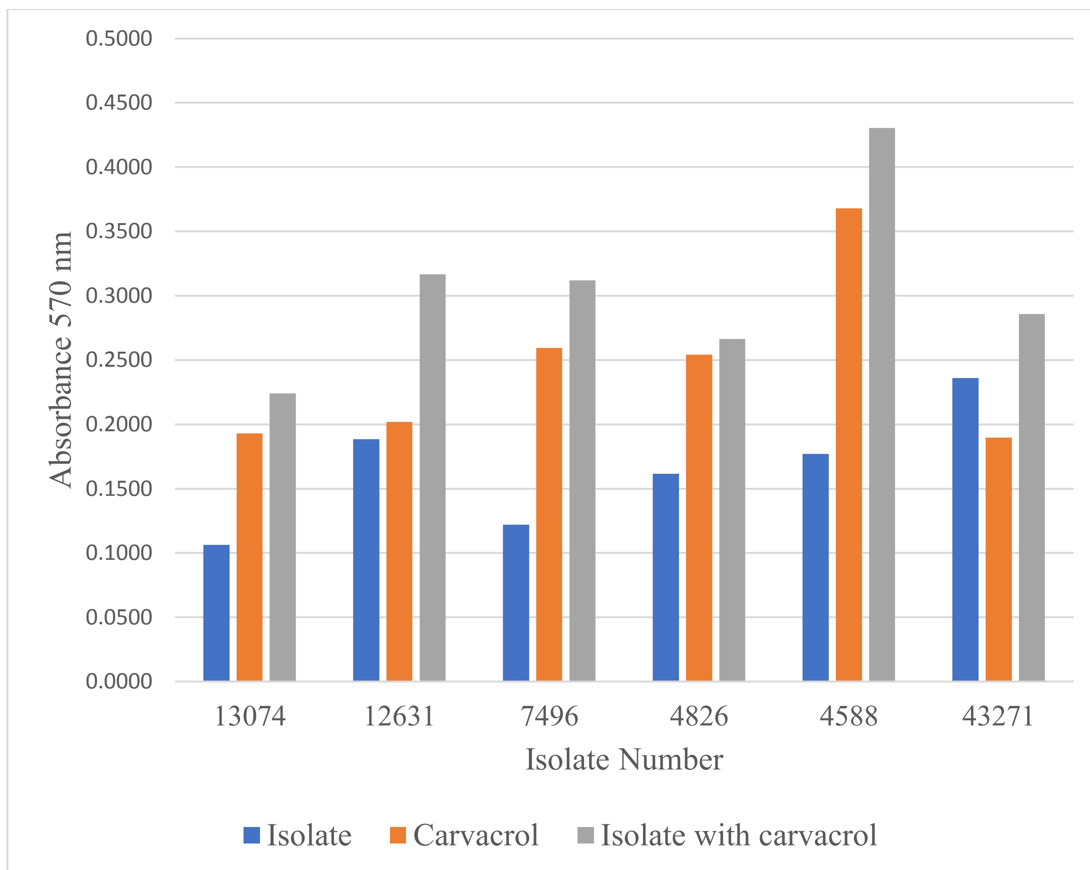


Figure 30. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 μL of 0.0176 g/ml carvacrol solution

The effect of 100 μL of 0.0176 g/ml carvacrol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed again that 100 μL of 0.0176 g/ml carvacrol solution, which contained 176×10^{-5} g of carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 12, Figure 31 and Figure 32).

Table 12. Effect of 100 μL volume of carvacrol solution of concentration 0.0176 g/ml on inhibition of *Staphylococcus aureus* biofilm formation a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.0176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 100 μL of 0.0176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1412	1.0614	0.7070	
2	13073	0.1985	0.5721	0.6082	
3	12989	0.1338	0.7860	0.5776	
4	12634	0.1209	0.4485	0.6107	
5	2483	0.1821	0.7022	0.4672	
6	2484	0.2237	0.7820	0.5469	
7	2564	0.2810	0.4965	0.8168	
8	2553	0.1307	0.3630	0.5875	
9	7353	0.1338	0.7257	0.7408	
10	6281	0.1781	0.2599	0.2946	
11	45139	0.2605	0.3565	0.9031	
12	48865	0.1736	0.5354	0.4814	
13	10762	0.1304	0.6786	0.5405	
14	5862	0.1749	0.8136	0.5049	
15	14102	0.1833	0.6538	0.5885	

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	Absorbance$_{570\ nm}$ of 100 μL of 0.0176 g/ml carvacrol solution	Absorbance$_{570\ nm}$ of 100 μL of 0.0176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1018	0.4979	0.7984	
2	12631	0.1854	0.4700	0.6320	
3	7496	0.1207	0.7319	0.5408	
4	4826	0.1521	0.9924	0.6575	
5	4588	0.1735	0.7989	0.6434	
6	43271	0.2461	0.4982	0.4725	

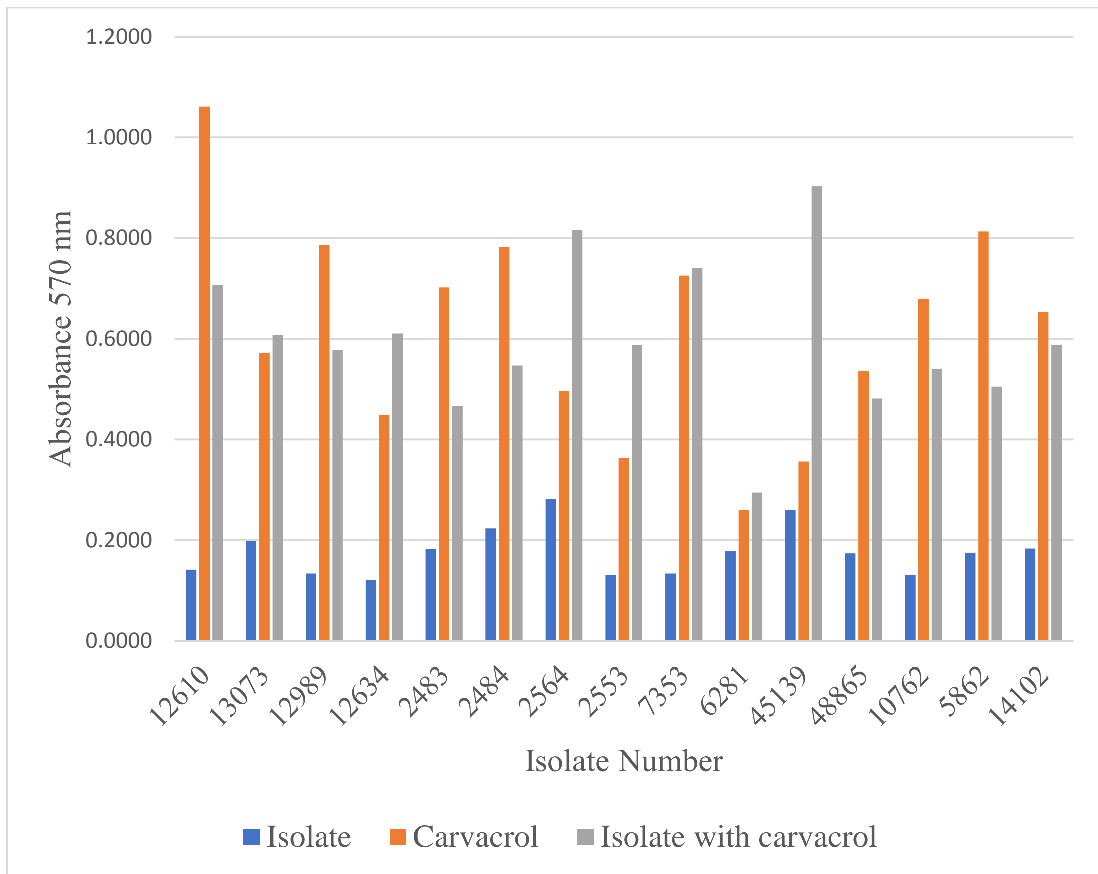


Figure 31. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.0176 g/ml carvacrol solution

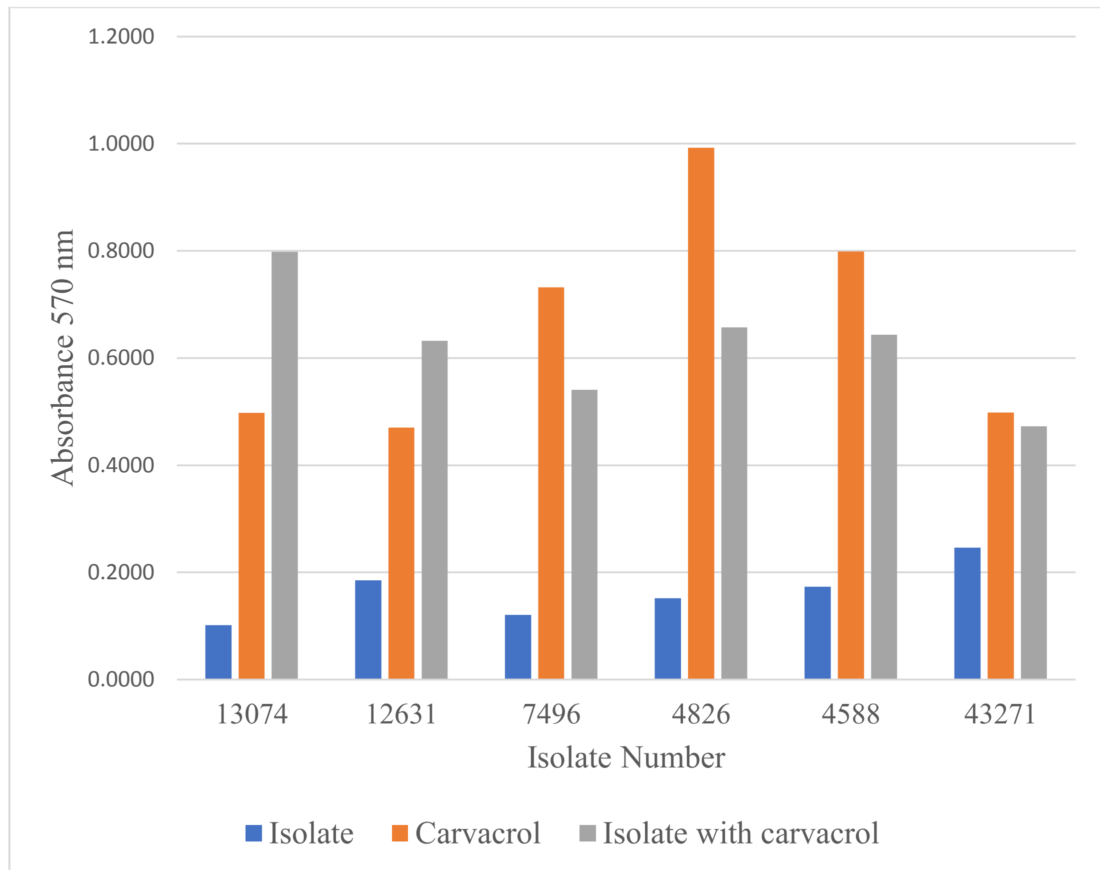


Figure 32. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.0176 g/ml carvacrol solution

3.7.1.2. The effect of carvacrol solution of concentration 0.088 g/ml, reflecting a concentration five times higher than the concentration of carvacrol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 µL of 0.088 g/ml carvacrol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed again that 50 µL of 0.088 g/ml carvacrol solution, which contained 44 $x10^{-4}$ g of carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains even at a concentration five times higher than the concentration of carvacrol in the *Origanum syriacum* methanol extract (Table 13, Figure 33 and Figure 34).

Table 13. Effect of 50 µL volume of carvacrol solution of concentration 0.088 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.088 g/ml carvacrol solution	$O.D_{570\ nm}$ of 50 µL of 0.088 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1176	1.5324	0.9965	
2	13073	0.1953	0.4589	0.4079	
3	12989	0.1930	0.4560	0.4073	
4	12634	0.1297	0.6447	0.2806	
5	2483	0.1302	0.6620	0.6785	
6	2484	0.1032	0.6621	0.5084	
7	2564	0.1091	0.9616	0.6335	
8	2553	0.1696	0.7165	0.8230	
9	7353	0.1226	1.9174	1.7770	
10	6281	0.1403	1.0028	0.8192	
11	45139	0.1114	1.4826	1.3066	
12	48865	0.2224	0.4473	0.3663	
13	10762	0.1725	0.3925	0.3606	
14	5862	0.2189	0.5350	0.5212	
15	14102	0.1422	2.1005	0.8446	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.088 g/ml carvacrol solution	$O.D_{570\ nm}$ of 50 µL of 0.088 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1510	0.3958	0.4638	
2	12631	0.1742	0.5204	0.5321	
3	7496	0.2074	0.5497	0.4732	
4	4826	0.1780	0.9428	0.6639	
5	4588	0.1775	1.4296	0.7592	
6	43271	0.2892	0.5602	0.4203	

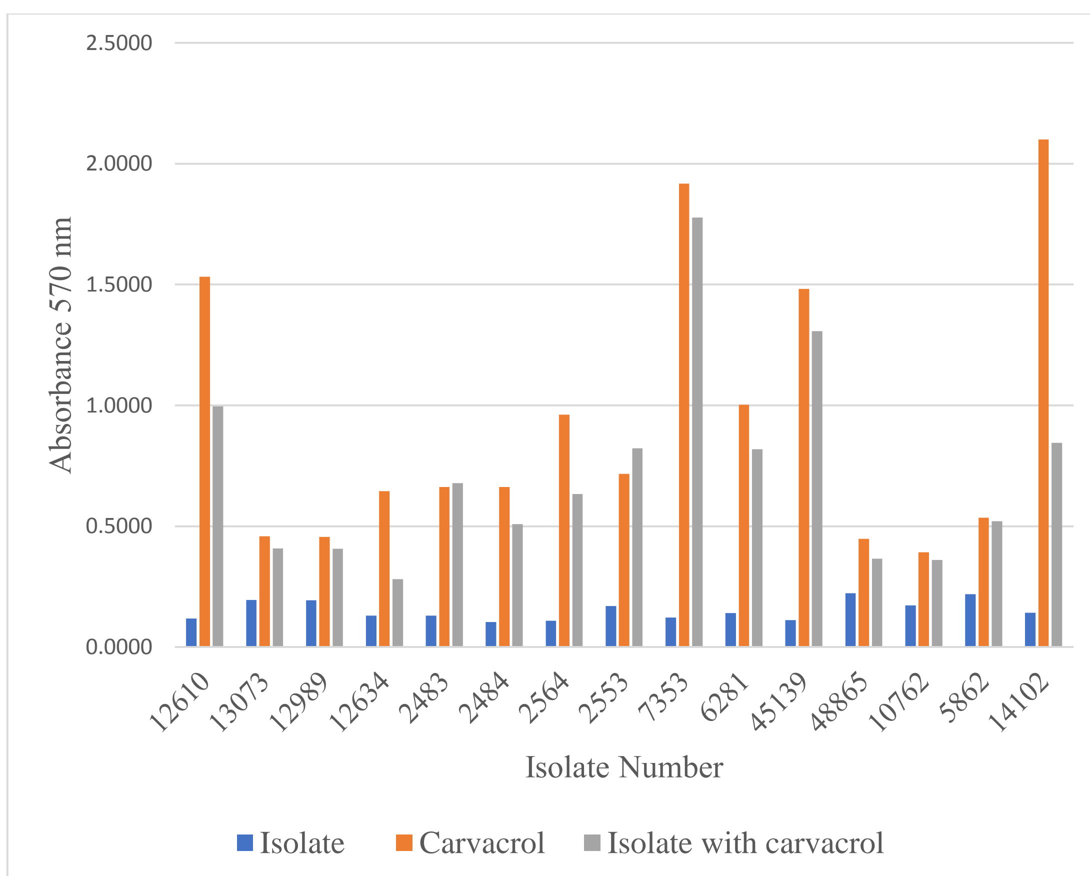


Figure 33. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.088 g/ml carvacrol solution

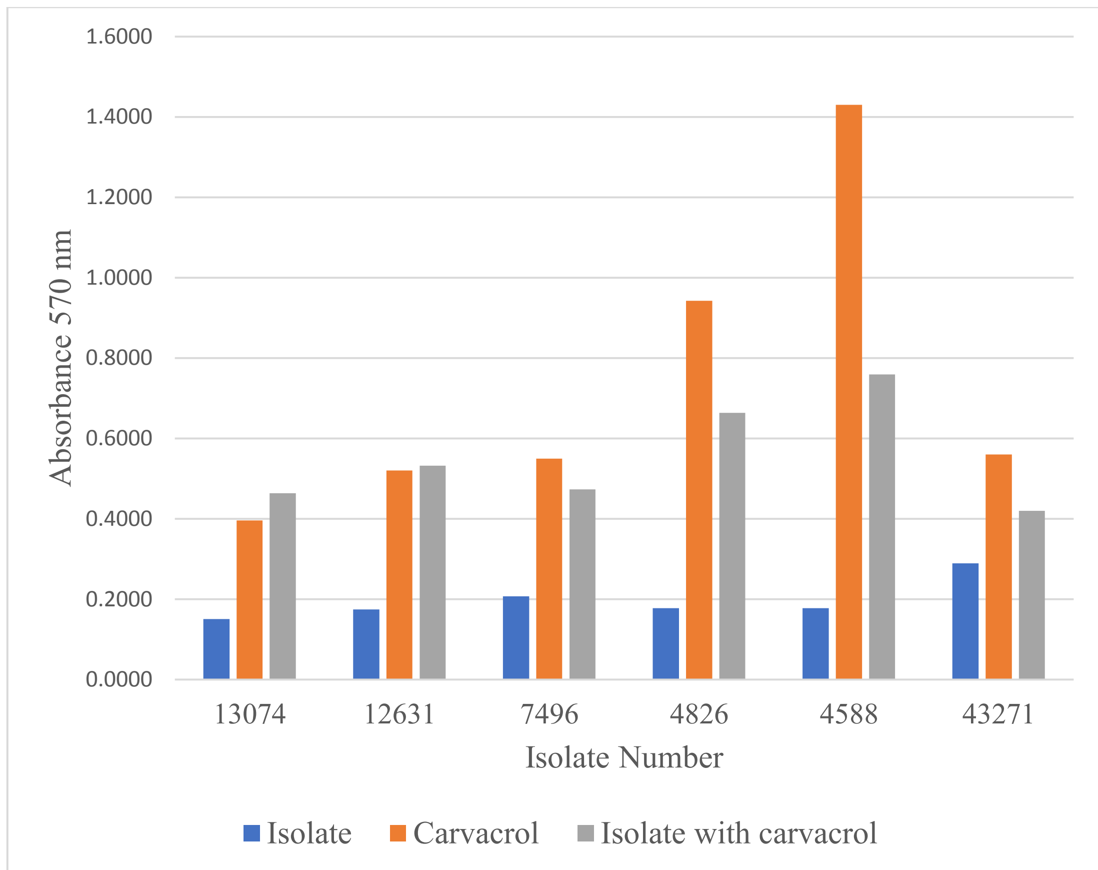


Figure 34. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 µL of 0.088 g/ml carvacrol solution

The effect of 100 µL of 0.088 g/ml carvacrol solution on inhibition of Staphylococcus aureus biofilm formation

In order to determine whether altering the volume to 100 µL of 0.088 g/ml carvacrol would exert an effect on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated each with carvacrol. The results showed that 100 µL of 0.088 g/ml carvacrol solution which contained 88 x 10^{-4} g carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 14 Figure 35 and Figure 36).

Table 14. Effect of 100 μL volume of carvacrol solution of concentration 0.088 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.088 g/ml carvacrol solution	$O.D_{570\ nm}$ of 100 μL of 0.088 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1298	1.7884	1.5404	
2	13073	0.1926	1.3674	1.7266	
3	12989	0.2003	1.2103	1.5675	
4	12634	0.1231	1.2636	1.6084	
5	2483	0.1318	2.6506	1.7642	
6	2484	0.1043	2.0633	1.6950	
7	2564	0.1087	2.3524	1.5725	
8	2553	0.1642	2.6189	1.6055	
9	7353	0.1295	1.7417	1.5494	
10	6281	0.1421	3.0534	2.4648	
11	45139	0.1270	1.3246	1.2435	
12	48865	0.2479	1.4343	1.5155	
13	10762	0.1339	1.4717	1.4603	
14	5862	0.2776	1.2840	1.7117	
15	14102	0.1763	1.7183	1.6575	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.088 g/ml carvacrol solution	$O.D_{570\ nm}$ of 100 µL of 0.088 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1443	1.0761	1.6351	
2	12631	0.1797	1.1576	1.6692	
3	7496	0.1849	1.2239	1.3679	
4	4826	0.1412	3.5977	1.9299	
5	4588	0.1755	3.7783	1.9337	
6	43271	0.2847	0.9369	1.6062	

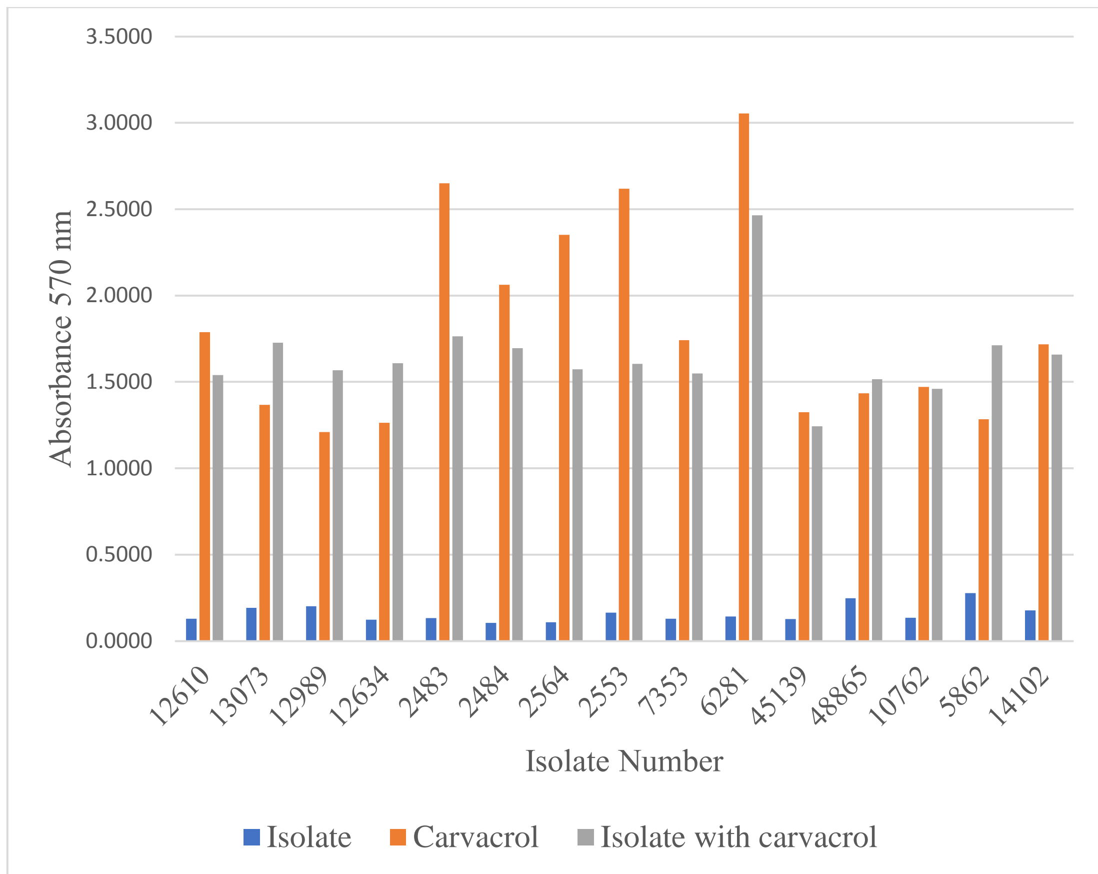


Figure 35. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.088 g/ml carvacrol solution

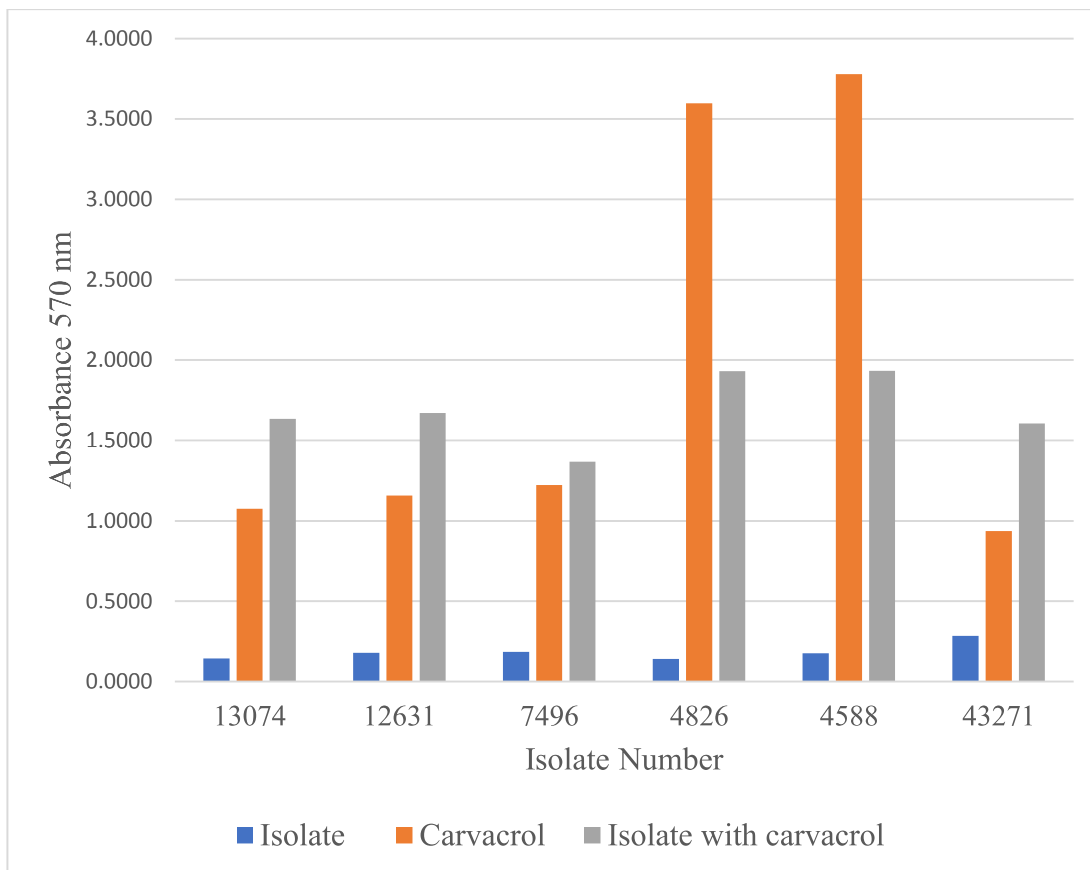


Figure 36. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 μL of 0.088 g/ml carvacrol solution

3.7.1.3. The effect of carvacrol solution of concentration 0.176 g/ml, reflecting a concentration ten times higher than the concentration of carvacrol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 μL of carvacrol solution of concentration 0.176 g/ml on inhibition of Staphylococcus aureus biofilm formation

In order to determine whether increasing the concentration of carvacrol even more would exhibit an effect on inhibition of *S. aureus* biofilm formation, the concentration of carvacrol was increased by ten times to 0.176 g/ml. Confirming the previously observed results with 100 μL of 0.088 g/ml carvacrol solution (Table 14, Figure 35 and Figure 36), 50 μL of 0.176 g/ml carvacrol solution which contained 88 x10^{-4} g

carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains even at a concentration ten times higher than the concentration of carvacrol in the *Origanum syriacum* methanol extract (Table 15, Figure 37 and Figure 38).

Table 15. Effect of 50 μL volume of carvacrol solution of concentration 0.176 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 μL of 0.176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 50 μL of 0.176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1263	2.8353	2.3267	
2	13073	0.2173	2.7841	2.5370	
3	12989	0.2051	3.3491	2.4121	
4	12634	0.1364	3.2233	2.3047	
5	2483	0.1306	3.8218	3.5110	
6	2484	0.1145	4.0124	3.2169	
7	2564	0.1133	4.3987	3.4268	
8	2553	0.1737	3.7971	2.9615	
9	7353	0.1256	3.7833	3.0672	
10	6281	0.1334	2.9713	3.2204	
11	45139	0.1184	2.8400	2.3649	
12	48865	0.1917	2.6671	2.5865	
13	10762	0.1745	1.7867	2.0658	
14	5862	0.2296	1.9967	2.3424	
15	14102	0.1329	3.1645	2.8864	

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 50 µL of 0.176 g/ml carvacrol solution	O.D$_{570\ nm}$ of 50 µL of 0.176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1585	3.1848	2.2150	
2	12631	0.1709	2.4106	2.9412	
3	7496	0.1982	1.8004	2.0119	
4	4826	0.1714	3.7236	3.8325	
5	4588	0.1634	3.7098	3.4792	
6	43271	0.2664	3.2333	2.5030	

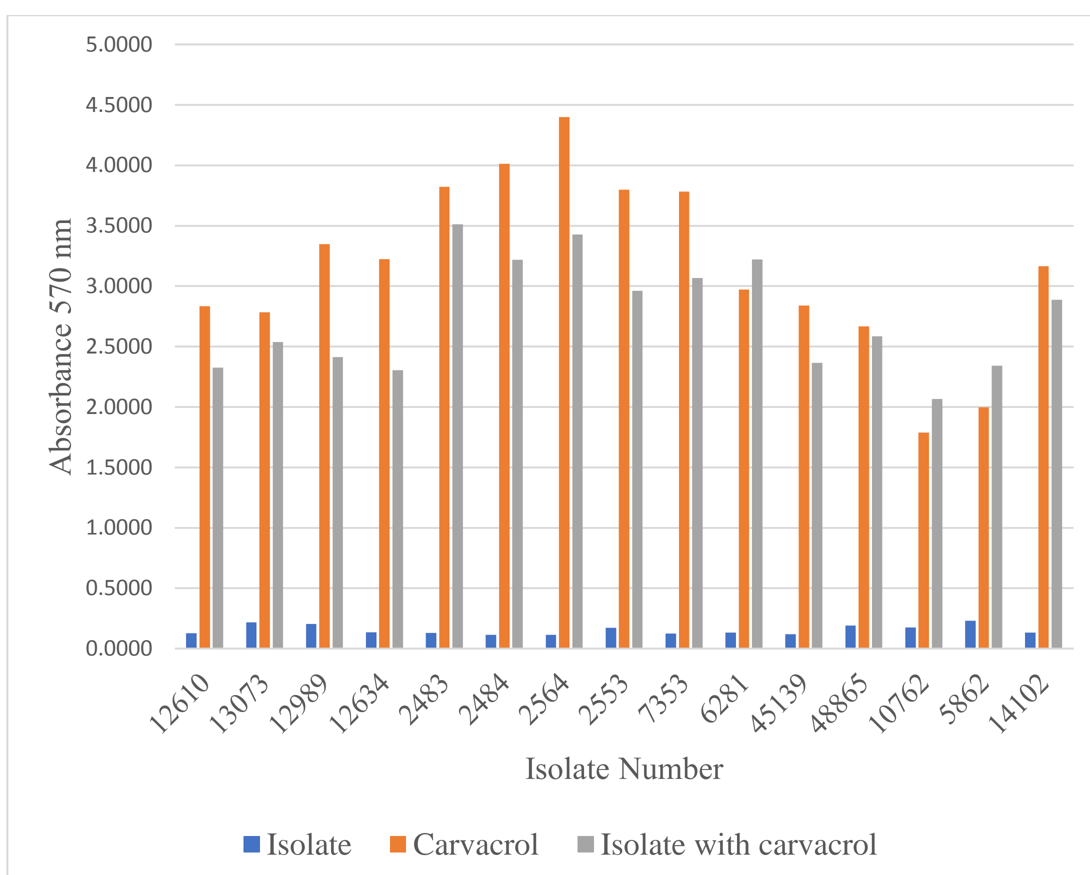


Figure 37. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.176 g/ml carvacrol solution

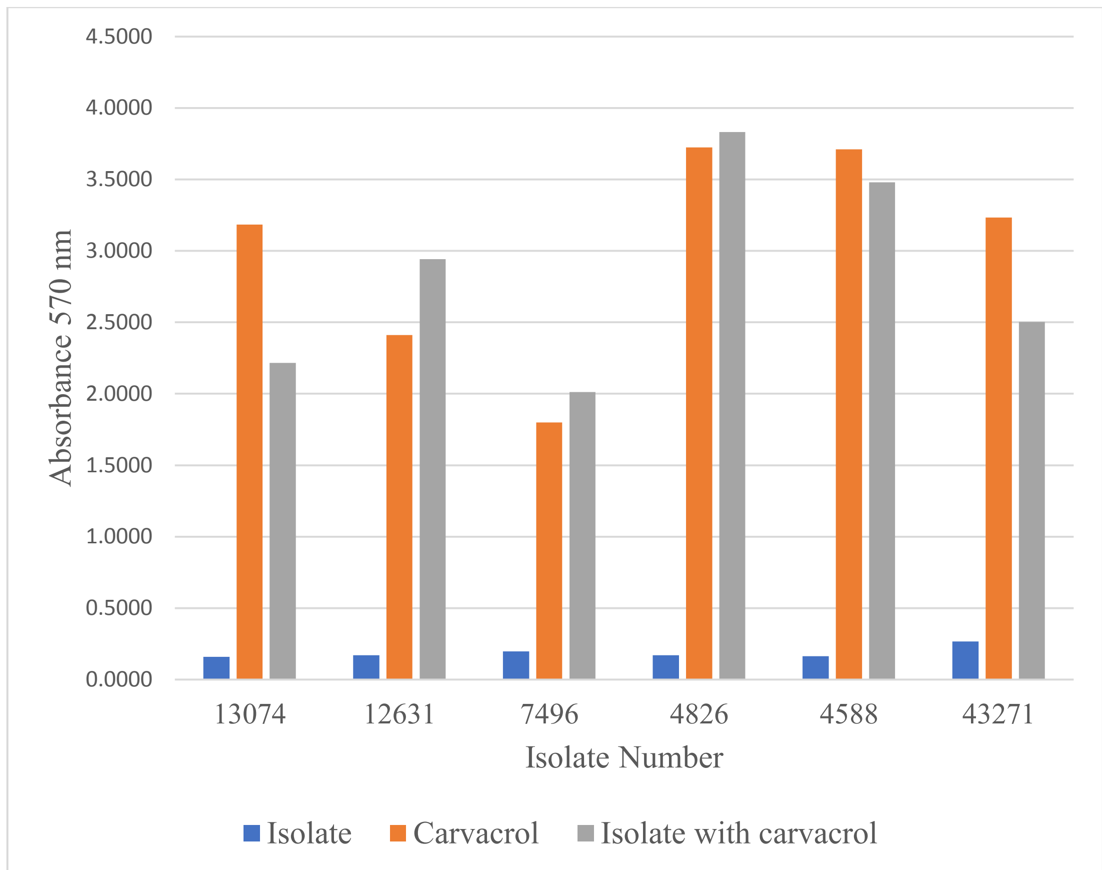


Figure 38. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 μL of 0.176 g/ml carvacrol solution

The effect of 100 μL of carvacrol solution of concentration 0.176 g/ml on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 μL of 0.176 g/ml carvacrol solution which contained 0.0176 g carvacrol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 16, Figure 39 and Figure 40).

Table 16. Effect of 100 μL volume of carvacrol solution of concentration 0.176 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 100 μL of 0.176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1199	3.0333	3.9953	
2	13073	0.1994	3.8776	3.3021	
3	12989	0.1950	3.4003	4.3197	
4	12634	0.1304	4.1176	3.1549	
5	2483	0.1396	4.5076	4.8068	
6	2484	0.1029	4.1162	4.3691	
7	2564	0.1151	4.0819	3.9469	
8	2553	0.1233	4.5943	3.8307	
9	7353	0.1210	3.7075	4.9288	
10	6281	0.1378	3.4837	3.9295	
11	45139	0.1262	3.1006	4.8301	
12	48865	0.1957	4.3557	3.1540	
13	10762	0.1765	3.5699	3.5836	
14	5862	0.2163	3.9760	4.2111	
15	14102	0.1376	3.9023	3.8253	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.176 g/ml carvacrol solution	$O.D_{570\ nm}$ of 100 µL of 0.176 g/ml carvacrol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1592	3.5790	3.7146	
2	12631	0.1993	4.1669	3.7476	
3	7496	0.1849	3.5390	2.9953	
4	4826	0.1750	4.2825	3.9311	
5	4588	0.1747	4.1123	3.6569	
6	43271	0.2878	4.4048	2.8876	

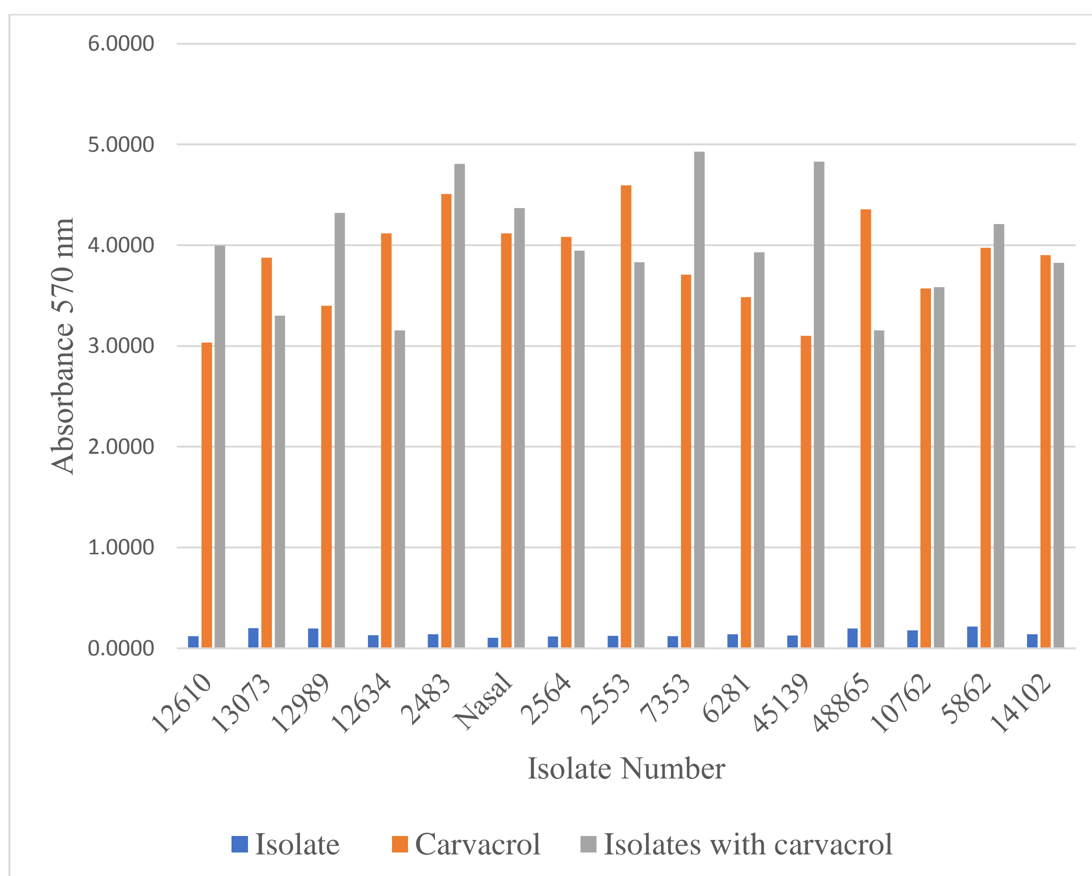


Figure 39. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.176 g/ml carvacrol solution

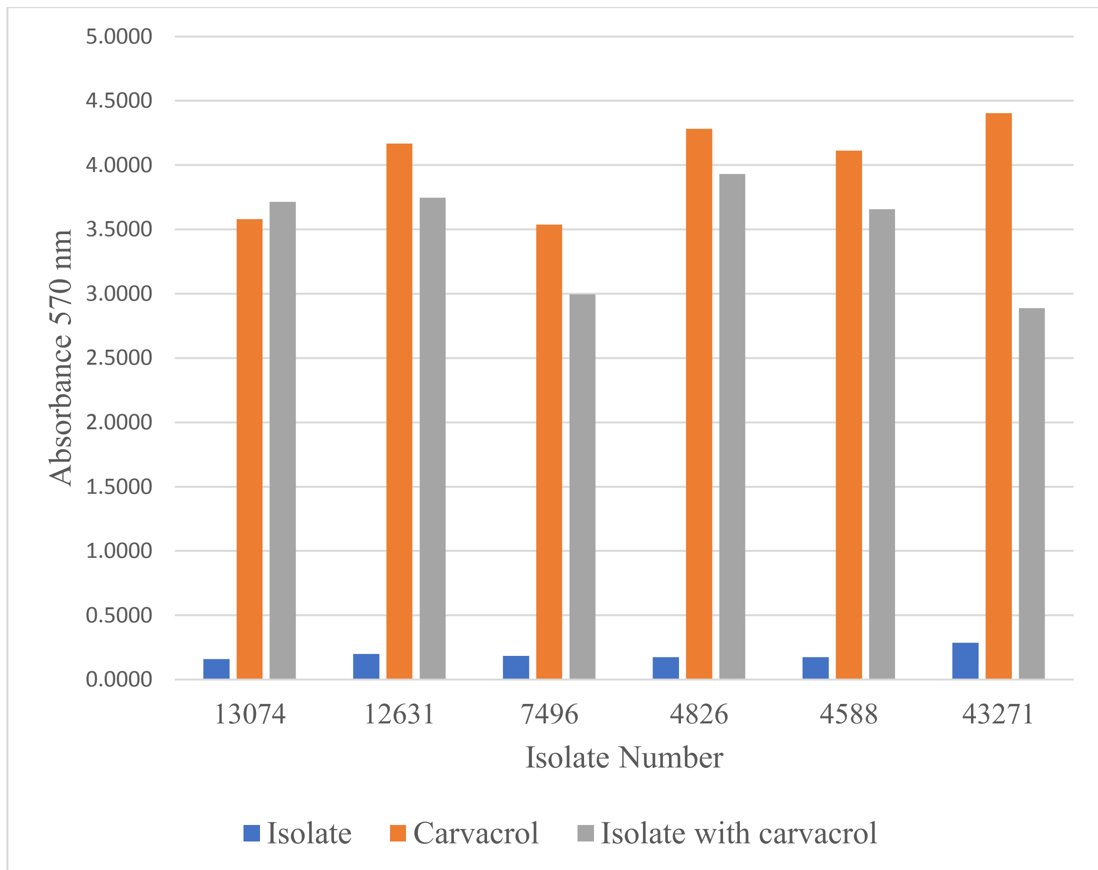


Figure 40. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 μL of 0.176 g/ml carvacrol solution

3.7.2. The effect of Thymol on inhibition of *Staphylococcus aureus* biofilm formation

In order to determine the effect of thymol which is one of the major components of *Origanum syracum* on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated with different volumes of thymol at different concentrations.

3.7.2.1. The effect of thymol solution of concentration 0.0247 g/ml, reflecting a concentration equivalent to the concentration of thymol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 μL of 0.0247 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 50 μL of 0.0247 g/ml thymol solution which contained 124 x 10^{-5} g thymol lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 17, Figure 41 and Figure 42).

Table 17. Effect of 50 µL volume of thymol solution of concentration 0.0247 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 50 µL of 0.0247 g/ml thymol solution	O.D$_{570\ nm}$ of 50 µL of 0.0247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1215	0.3021	0.3746	
2	13073	0.1288	0.3406	0.3556	
3	12989	0.1530	0.2675	0.3769	
4	12634	0.1073	0.5725	0.4844	
5	2483	0.1235	0.2517	0.3527	
6	2484	0.1827	0.3806	0.5020	
7	2564	0.1856	0.5399	0.6239	
8	2553	0.0950	0.4057	0.4954	
9	7353	0.0987	0.7725	0.4437	
10	6281	0.1229	0.3234	0.3843	
11	45139	0.1234	0.4547	0.4821	
12	48865	0.1848	0.5082	0.4523	
13	10762	0.1676	0.3200	0.4098	
14	5862	0.1412	0.4614	0.5705	
15	14102	0.1481	0.5610	0.5321	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.0247 g/ml thymol solution	$O.D_{570\ nm}$ of 50 µL of 0.0247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.0926	0.4665	0.4831	
2	12631	0.0980	1.5720	1.2434	
3	7496	0.1398	1.1992	0.6025	
4	4826	0.1613	0.4485	0.4479	
5	4588	0.1218	0.8485	0.6162	
6	43271	0.1962	0.3276	0.3247	

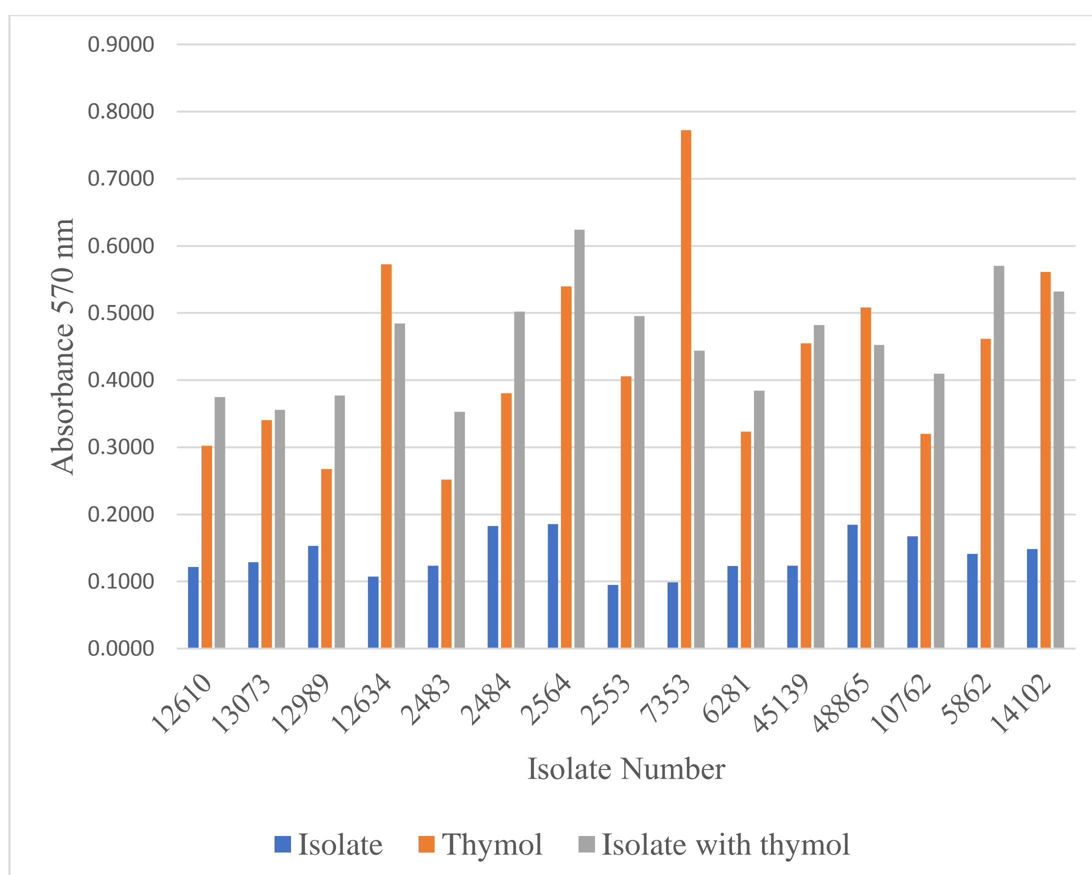


Figure 41. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.0247 g/ml thymol solution

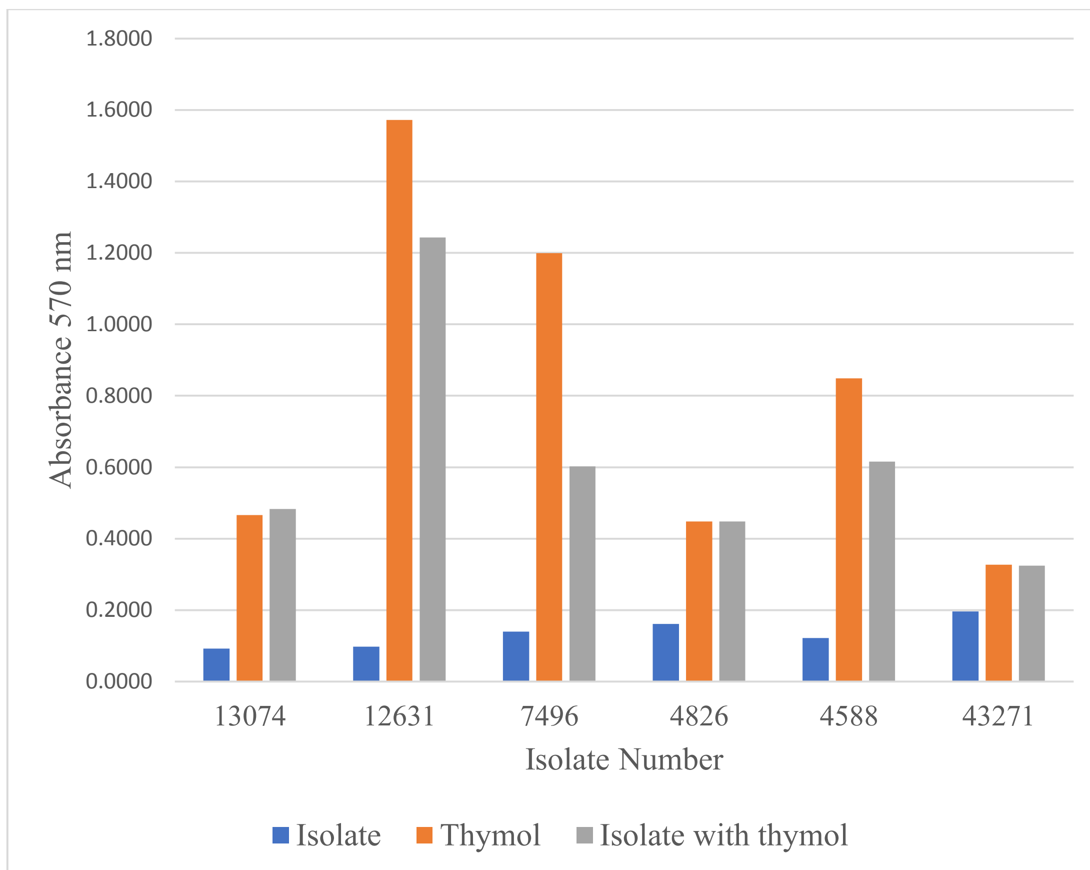


Figure 42. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 µL of 0.0247 g/ml thymol solution

The effect of 100 µL of 0.0247 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 µL of 0.0247 g/ml thymol solution which contained 247 x 10^{-5} g of thymol lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 18, Figure 43 and Figure 44).

Table 18. Effect of 100 μL volume of thymol solution of concentration 0.0247 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.0247 g/ml thymol solution	$O.D_{570\ nm}$ of 100 μL of 0.0247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1306	1.7592	1.6568	
2	13073	0.1259	1.7225	1.0842	
3	12989	0.1483	1.2965	1.0875	
4	12634	0.1119	1.5225	1.5229	
5	2483	0.1216	1.3997	1.0620	
6	2484	0.1862	1.4274	0.9219	
7	2564	0.1890	1.5091	1.2000	
8	2553	0.1022	1.7226	1.3161	
9	7353	0.1073	3.1991	2.0288	
10	6281	0.1183	1.3921	1.3028	
11	45139	0.1175	1.4539	1.4245	
12	48865	0.1805	1.4288	1.3068	
13	10762	0.1582	1.4177	0.9753	
14	5862	0.1507	1.6901	1.1303	
15	14102	0.1305	1.3539	1.1182	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.0247 g/ml thymol solution	$O.D_{570\ nm}$ of 100 µL 0.0247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.0912	1.5332	1.3443	
2	12631	0.0986	0.2884	0.3720	
3	7496	0.1970	3.4529	1.8805	
4	4826	0.1681	3.2635	1.4439	
5	4588	0.1211	2.8669	1.5546	
6	43271	0.1653	1.7655	0.8744	

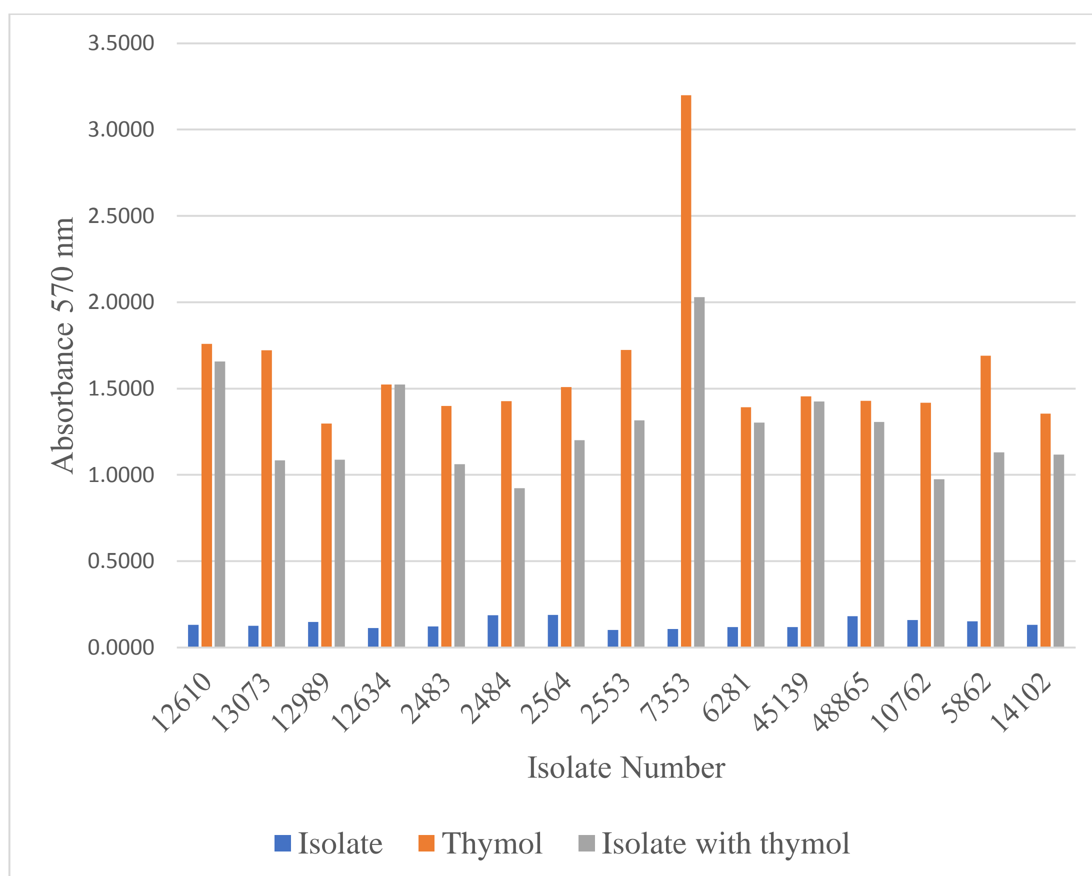


Figure 43. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.0247 g/ml thymol solution

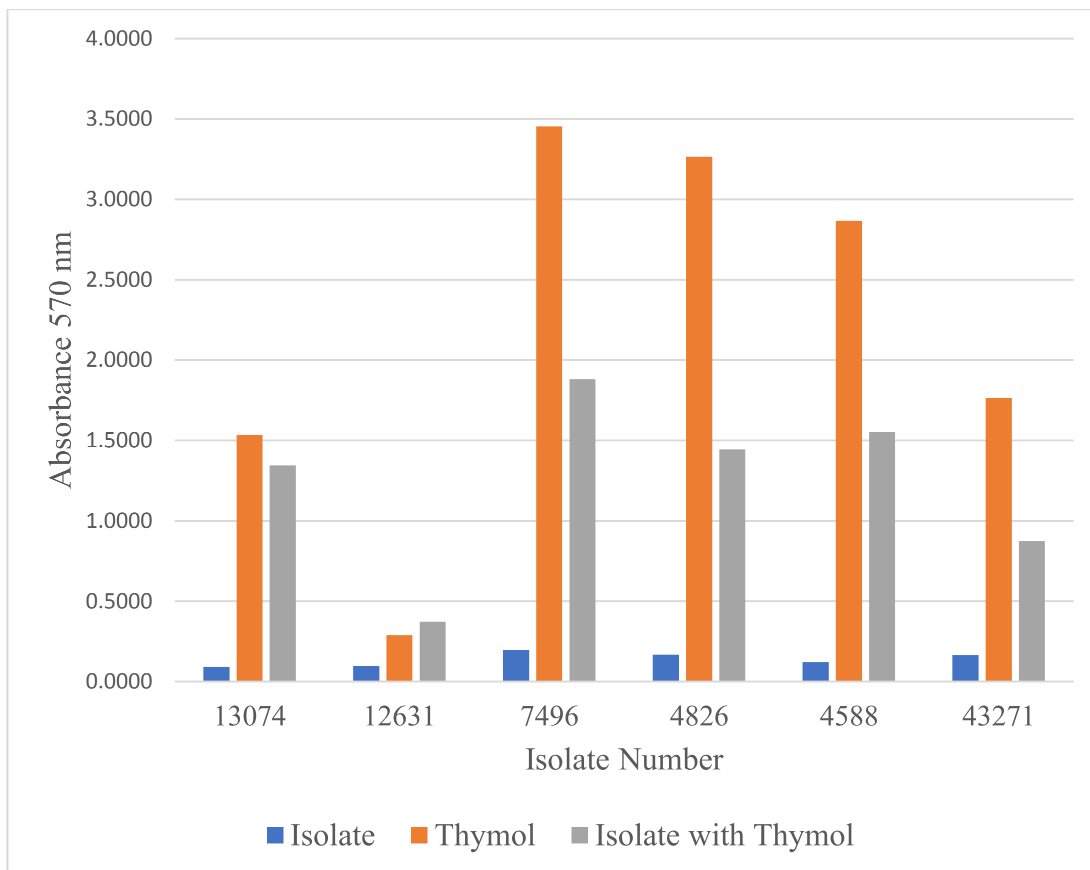


Figure 44. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.0247 g/ml thymol solution

3.7.2.2. The effect of thymol solution of concentration 0.124 g/ml, reflecting a concentration five times higher than the concentration of thymol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 µL of 0.124 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

In order to determine whether increasing the concentration of thymol would exhibit an effect on inhibition of *S. aureus* biofilm formation, the concentration of thymol was increased by five times to 0.124 g/ml. The results showed that 50 µL of 0.124 g/ml thymol solution which contained 62 x10^{-4} g of thymol solution lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant

S. aureus strains even at a concentration five times higher than the concentration of thymol in the *Origanum syriacum* methanol extract (Table 19, Figure 45 and Figure 46).

Table 19. Effect of 50 μL volume of thymol solution of concentration 0.124 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 50 μL of 0.124 g/ml thymol solution	O.D$_{570\ nm}$ of 50 μL of 0.124 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1683	0.7397	0.5566	
2	13073	0.1418	0.8383	0.5513	
3	12989	0.1755	0.7102	0.5204	
4	12634	0.1483	0.4445	0.3167	
5	2483	0.1006	0.4897	0.2962	
6	2484	0.1029	0.9837	0.4266	
7	2564	0.1212	1.7949	0.5997	
8	2553	0.1159	0.4252	0.3028	
9	7353	0.1177	0.4872	0.3203	
10	6281	0.1200	0.4876	0.2767	
11	45139	0.1753	0.9983	0.5594	
12	48865	0.1117	1.1313	0.7469	
13	10762	0.1478	0.6691	0.3097	
14	5862	0.1379	1.0986	0.5332	
15	14102	0.1483	0.4180	0.2838	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 µL of 0.124 g/ml thymol solution	$O.D_{570\ nm}$ of 50 µL of 0.124 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1465	0.6550	0.4816	
2	12631	0.1433	0.7278	0.5676	
3	7496	0.1267	0.5323	0.3050	
4	4826	0.1550	0.4489	0.2367	
5	4588	0.1212	0.4744	0.2965	
6	43271	0.1657	0.8694	0.5349	

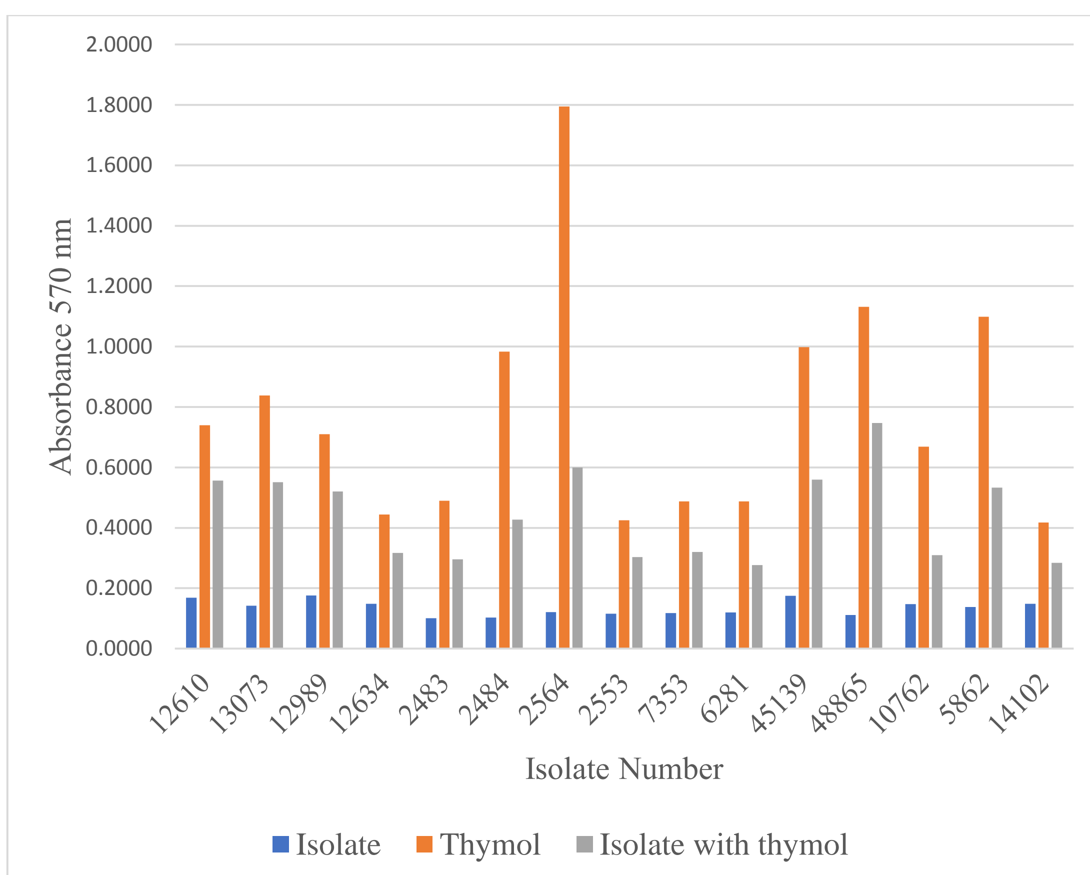


Figure 45. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.124 g/ml thymol solution

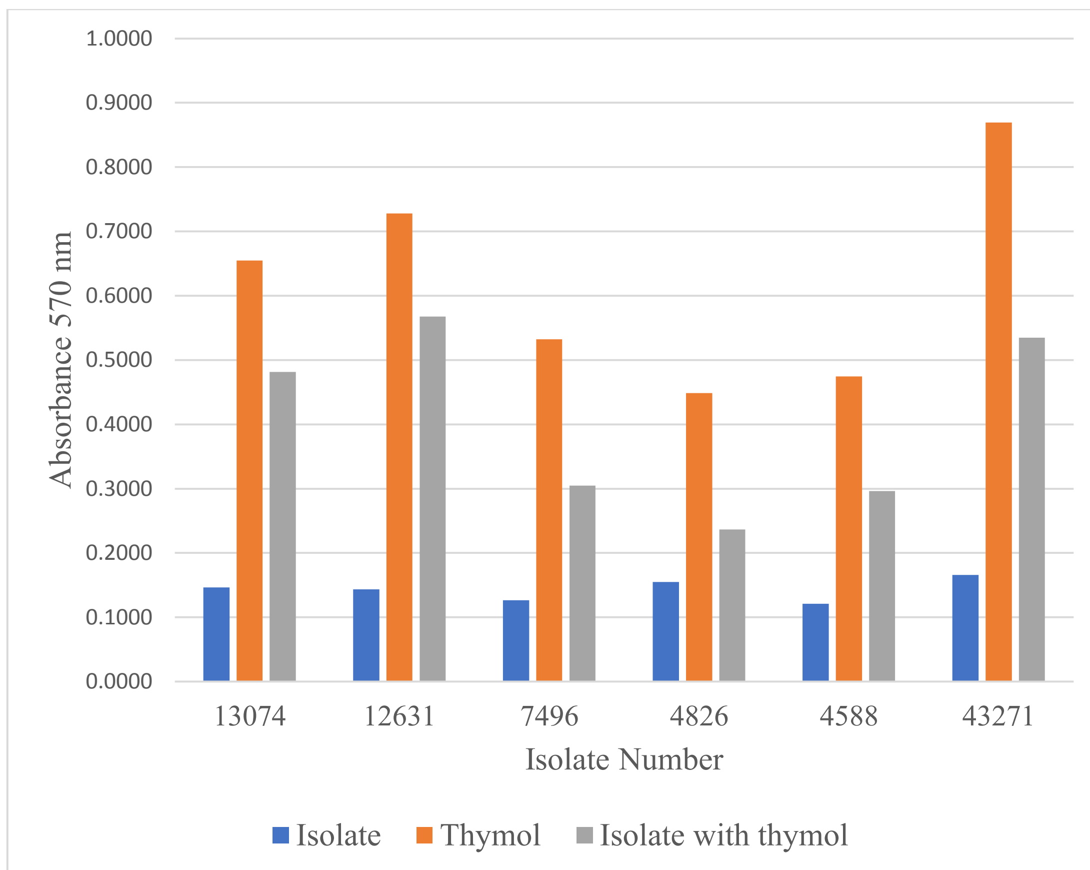


Figure 46. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 µL of 0.124 g/ml thymol solution

The effect of 100 µL of 0.124 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

In order to determine whether altering the volume to 100 µL of 0.124 g/ml thymol would exert an effect on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated each with thymol. The results showed that 100 µL of 0.124 g/ml thymol solution which contained 124 x 10^{-4} g of thymol lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 20, Figure 47 and Figure 48).

Table 20. Effect of 100 µL volume of thymol solution of concentration 0.124 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.124 g/ml thymol solution	$O.D_{570\ nm}$ of 100 µL of 0.124 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1811	1.1631	0.9287	
2	13073	0.1401	2.0948	1.0535	
3	12989	0.2142	1.2982	1.2035	
4	12634	0.1200	0.8676	0.6680	
5	2483	0.1095	0.7016	0.6548	
6	2484	0.1223	1.4477	0.8707	
7	2564	0.1280	1.7255	1.1656	
8	2553	0.1105	0.7218	0.5879	
9	7353	0.1184	1.3465	0.7162	
10	6281	0.1252	0.9801	0.5784	
11	45139	0.1952	1.7013	1.1723	
12	48865	0.1223	1.5754	1.1650	
13	10762	0.1409	1.5871	0.8948	
14	5862	0.1503	1.8554	1.3051	
15	14102	0.1410	0.7842	0.5889	

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 100 µL of 0.124 g/ml thymol solution	O.D570 nm of 100 µL 0.124 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1660	1.7783	1.0999	
2	12631	0.1333	1.7739	1.1822	
3	7496	0.1203	1.2714	0.8948	
4	4826	0.1482	0.9503	0.4678	
5	4588	0.1247	0.9930	0.7293	
6	43271	0.1746	1.6295	1.1380	

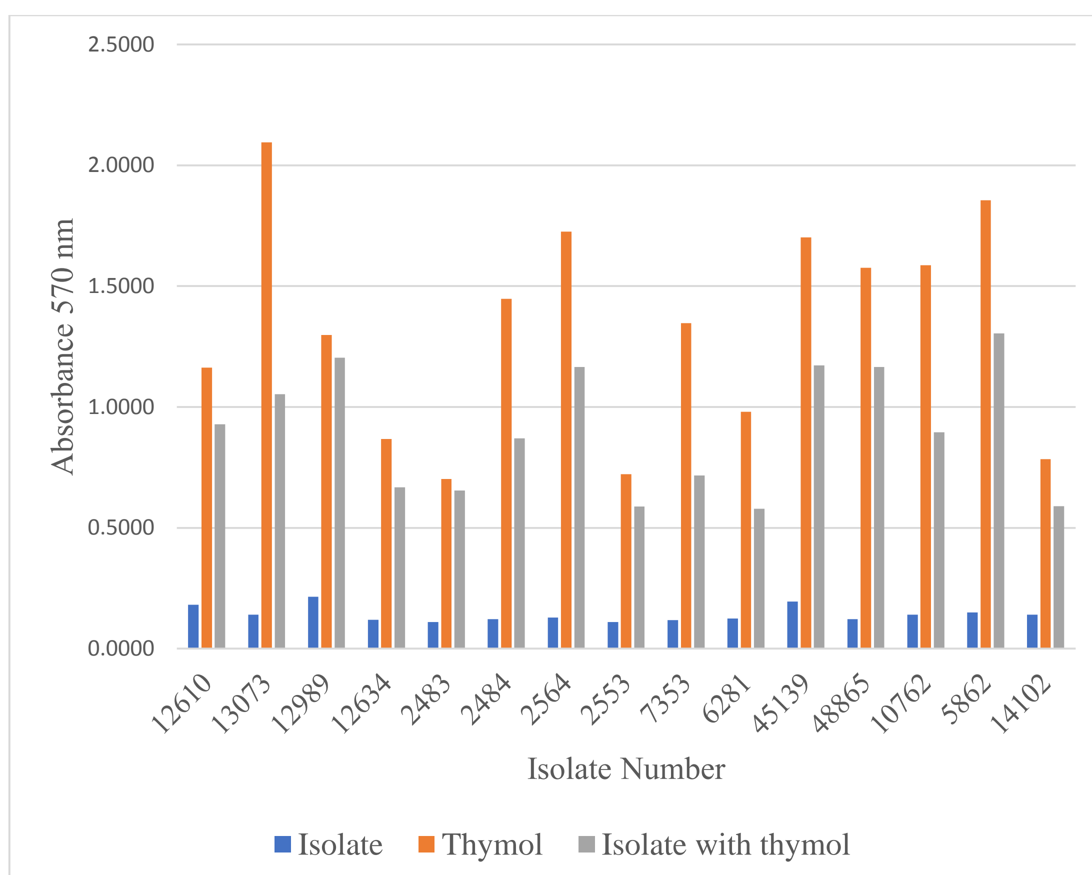


Figure 47. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.124 g/ml thymol solution

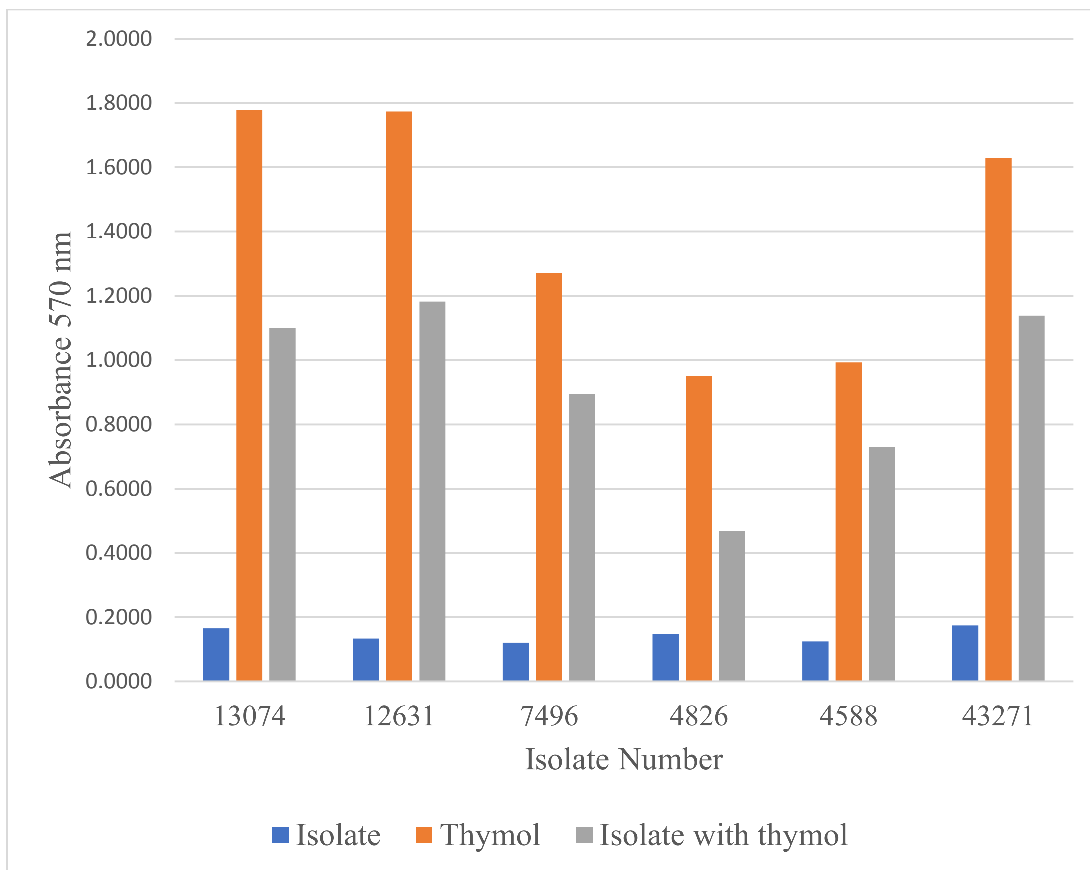


Figure 48. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.124 g/ml thymol solution

3.7.2.3. The effect of thymol solution of concentration 0.247 g/ml, reflecting a concentration ten times higher than the concentration of thymol in *Origanum syriacum* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 50 µL of 0.247 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

Confirming the previously observed results with 100 µL of 0.124 g/ml thymol solution (Table 20, Figure 47 and Figure 48), 50 µL of 0.247 g/ml thymol solution solution which contained 124 x10^{-4} g of thymol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains

even at a concentration ten times higher than the concentration of thymol in the *Origanum syriacum* methanol extract (Table 21, Figure 49 and Figure 50).

Table 21. Effect of 50 μL volume of thymol solution of concentration 0.247 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 50 μL of 0.247 g/ml thymol solution	$O.D_{570\ nm}$ of 50 μL of 0.247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1816	2.0882	1.9117	
2	13073	0.1336	2.5778	2.2995	
3	12989	0.2202	1.9532	1.9846	
4	12634	0.1069	2.6709	1.9998	
5	2483	0.1372	1.6855	1.5224	
6	2484	0.1055	3.7670	2.5109	
7	2564	0.1446	3.5968	2.9083	
8	2553	0.1075	1.7964	1.4521	
9	7353	0.1074	2.8036	2.2034	
10	6281	0.1203	1.8655	1.5081	
11	45139	0.1574	2.9299	2.4029	
12	48865	0.1132	3.3692	2.7783	
13	10762	0.1422	3.6306	2.2786	
14	5862	0.1515	3.2537	2.6919	
15	14102	0.1263	1.7964	1.4949	

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 50 µL of 0.247 g/ml thymol solution	O.D570 nm of 50 µL of 0.247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1481	2.2458	1.9517	
2	12631	0.1312	2.5209	1.9885	
3	7496	0.1155	2.9205	2.2585	
4	4826	0.1544	2.2851	1.6473	
5	4588	0.1078	2.0287	1.5541	
6	43271	0.1692	3.0052	2.4649	

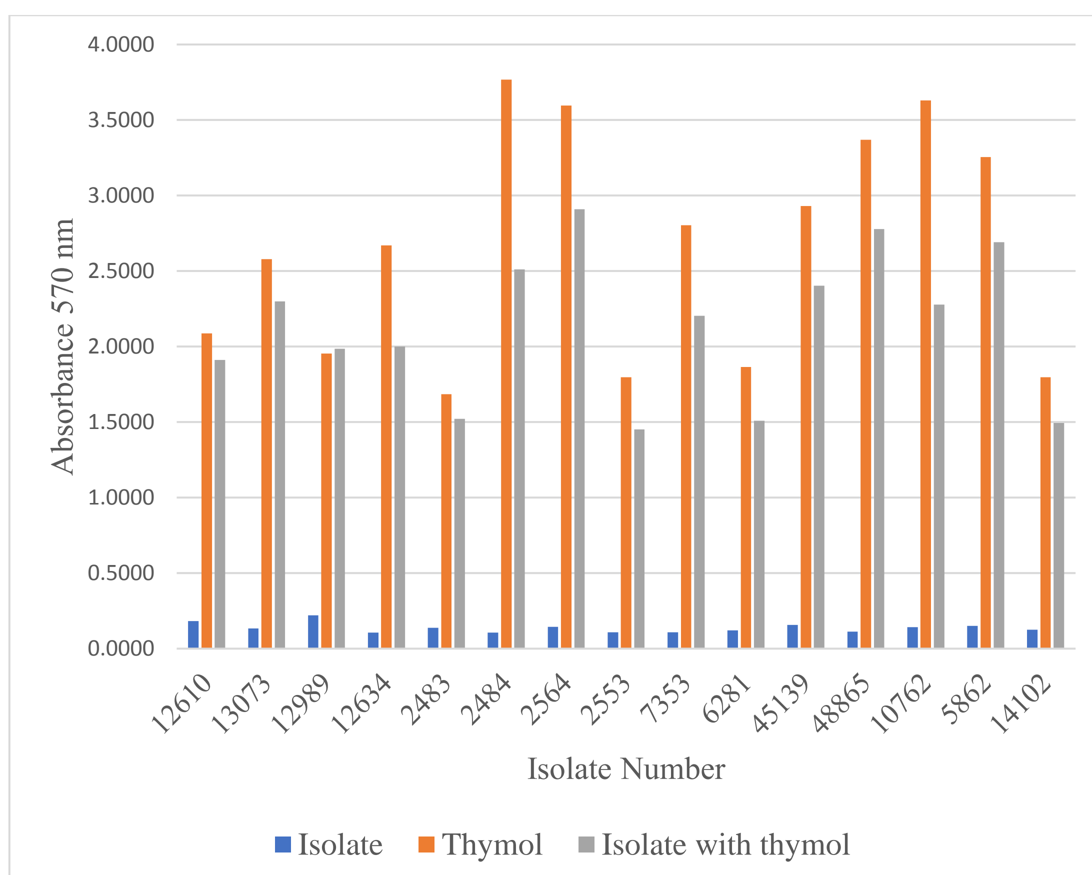


Figure 49. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 50 µL of 0.247 g/ml thymol solution

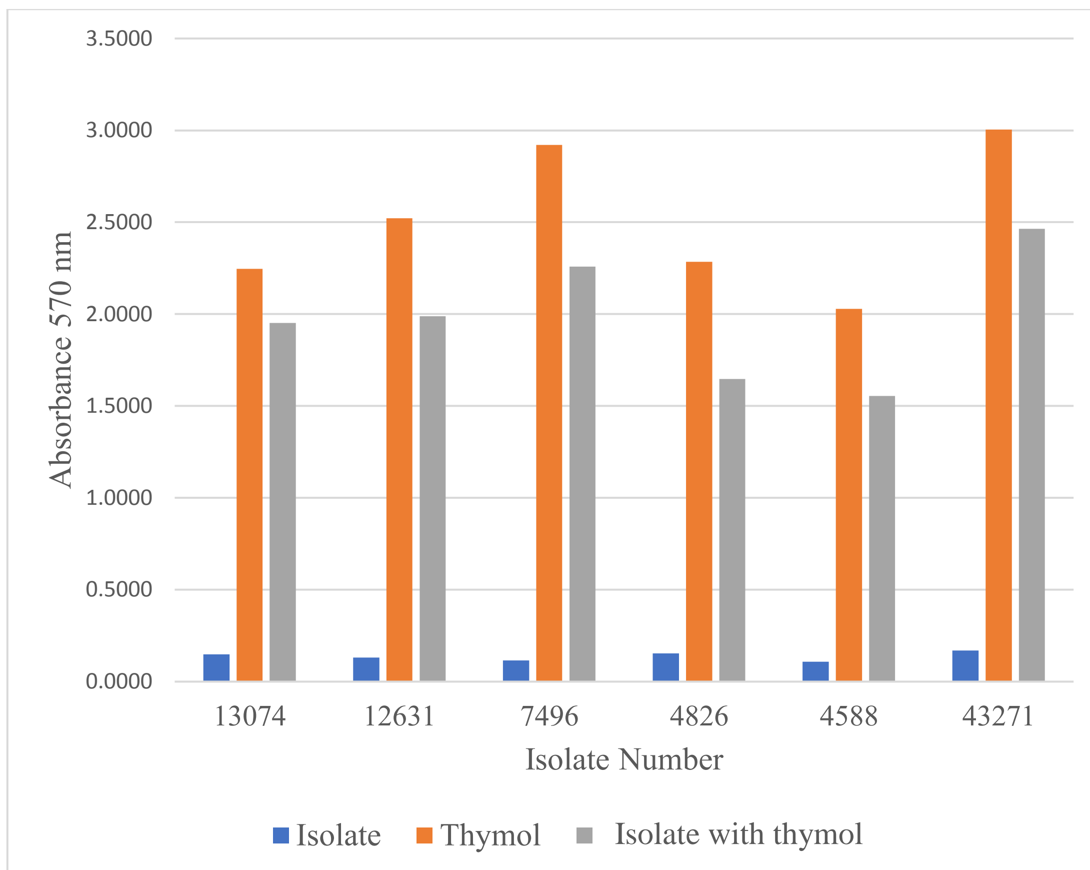


Figure 50. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 50 μL of 0.247 g/ml thymol solution

The effect of 100 μL of 0.247 g/ml Thymol solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 μL of 0.247 g/ml thymol solution which contained 247 x 10^{-4} g of thymol, lacked any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 22, Figure 51 and Figure 52).

Table 22. Effect of 100 μL volume of thymol solution of concentration 0.247 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.247 g/ml thymol solution	$O.D_{570\ nm}$ of 100 μL of 0.247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1604	3.9151	3.6532	
2	13073	0.1419	4.8658	3.7239	
3	12989	0.2142	4.0362	4.5705	
4	12634	0.1379	4.3131	3.4525	
5	2483	0.1105	3.3560	3.2854	
6	2484	0.1143	3.3450	3.4689	
7	2564	0.1225	3.6013	3.7469	
8	2553	0.1018	2.6013	2.8578	
9	7353	0.1188	3.8874	4.3601	
10	6281	0.1226	2.8608	3.8649	
11	45139	0.1491	4.3897	4.3403	
12	48865	0.1167	4.4487	4.3803	
13	10762	0.1445	3.2767	3.3702	
14	5862	0.1424	4.4104	3.9238	
15	14102	0.1460	3.5654	3.5066	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.247 g/ml thymol solution	$O.D_{570\ nm}$ of 100 µL of 0.247 g/ml thymol solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1444	4.0331	4.2324	
2	12631	0.1384	4.3635	4.0262	
3	7496	0.1232	3.8216	3.9190	
4	4826	0.1490	2.7842	3.7068	
5	4588	0.1281	3.5303	3.6752	
6	43271	0.1646	4.1944	4.5961	

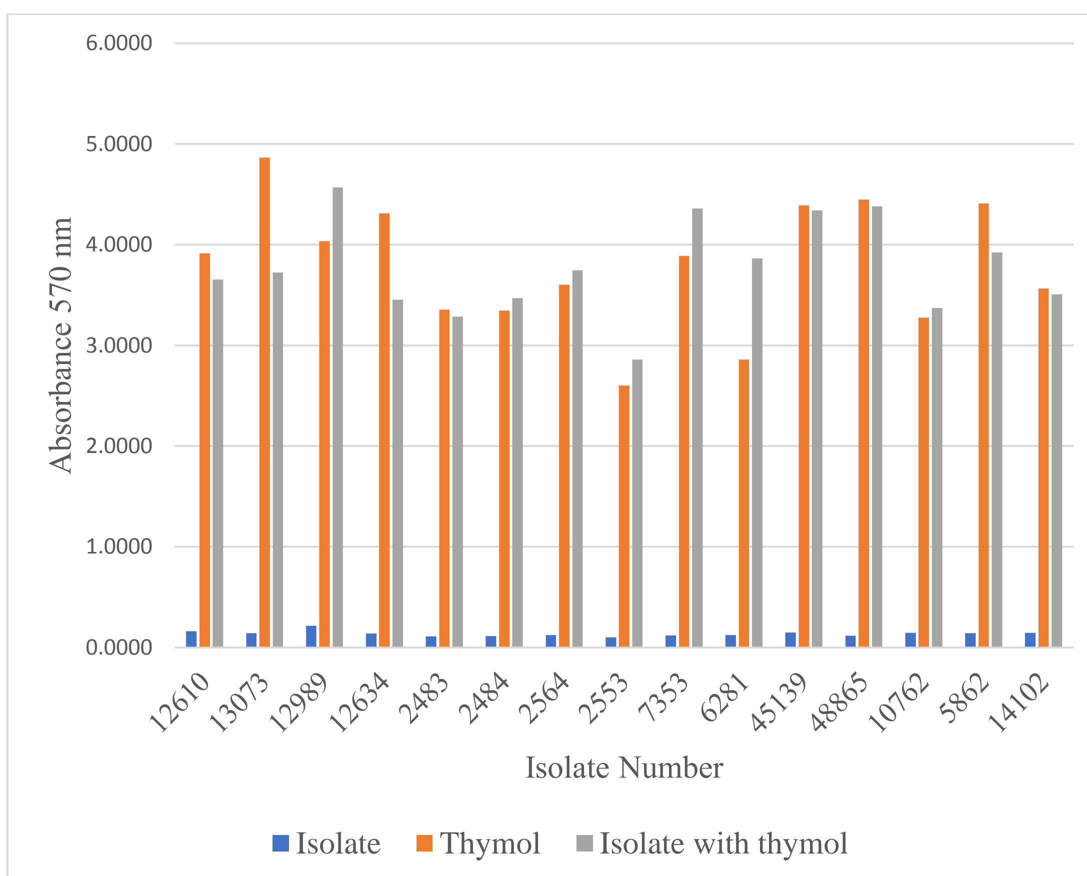


Figure 51. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.247 g/ml thymol solution

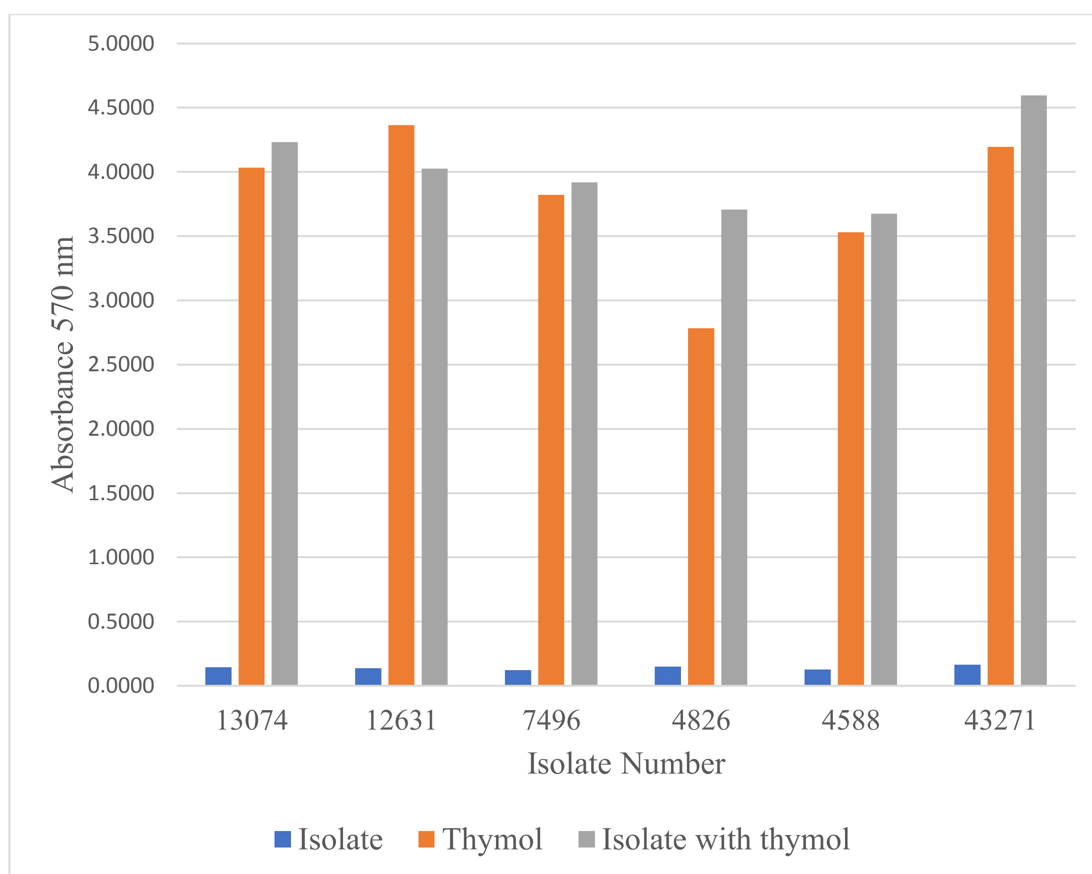


Figure 52. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.247 g/ml thymol solution

3.8. The effect of three major components of *Rosmarinus officinalis* on inhibition of *Staphylococcus aureus* biofilm formation

3.8.1. The effect of α-pinene on inhibition of *Staphylococcus aureus* biofilm formation

In order to determine the effect of α-pinene which is one of the major components of *Rosmarinus officinalis* on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated with different volumes of α-pinene at different concentrations.

3.8.1.1. The effect of α-pinene solution of concentration 0.0194 g/ml, reflecting a concentration equivalent to the concentration of α-pinene in *Rosmarinus officinalis* methanolic extract, on inhibition of Staphylococcus aureus biofilm formation

The effect of 100 μL of 0.0194 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 μL of 0.0194 g/ml α-pinene solution which contained 194 x 10^{-5} g of α-pinene, possessed an effect on inhibition of biofilm formation of all methicillin susceptible *S. aureus* isolate except for two isolates: 6281 and 2564 with less than 1% inhibition in biofilm formation. The inhibition of biofilm formation varied in its significance between one isolate and the other and ranged from minimal inhibition of biofilm formation with 6.44 % inhibition with isolate 2483 and up to 78.72 % inhibition in biofilm formation with isolate 45139 (Table 23.a and Figure 53). On the other hand, all methicillin resistant *S. aureus* strains showed an inhibition in biofilm formation except for isolate 4588 which was not affected by 100 μL of 0.0194 g/ml α-pinene (Table 23.b and Figure 54). The inhibition in biofilm formation of *S. aureus* strains ranged from insignificant inhibition with around 25 % inhibition with isolates 13074 and 43271 and up to significant inhibition in biofilm formation with isolate 4826 with 83 % inhibition in biofilm formation.

Table 23. Effect of 100 μL volume of α-pinene solution of concentration 0.0194 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 100 μL of 0.0194 g/ml α-pinene solution	O.D$_{570\ nm}$ of 100 μL of 0.0194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1730	0.1216	0.1237	28.47
2	13073	0.1955	0.1397	0.1352	30.84
3	12989	0.4063	0.1238	0.1575	61.24
4	12634	0.1931	0.1253	0.1205	37.61
5	2483	0.1861	0.1451	0.1741	6.44
6	2484	0.2147	0.1316	0.1166	45.70
7	2564	0.1557	0.1205	0.1545	0.78
8	2553	0.1912	0.1312	0.1494	21.89
9	7353	0.2693	0.1507	0.1466	45.55
10	6281	0.1517	0.1180	0.1540	
11	45139	0.7316	0.1532	0.1557	78.72
12	48865	0.1995	0.1495	0.1529	23.35
13	10762	0.2762	0.1418	0.1518	45.03
14	5862	0.2055	0.1456	0.1441	29.84
15	14102	0.3042	0.1308	0.1797	40.93

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 100 μL of 0.0194 g/ml α-pinene solution	O.D$_{570\ nm}$ of 100 μL of 0.0194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.2627	0.1419	0.1535	41.57
2	12631	0.2068	0.1458	0.1478	28.51
3	7496	0.1662	0.1249	0.1252	24.70
4	4826	0.7596	0.1219	0.1223	83.90
5	4588	0.1745	0.1576	0.1926	
6	43271	0.2274	0.1595	0.1997	12.14

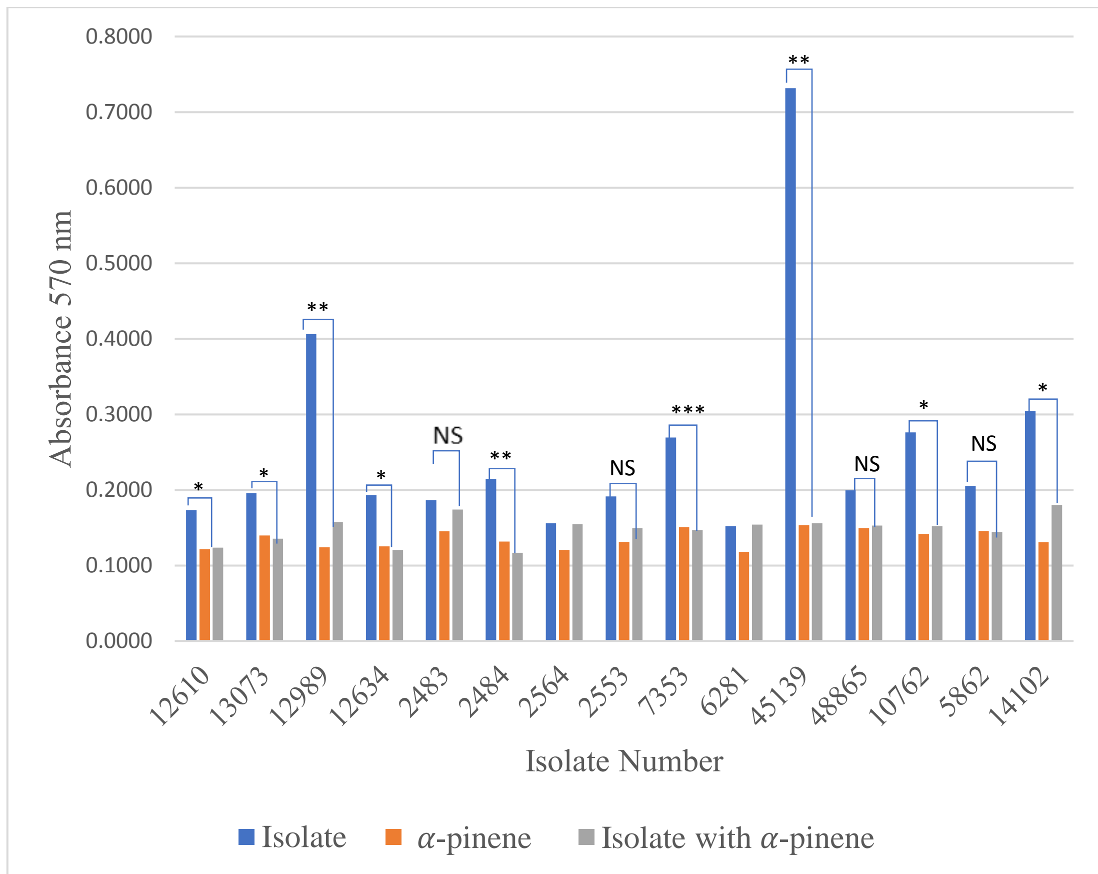


Figure 53. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.0194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

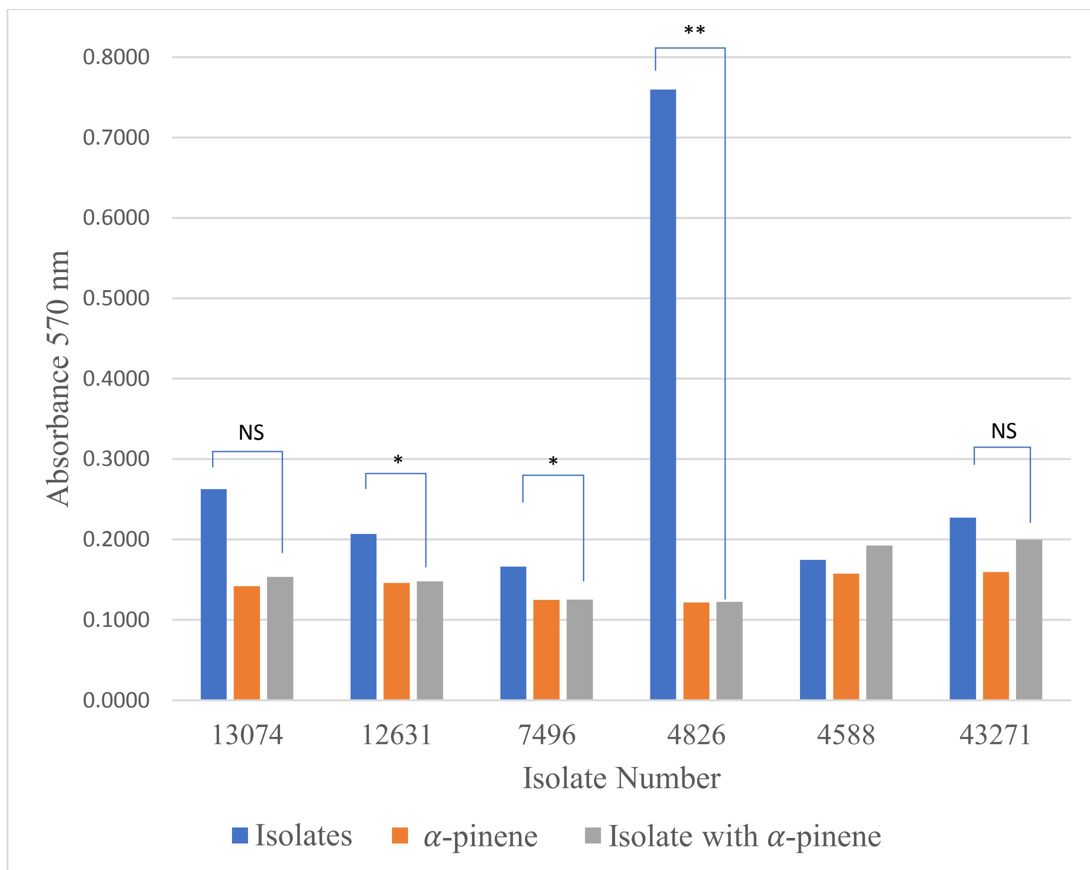


Figure 54. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.0194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 µL of 0.0194 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 150 µL of 0.0194 g/ml α-pinene solution which contained 29 x 10^{-4} g of α-pinene, inhibited biofilm formation of all methicillin susceptible *S. aureus* isolates except for isolate 2564 that only displayed a 1.66 % decrease in biofilm formation (Table 24.a, Figure 55). The result observed with isolate 2564 at 150 µL of 0.0194 g/ml α-pinene solution was consistent with the result observed with 100 µL of 0.0194 g/ml α-pinene. The percentage of inhibition of biofilm formation of MSSA strains ranged from 20.43 % decrease in biofilm formation with isolate 2484 and up to

a significant decrease of 50 % in biofilm formation of isolates 5862 and 13073 and 80 % decrease in biofilm formation of isolate 45139. Isolate 6281, whose biofilm formation was not previously affected by 100 μL of 0.0194 g/ml α-pinene, displayed a significant inhibition of biofilm formation by 28.41 %. On the other hand, all methicillin resistant *S. aureus* strains experienced a similar or a higher inhibition in biofilm formation with 150 μL of 0.0194 g/ml α-pinene as compared with 100 μL of 0.0194 g/ml α-pinene except for isolate 7496 whose biofilm inhibition was less than that observed with 100 μL of 0.0194 g/ml α-pinene (Table 24.b and and Figure 56). Isolate 4588 whose biofilm formation was not affected by 100 μL of 0.0194 g/ml α-pinene was also not affected by increase in the volume of α-pinene solution to 150 μL.

Table 24. Effect of 150 μL volume of α-pinene solution of concentration 0.0194 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.0194 g/ml α-pinene solution	$O.D_{570\ nm}$ of 150 μL of 0.0194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1752	0.1148	0.1580	9.79
2	13073	0.1950	0.0983	0.1032	47.08
3	12989	0.3828	0.1041	0.0951	75.15
4	12634	0.1959	0.1244	0.1238	36.79
5	2483	0.2200	0.1343	0.1661	24.51
6	2484	0.2204	0.1243	0.1753	20.43
7	2564	0.1594	0.1294	0.1567	1.66
8	2553	0.2093	0.1430	0.1234	41.03
9	7353	0.2982	0.1462	0.1626	45.48
10	6281	0.1511	0.1321	0.1082	28.41
11	45139	0.7583	0.1148	0.1133	85.06
12	48865	0.1992	0.1159	0.1185	40.49
13	10762	0.2150	0.1165	0.1127	47.61
14	5862	0.2253	0.1095	0.1118	50.40
15	14102	0.3612	0.1278	0.1776	50.83

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 150 µL of 0.0194 g/ml α-pinene solution	O.D$_{570\ nm}$ of 150 µL of 0.0194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.2753	0.1154	0.1463	46.86
2	12631	0.2398	0.1671	0.1295	46.02
3	7496	0.1653	0.1153	0.1568	5.14
4	4826	0.7584	0.1290	0.1824	75.95
5	4588	0.1674	0.1634	0.2002	
6	43271	0.2337	0.1440	0.1047	55.20

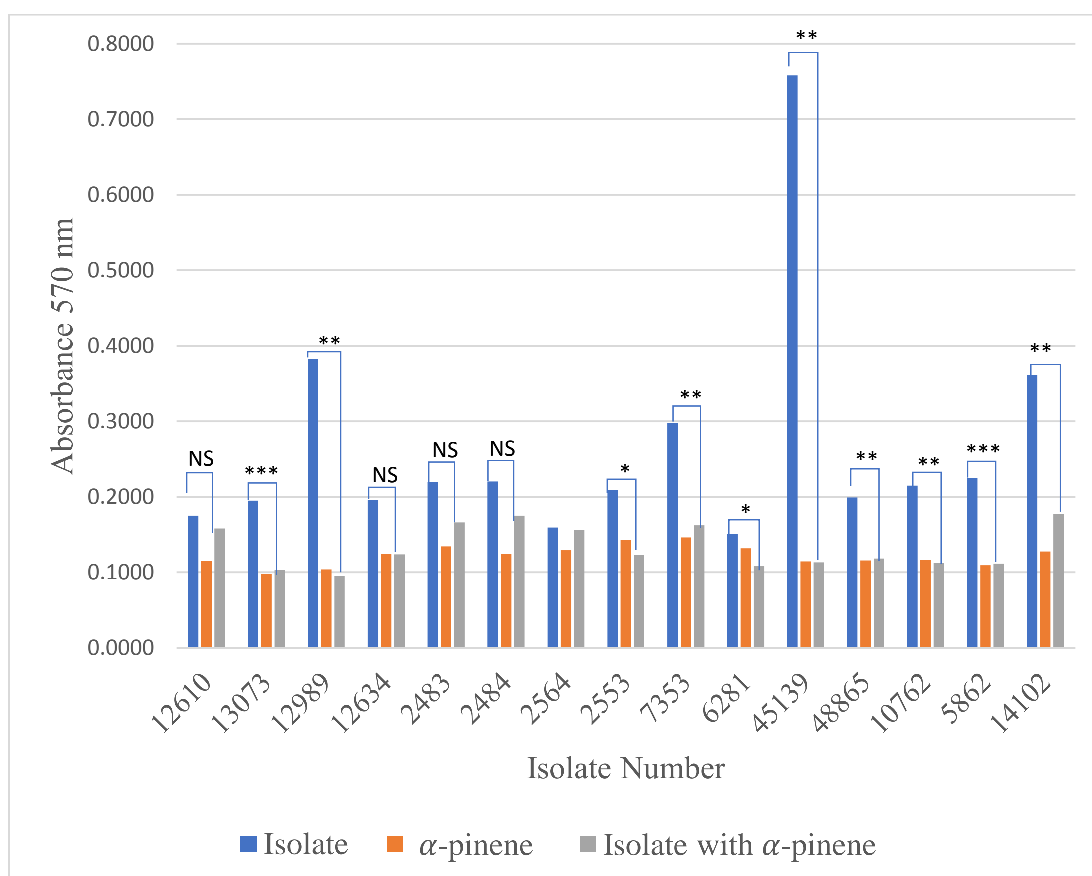


Figure 55. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.0194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

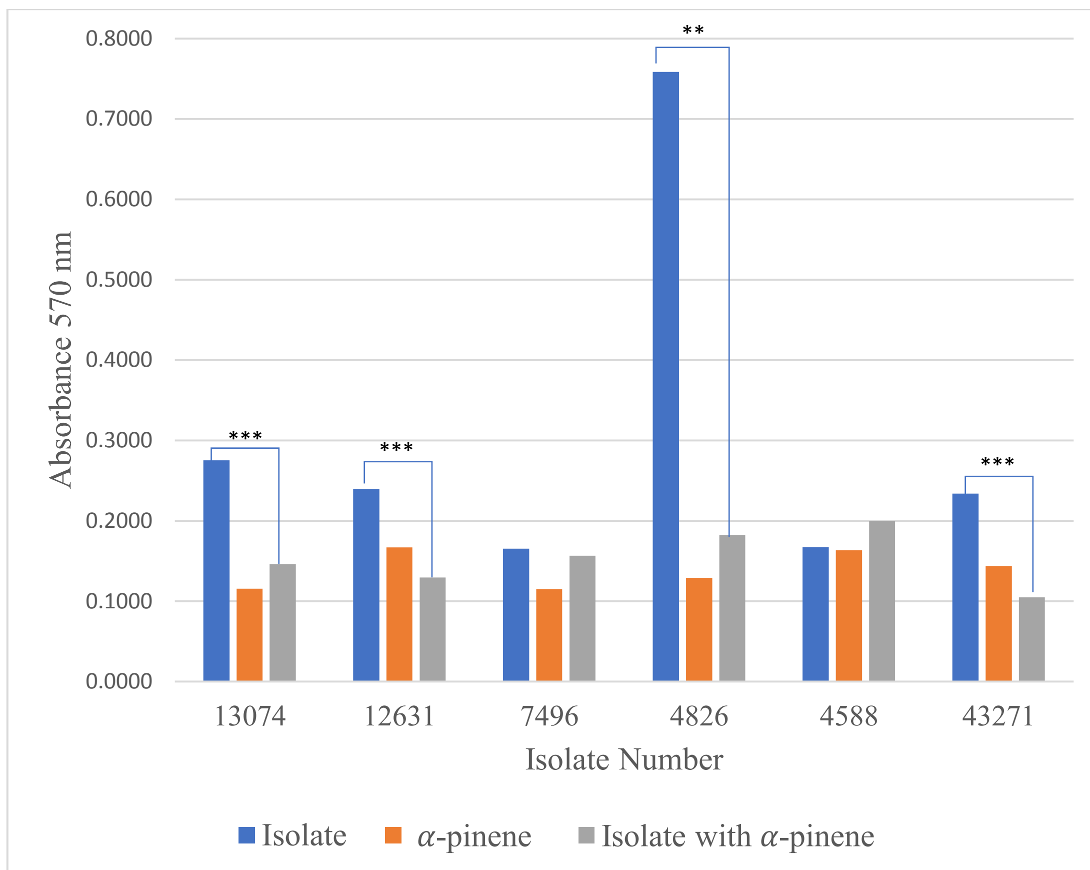


Figure 56. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.0194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.1.2 The effect of α-pinene solution of concentration 0.097 g/ml, reflecting a concentration five times higher than the concentration of α-pinene in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 µL of 0.097 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 µL of 0.097 g/ml which contained 97 x 10^{-4} g of α-pinene, inhibited biofilm formation of the majority of methicillin susceptible *S. aureus* isolates (Table 25.a and Figure 57). Biofilm formation of isolate 2483 was not affected by 100

μL of 0.097 g/ml. Isolates 12610, 12634 and 2564 showed a negligible decrease in biofilm formation ranging from less than 1 % inhibition of biofilm formation to 3 %. The remaining MSSA strains, however, displayed an inhibition in biofilm formation ranging from 6.79 % with isolate 6281 and up to highly significant decrease in biofilm formation with isolates 2484 and 45139 with 50 % and 80 % inhibition in biofilm formation, respectively. Isolate 2564 still did not show inhibition in biofilm formation inspite increasing the concentration of α-pinene to 0.097 g/ml. However, isolate 6281 whose biofilm formation was previously not affected by 100 μL of 0.0194 g/ml α-pinene, showed a 6.79 % decrease in biofilm formation with 100 μL of the higher concentration of 0.097 g/ml of α-pinene solution. On the other hand, all methicillin resistant *S. aureus* strains exhibited an inhibition in biofilm formation with 100 μL of 0.097 g/ml α-pinene solution including isolate 4588 which showed no inhibition in biofilm formation at the lower concentration of 0.0194 g/ml α-pinene solution (Table 25.b and Figure 58). Yet, isolate 7496 exhibited a nearly negligible variation of only 2 % inhibition in biofilm formtation.

Table 25. Effect of 100 μL volume of α-pinene solution of concentration 0.097 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.097 g/ml α-pinene solution	$O.D_{570\ nm}$ of 100 μL of 0.097 g/ml diluted α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1708	0.1019	0.1699	0.56
2	13073	0.2297	0.1110	0.1678	26.95
3	12989	0.3389	0.1381	0.1761	48.03
4	12634	0.1835	0.1497	0.1791	2.38
5	2483	0.1894	0.1004	0.2001	
6	2484	0.2115	0.0999	0.1246	41.08
7	2564	0.1568	0.1142	0.1520	3.06
8	2553	0.1967	0.1164	0.1669	15.12
9	7353	0.2387	0.1254	0.1633	31.58
10	6281	0.1553	0.1175	0.1448	6.79
11	45139	0.7836	0.1090	0.1693	78.40
12	48865	0.1909	0.1275	0.1874	1.84
13	10762	0.2092	0.1198	0.1948	6.88
14	5862	0.2035	0.1288	0.1718	15.58
15	14102	0.3045	0.1177	0.1589	47.81

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 100 µL of 0.097 g/ml α-pinene solution	O.D$_{570\ nm}$ of 100 µL of 0.097 g/ml diluted α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.2500	0.1351	0.1589	36.44
2	12631	0.1959	0.1194	0.1627	16.98
3	7496	0.1444	0.1113	0.1415	2.02
4	4826	0.4772	0.1118	0.1541	67.70
5	4588	0.1753	0.1276	0.1534	12.50
6	43271	0.2011	0.1306	0.1778	11.59

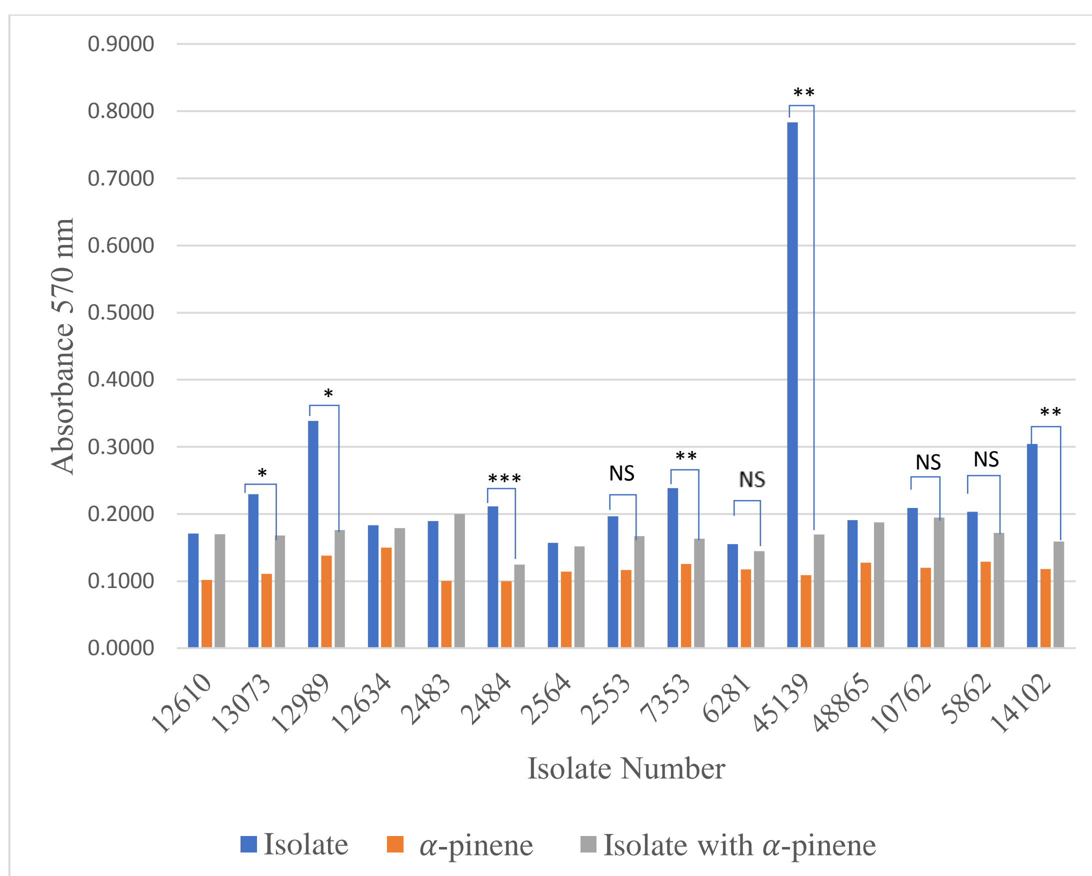


Figure 57. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.097 g/ml *α*-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

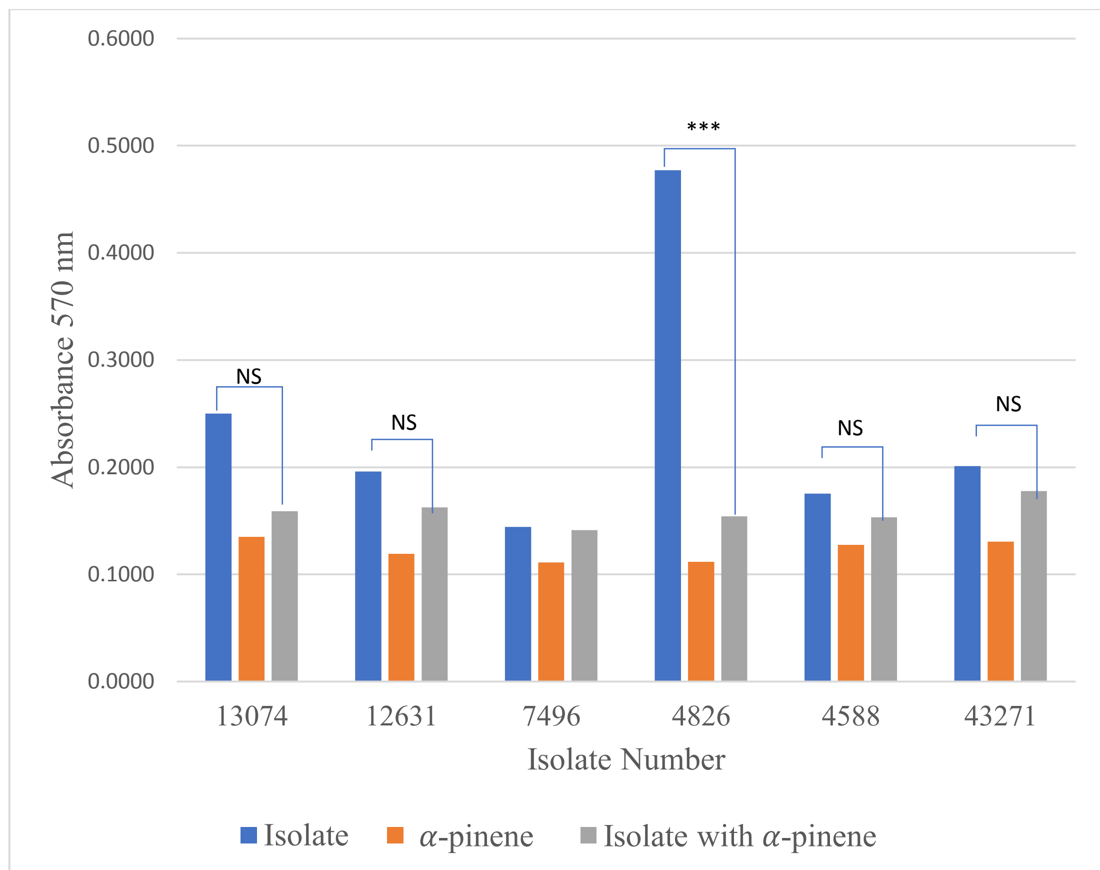


Figure 58. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.097 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 µL of 0.097 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that increasing the volume of 0.097 g/ml α-pinene solution to 150 µL, which contained 15 x 10^{-3} g of α-pinene, inhibited biofilm formation of only 10 out of 15 MSSA isolates. This inhibition ranged from an insignificant decrease in biofilm formation of only 9% to a highly significant decrease of 76.26 % with isolate 45139 (Table 26.a and Figure 59). On the other hand, 150 µL of 0.097 g/ml α-pinene showed an effect on inhibition of *S. aureus* biofilm formation of all but one isolate which is isolate 4588 which had previously, a 12.5 % inhibition in biofilm formation

with 100 μL of 0.097 g/ml α-pinene solution which was not considered to be a significant decrease (Table 26.b and Figure 60). Isolate 7496 showed a significant increase in inhibition of biofilm formation with 150 μL of 0.097 g/ml α-pinene solution as compared to 100 μL of 0.097 g/ml α-pinene solution.

Table 26. Effect of 150 μL volume of α-pinene solution of concentration 0.097 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.097 g/ml α-pinene solution	$O.D_{570\ nm}$ of 150 μL of 0.097 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1750	0.1475	0.1895	
2	13073	0.1684	0.1392	0.1702	
3	12989	0.3492	0.1245	0.1722	50.69
4	12634	0.1872	0.1370	0.1702	9.07
5	2483	0.2110	0.1601	0.1661	21.31
6	2484	0.2304	0.1798	0.1638	28.93
7	2564	0.1604	0.2274	0.1939	
8	2553	0.1929	0.1750	0.1716	11.04
9	7353	0.2854	0.1422	0.2007	29.68
10	6281	0.1538	0.1555	0.1918	
11	45139	0.7341	0.1901	0.1743	76.26
12	48865	0.2156	0.1966	0.2296	
13	10762	0.2293	0.1870	0.1733	24.42
14	5862	0.2555	0.1774	0.1808	29.22
15	14102	0.3207	0.1648	0.2037	36.49

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 150 µL of 0.097 g/ml α-pinene solution	O.D$_{570\ nm}$ of 150 µL of 0.097 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.2634	0.1893	0.1740	33.94
2	12631	0.1943	0.1645	0.1825	6.11
3	7496	0.1458	0.1226	0.0997	31.58
4	4826	0.7418	0.1418	0.1978	73.33
5	4588	0.1729	0.1925	0.2064	
6	43271	0.2149	0.1749	0.1810	15.76

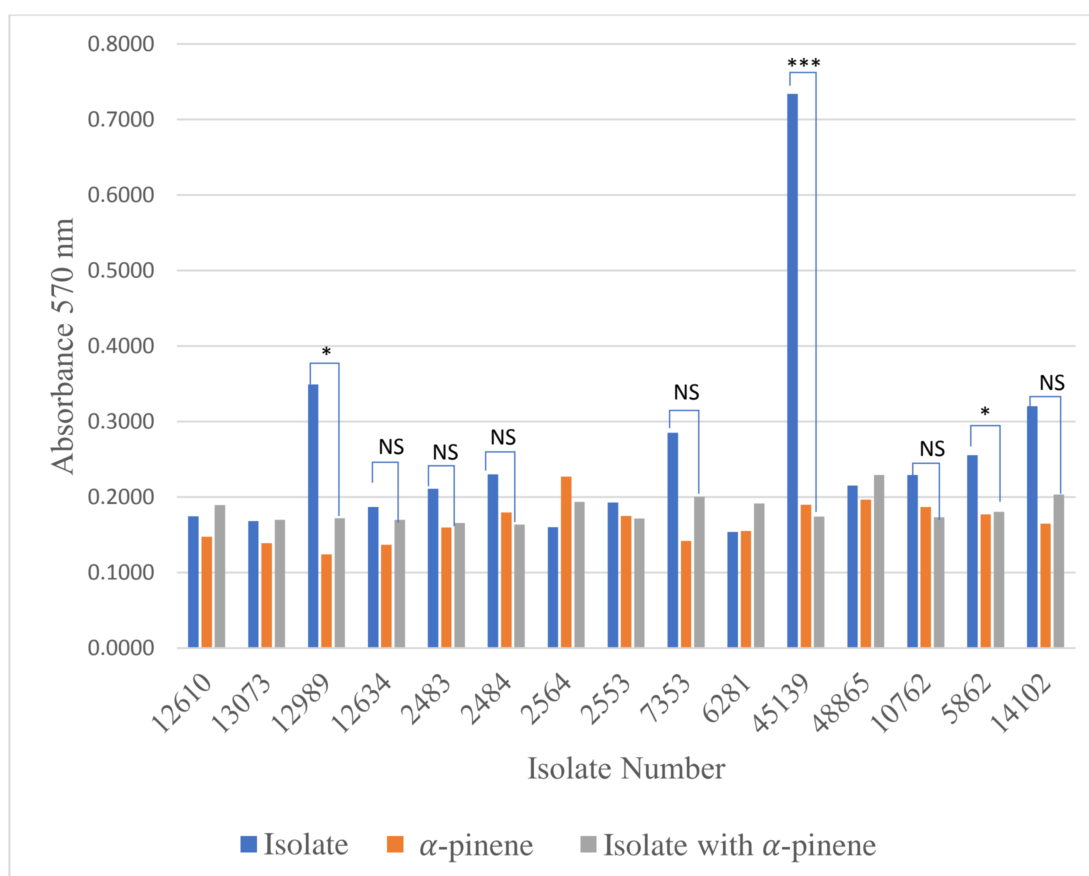


Figure 59. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.097 g/ml *α*-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

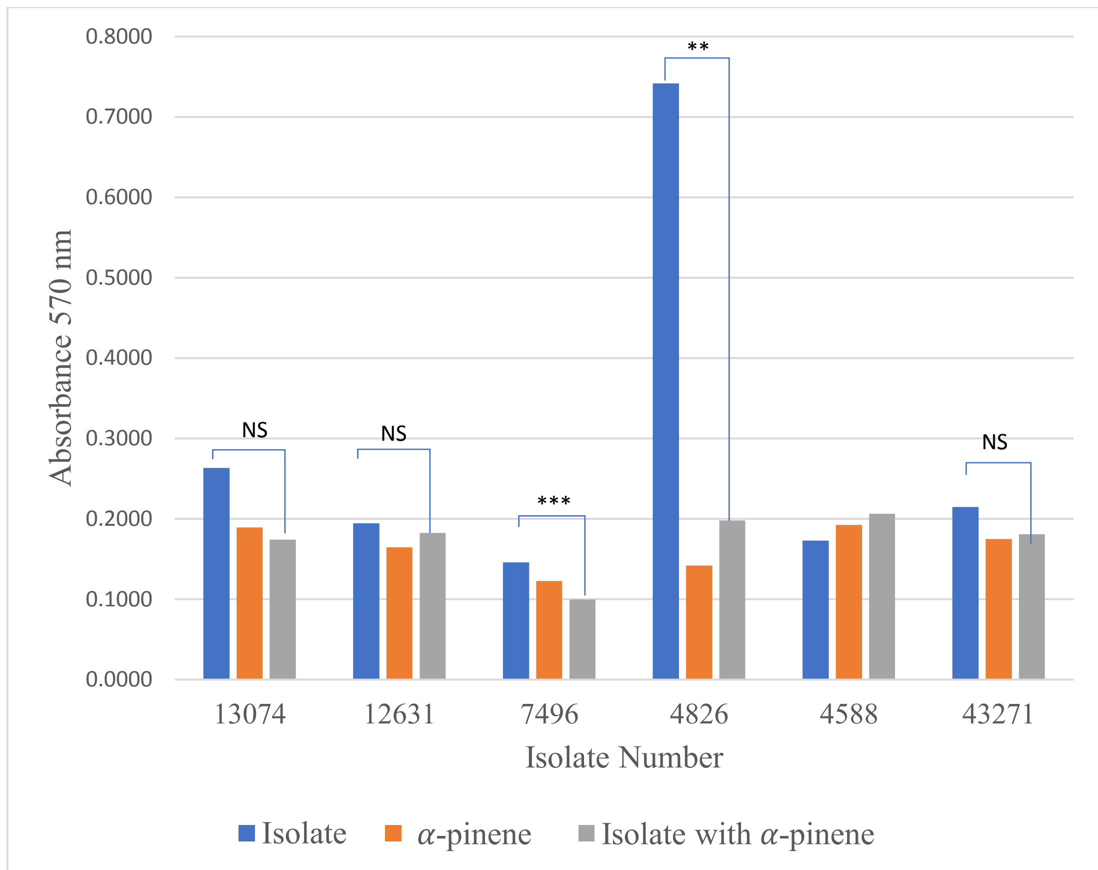


Figure 60. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 μL of 0.097 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.1.3. The effect of α-pinene solution of concentration 0.194 g/ml, reflecting a concentration ten times higher than the concentration of α-pinene in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 μL of 0.194 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that the majority of methicillin susceptible *S. aureus* isolates displayed an inhibition in their biofilm formation with 100 μL of 0.194 g/ml α-pinene solution which contained 0.019 g of α-pinene and the significance of this inhibition

was comparable with the siginificance of inhibition observed at the lower 0.097 g/ml concentration of α-pinene solution at the same volume (Table 27.a and Figure 61). Similar to what was observed at the lower 0.097 g/ml concentration of α-pinene, isolates 12634 and 2483 lacked an inhibition in their biofilm formation with 0.194 g/ml α-pinene solution. However, islolate 12989 whose biofilm was siginificantly inhibited at the lower 0.097 g/ml concentration of α-pinene was not affected by 0.194 g/ml α-pinene solution. On the other hand, inhibition of biofilm of methicillin resistant *S. aureus* was significant with four isolates but no inhibition in biofilm formation was observed with isolates 13074 and 7496 (Table 27.b and Figure 62). This result is similar to what was observed with isolate 7496 at lower concentration of 0.097 g/ml α-pinene solution at the same volume of 100 μL.

Table 27. Effect of 100 μL volume of α-pinene solution of concentration 0.194 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.194 g/ml α-pinene solution	$O.D_{570\ nm}$ of 100 μL of 0.194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1689	0.1081	0.1442	14.66
2	13073	0.1968	0.1514	0.1071	45.56
3	12989	0.1672	0.2067	0.2677	
4	12634	0.1359	0.1575	0.1621	
5	2483	0.1444	0.1540	0.1419	1.76
6	2484	0.2486	0.1588	0.1820	26.78
7	2564	0.2336	0.0990	0.1271	45.60
8	2553	0.2959	0.1204	0.2062	30.29
9	7353	0.2260	0.1328	0.1725	23.66
10	6281	0.2013	0.1149	0.1125	44.14
11	45139	0.2654	0.1253	0.1589	40.14
12	48865	0.2750	0.1255	0.1533	44.25
13	10762	0.1239	0.1491	0.1201	3.10
14	5862	0.2179	0.1402	0.1845	15.30
15	14102	0.1906	0.1847	0.2178	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.194 g/ml α-pinene solution	$O.D_{570\ nm}$ of 100 µL of 0.194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1262	0.1465	0.1903	
2	12631	0.2334	0.1035	0.0975	58.21
3	7496	0.1566	0.1111	0.2480	
4	4826	0.2347	0.1093	0.1670	28.83
5	4588	0.2215	0.0967	0.1219	44.96
6	43271	0.2397	0.1245	0.1425	40.56

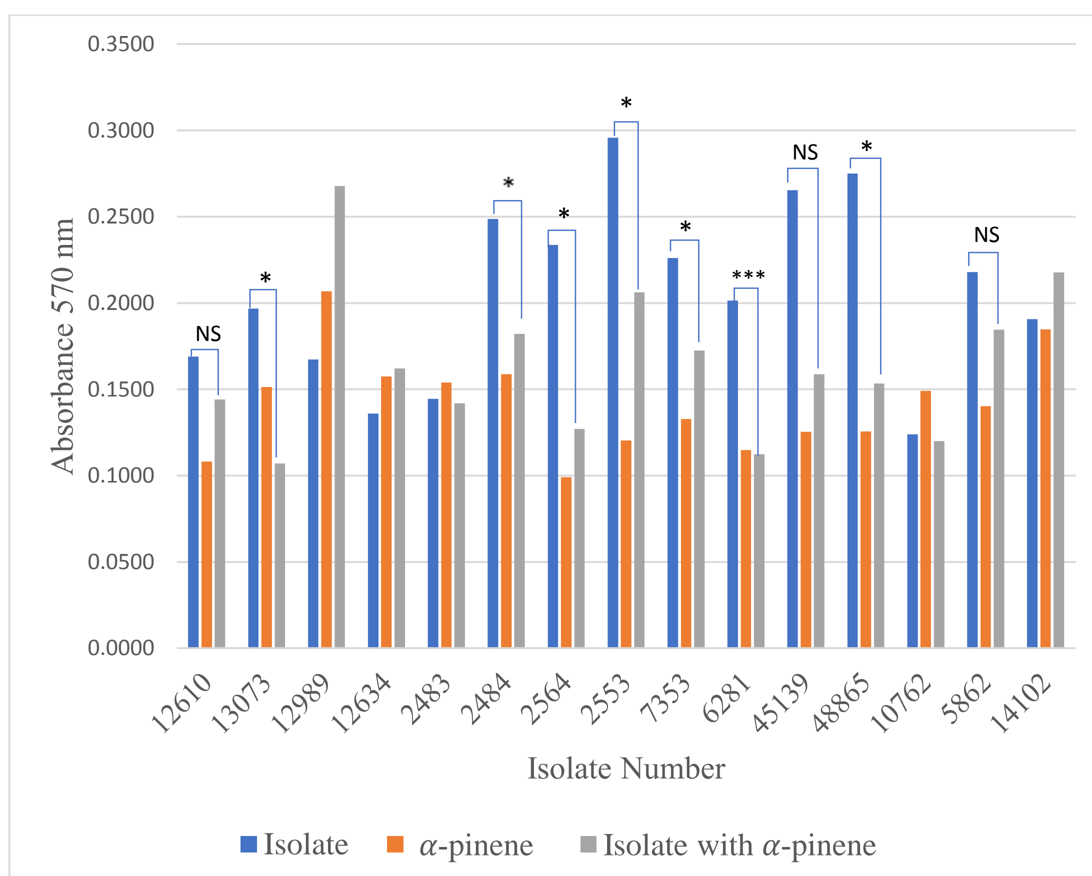


Figure 61. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.194 g/ml *α*-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

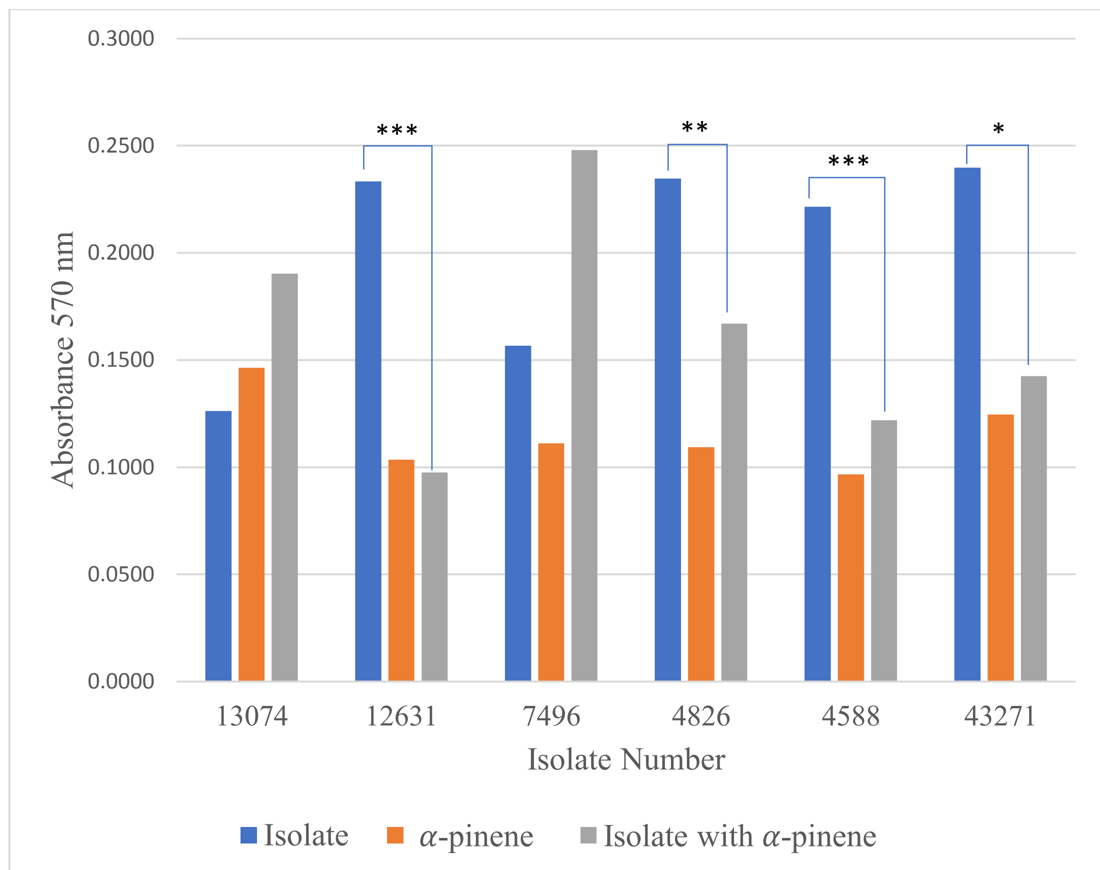


Figure 62. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 μL of 0.194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 μL of 0.194 g/ml α-pinene solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that eight out of the 15 methicillin susceptible *S. aureus* lacked any inhibition in biofilm formation with 150 μL of 0.194 g/ml α-pinene solution which contained 0.03 g of α-pinene (Table 28 and Figure 63). Similarly, only two methicillin resistant *S. aureus* strains which are isolates 4826 and 4588 displayed inhibition in biofilm formation at 150 μL of 0.194 g/ml α-pinene solution. Yet, inhibition of biofilm formation of isolate 4588 was not considered significant. In comparison with 100 μL of 0.194 g/ml α-pinene solution and with the lower concentrations of α-pinene

solution this condition was the least effective on inhibition of *S. aureus* biofilm formation (Figure 64).

Table 28. Effect of 150 μL volume of α-pinene solution of concentration 0.194 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.194 g/ml α-pinene solution	$O.D_{570\ nm}$ of 150 μL of 0.194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	12610	0.1310	0.1468	0.1947	
2	13073	0.1977	0.1215	0.1087	45.02
3	12989	0.1688	0.2071	0.2326	
4	12634	0.1324	0.1985	0.1440	
5	2483	0.1383	0.2998	0.1694	
6	2484	0.1746	0.2050	0.2348	
7	2564	0.2115	0.1005	0.0915	56.76
8	2553	0.2408	0.1667	0.1911	20.65
9	7353	0.2198	0.2316	0.3807	
10	6281	0.2197	0.1085	0.0954	56.60
11	45139	0.2284	0.1580	0.1720	24.71
12	48865	0.2238	0.1377	0.1793	19.91
13	10762	0.2240	0.1505	0.1850	17.42
14	5862	0.2026	0.2021	0.2048	
15	14102	0.1893	0.2345	0.1921	

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 150 µL of 0.194 g/ml α-pinene solution	O.D$_{570\ nm}$ of 150 µL of 0.194 g/ml α-pinene solution with bacterium	% Inhibition of biofilm formation
1	13074	0.1280	0.1370	0.1779	
2	12631	0.1812	0.2648	0.2193	
3	7496	0.1495	0.0952	0.1837	
4	4826	0.2211	0.1930	0.1792	18.94
5	4588	0.2118	0.2197	0.1540	27.31
6	43271	0.2113	0.1717	0.2352	

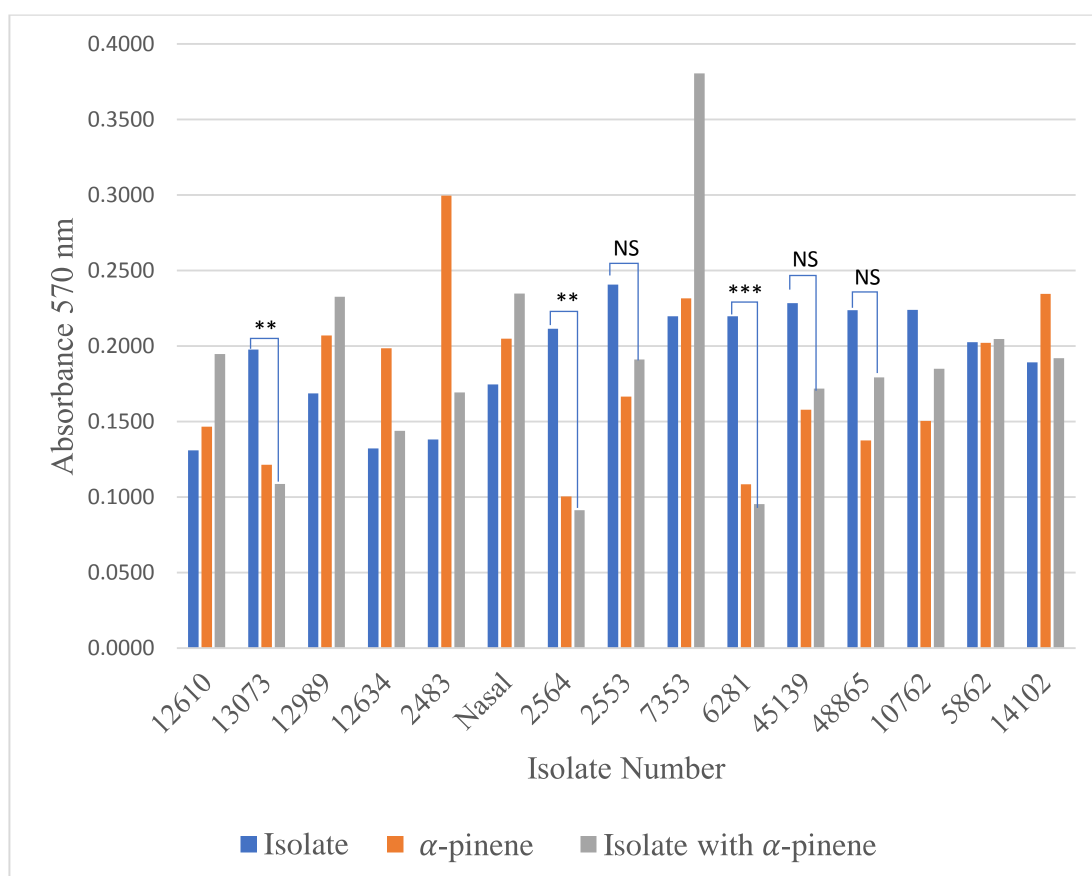


Figure 63. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.194 g/ml *α*-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

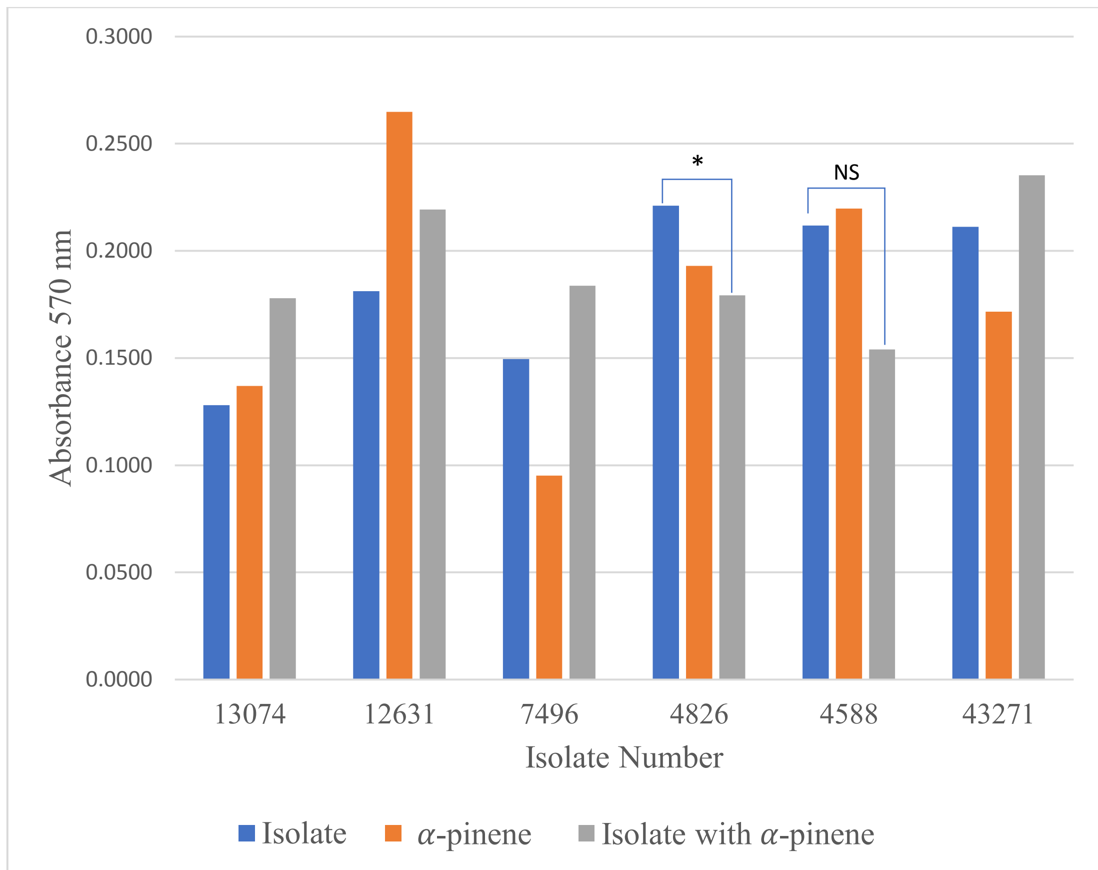


Figure 64. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 μL of 0.194 g/ml α-pinene solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.2. The effect of camphor on inhibition of *Staphylococcus aureus* biofilm formation

In order to determine the effect of camphor which is one of the major components of *Rosmarinus officinalis* on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated with different volumes of camphor at different concentrations

3.8.2.1. The effect of camphor solution of concentration 0.0143 g/ml, reflecting a concentration equivalent to the concentration of camphor in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 μL of 0.0143 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that the majority of the methicillin susceptible strains exhibited inhibition in biofilm formation with 100 μL of 0.0143 g/ml which contained 143×10^{-5} g of camphor (Table 29.a amd Figure 65). However, isolate 2564 biofilm was not affected by 100 μL of 0.0143 g/ml camphor solution and isolates 12634, 2553 and 10762 inhibition of biofilm formation was negligable (around 3 %). The inhibition in biofilm formation ranged from low insiginifcance in biofilm formation of 12.81 % with isolate 2483 and up to 52.59 % with isolate 12610 (Table 29.a and Figure 65). On the other hand, all methicillin resistant *S. aureus* strains were inhibited by 100 μL of 0.0143 g/ml camphor solution and this inhibition ranged from nearly insignifacnt decrease in biofilm formation with 10.28 % with isolate 13074 and up to significant inhibition in biofilm formation with isolate 12631 with 35.62 % inhibition in biofilm formation (Table 29.b and Figure 66)

Table 29. Effect of 100 μL volume of camphor solution of concentration 0.0143 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.0143 g/ml camphor solution	$O.D_{570\ nm}$ of 100 μL of 0.0143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.3445	0.2908	0.1633	52.59
2	13073	0.3594	0.1568	0.3039	15.44
3	12989	0.2976	0.1319	0.2430	18.33
4	12634	0.2395	0.1309	0.2316	3.32
5	2483	0.3002	0.1217	0.2617	12.81
6	2484	0.1806	0.1088	0.1433	20.61
7	2564	0.1609	0.1191	0.1776	
8	2553	0.1772	0.1271	0.1745	1.56
9	7353	0.1970	0.1227	0.1658	15.87
10	6281	0.1639	0.1307	0.1416	13.58
11	45139	0.4127	0.1107	0.2723	34.01
12	48865	0.1798	0.1182	0.1488	17.27
13	10762	0.1779	0.1195	0.1702	4.34
14	5862	0.2305	0.1363	0.1521	34.01
15	14102	0.2764	0.1222	0.1780	35.60

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.0143 g/ml camphor solution	$O.D_{570\ nm}$ of 100 µL of 0.0143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.3445	0.1450	0.3091	10.28
2	12631	0.2271	0.1331	0.1462	35.62
3	7496	0.1256	0.0880	0.1070	14.82
4	4826	0.4626	0.1369	0.3114	32.68
5	4588	0.2204	0.1392	0.1797	18.50
6	43271	0.1996	0.1155	0.1409	29.43

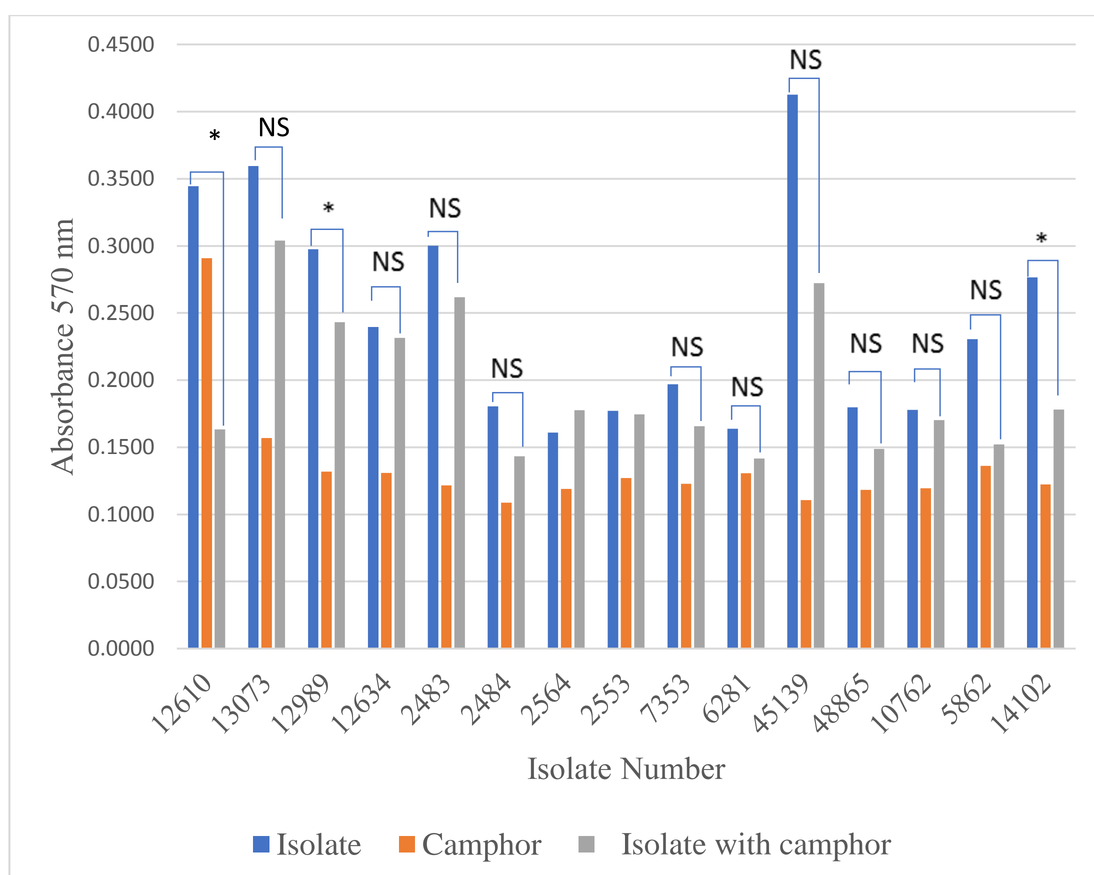


Figure 65. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.0143 g/ml camphor solution (0.01 < p ≤ 0.05 (*), 0.001 < p ≤ 0.01 (**), p ≤ 0.001 (***))

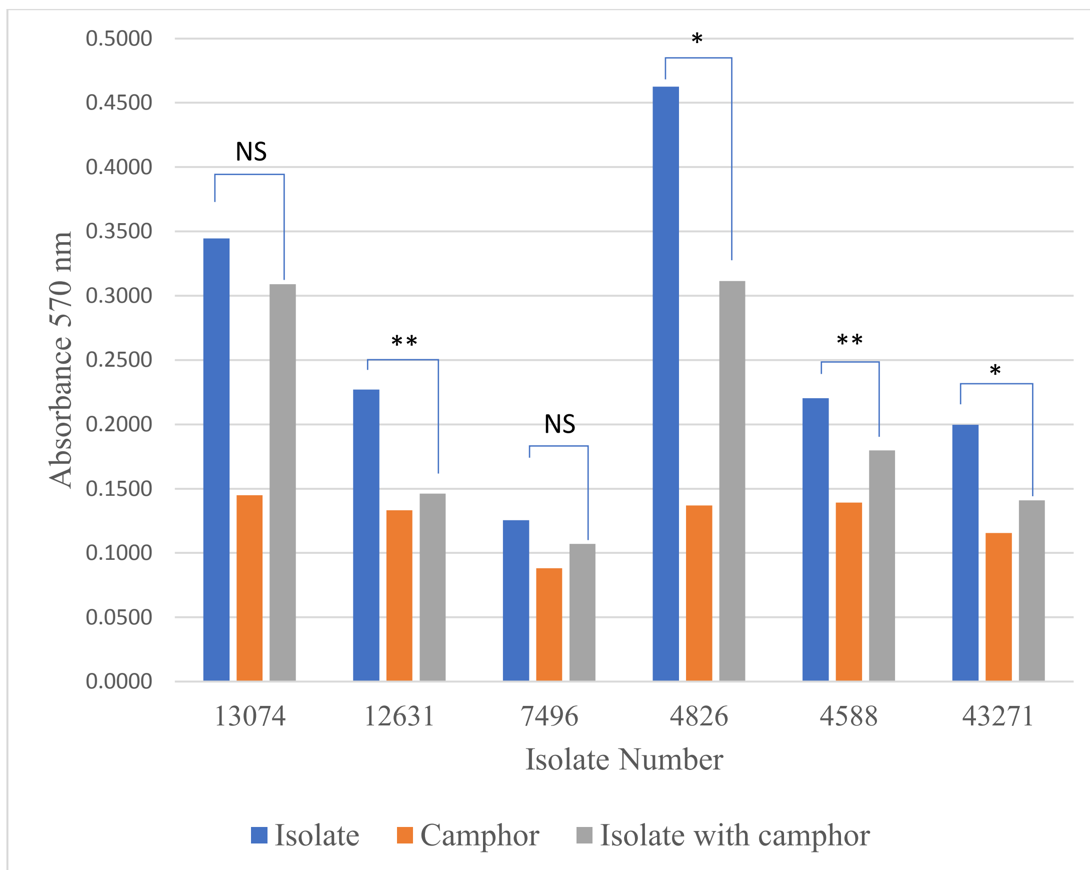


Figure 66. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 μL of 0.0143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 μL of 0.0143 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that with 150 μL of 0.0143 g/ml camphor solution which contained 21 x 10^{-4} g of camphor, that all methicillin susceptible *S. aureus* strains exhibited a decrease in biofilm formation and this inhibition in biofilm formation varied between the isolates from a slight insignificant decrease in biofilm formation of 8.33 % inhibition with isolate 12989 and up to highly sifnificant inhibition in biofilm formation with 59.74 % and 46.14 % with isolates 12610 and 14102, respectively (Table 30.a and Figure 67). Isolate 10762 exhibited a negligable decrease of only 4 % inhibition in its biofilm formation which is the same inhibition previously observed

with 100 μL of 0.0143 g/ml camphor solution. The results also showed that 150 μL of 0.0143 g/ml camphor solution exerted an increased effect on the inhibition of biofilm formation of the majority of *S. aureus* isolates and that this inhibition was highly significant for these isolates as compared to 100 μL of 0.0143 g/ml camphor solution. Isolate 2564 whose biofilm formation was previously not affected by 100 μL of 0.0143 g/ml camphor solution showed a slight inhibition of 9 % in its biofilm formation. On the other hand, all methicillin resistant *S. aureus* isolates showed an inhibition in their biofilm formation with 150 μL of 0.0143 g/ml camphor solution and the percentage of inhibition was increased with isolates 13074 and 7496 and this inhibition was more significant with isolate 4826 as compared with 100 μL of 0.0143 g/ml camphor solution (Table 30.b and Figure 68).

Table 30. Effect of 150 μL volume of camphor solution of concentration 0.0143 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.0143 g/ml camphor solution	$O.D_{570\ nm}$ of 150 μL of 0.0143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.3947	0.3067	0.1589	59.74
2	13073	0.4198	0.1477	0.2467	41.24
3	12989	0.2391	0.1290	0.2192	8.33
4	12634	0.2573	0.1307	0.1917	25.49
5	2483	0.3030	0.1415	0.1989	34.37
6	2484	0.1869	0.1036	0.1090	41.68
7	2564	0.1578	0.1096	0.1429	9.44
8	2553	0.2252	0.1159	0.1371	39.12
9	7353	0.2270	0.1288	0.1497	34.08
10	6281	0.1864	0.1394	0.1398	25.02
11	45139	0.3744	0.1141	0.2943	21.40
12	48865	0.1819	0.1554	0.1293	28.92
13	10762	0.1788	0.1360	0.1712	4.28
14	5862	0.2371	0.1430	0.1911	19.38
15	14102	0.2949	0.1177	0.1588	46.14

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.0143 g/ml camphor solution	$O.D_{570\ nm}$ of 150 µL of 0.0143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.3947	0.1589	0.3067	22.29
2	12631	0.1916	0.1633	0.1249	34.81
3	7496	0.1249	0.0937	0.1007	19.42
4	4826	0.4455	0.1190	0.1519	65.90
5	4588	0.2374	0.1395	0.2016	15.08
6	43271	0.1999	0.1040	0.1702	14.88

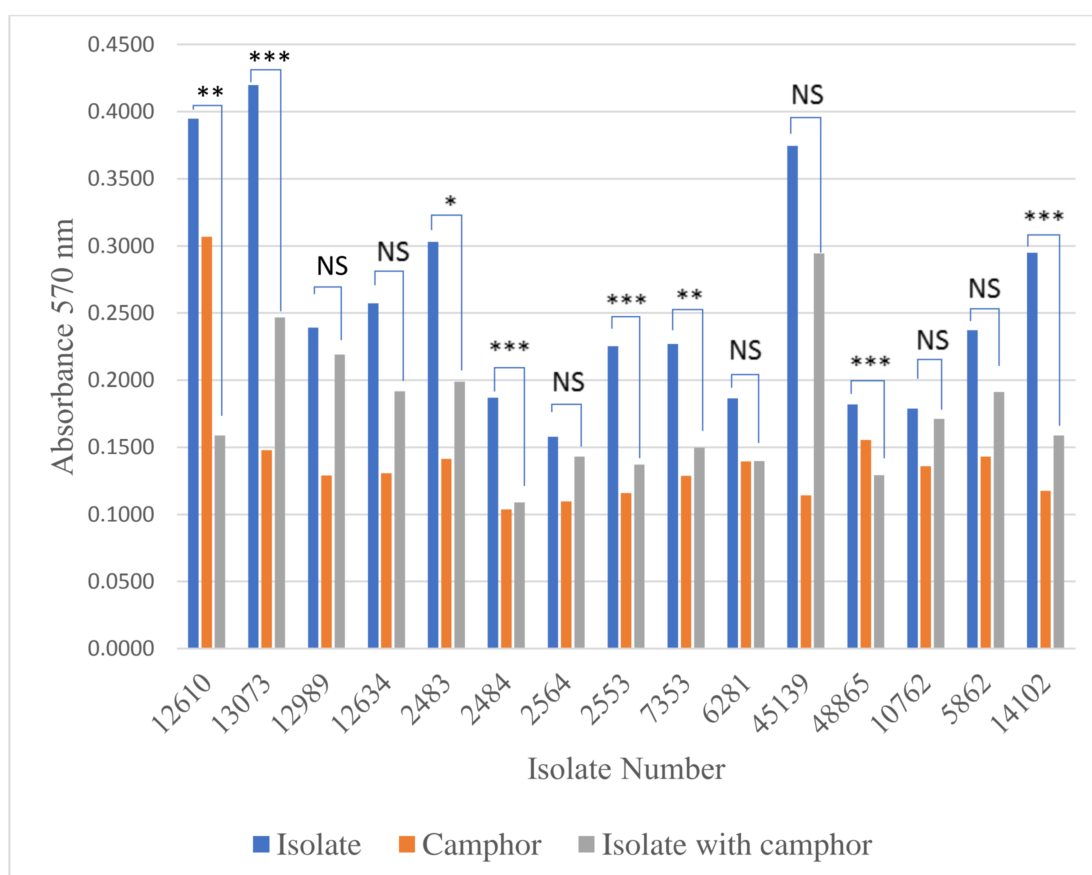


Figure 67. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.0143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

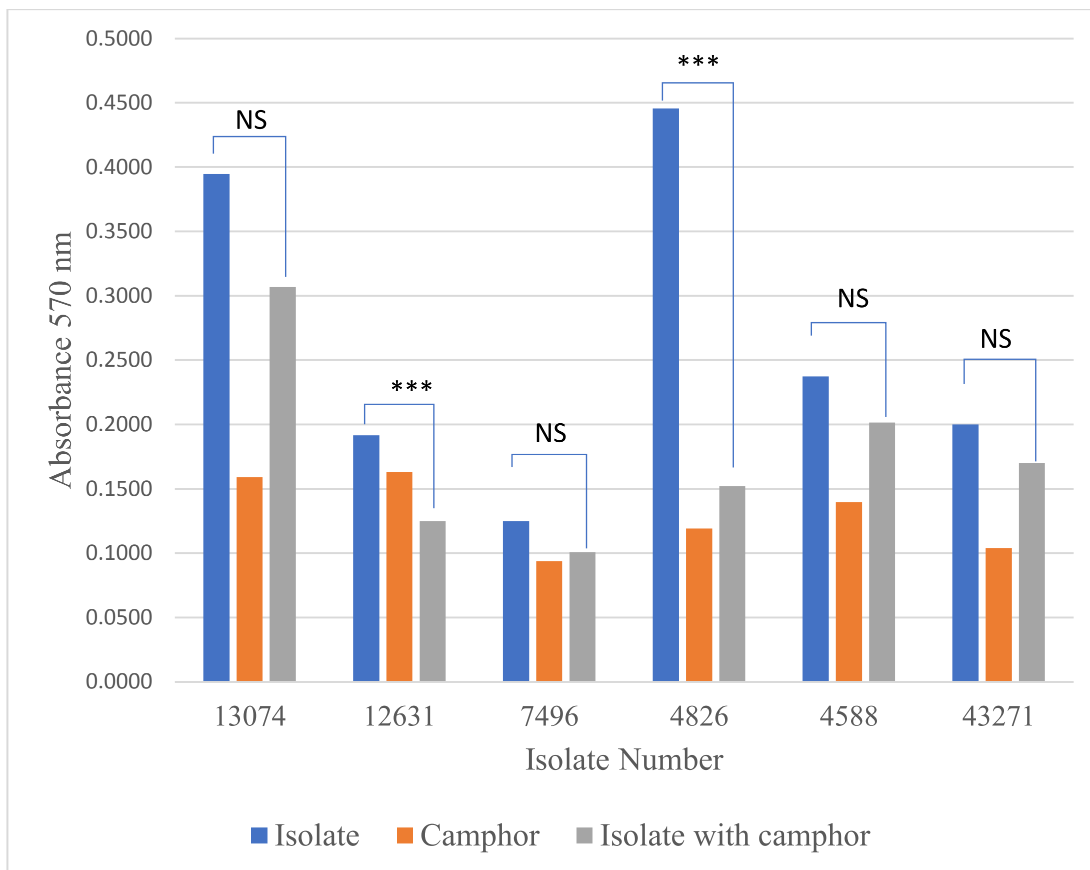


Figure 68. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 μL of 0.0143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.2.2. The effect of camphor solution of concentration 0.072 g/ml, reflecting a concentration five times higher than the concentration of camphor in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 μL of 0.072 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that increasing the concentration of camphor solution to 0.072 g/ml exhibited an increased inhibition in biofilm formation in the majority of tested methicillin susceptible *S. aureus* strains where isolates 13073, 12989, 12634 and 45139 showed an increased inhibition in their biofilm formation with 100 μL of 0.072

g/ml camphor solution as compared to the lower concentration of 0.0143 g/ml camphor solution at an equal volume of 100 µL (Table 31.a and Figure 69). The major increase in inhibition of biofilm formation was observed with isolate 2483 where inhibition in biofilm formation increased from 12.81 % which was considered insignificant inhibition to 40.99 % considered to be a significant inhibition of biofilm formation. In addition, isolates 2564, 2553 and 10762 whose biofilm formation was not affected by 100 µL of 0.0143 g/ml were still not altered by a higher concentration of camphor solution. On the other hand, all except one methicillin resistant strain exhibited an inhibition in biofilm formation and this inhibition ranged from 9.16 % with isolate 4588 and up to 47.94 % with isolate 13074 (Table 31.b and Figure 70). Isolate 7496 biofilm formation was not affected by increase in concentration of camphor solution and showed a similar inhibition of biofilm formation to that observed at the lower 0.0143 g/ml concentration of camphor solution. However, isolate 12631 whose biofilm was previously significantly inhibited at the lower concentration of 0.0143 g/ml camphor solution, lacked an inhibition in biofilm formation once the concentration of camphor solution was increased to 0.072 g/ml.

Table 31. Effect of 100 μL volume of camphor solution of concentration 0.072 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.072 g/ml camphor solution	$O.D_{570\ nm}$ of 100 μL of 0.072 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.3944	0.1288	0.2053	47.94
2	13073	0.3376	0.1566	0.2525	25.21
3	12989	0.2091	0.1086	0.1384	33.84
4	12634	0.2056	0.1249	0.1721	16.28
5	2483	0.2701	0.1174	0.1594	40.99
6	2484	0.1794	0.1225	0.1510	15.82
7	2564	0.1580	0.1134	0.1685	
8	2553	0.1601	0.1250	0.1747	
9	7353	0.1919	0.1301	0.1654	13.80
10	6281	0.1803	0.1421	0.1735	3.79
11	45139	0.4366	0.1589	0.1946	55.43
12	48865	0.1572	0.1335	0.1460	7.11
13	10762	0.1593	0.1242	0.1849	
14	5862	0.2112	0.1399	0.1592	24.65
15	14102	0.2556	0.1244	0.1675	34.49

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.072 g/ml camphor solution	$O.D_{570\ nm}$ of 100 µL of 0.072 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.3944	0.1333	0.2053	47.94
2	12631	0.1656	0.1209	0.1751	
3	7496	0.1193	0.0893	0.1040	12.84
4	4826	0.4165	0.1569	0.1922	53.85
5	4588	0.2211	0.1271	0.2009	9.16
6	43271	0.2088	0.1159	0.1554	25.59

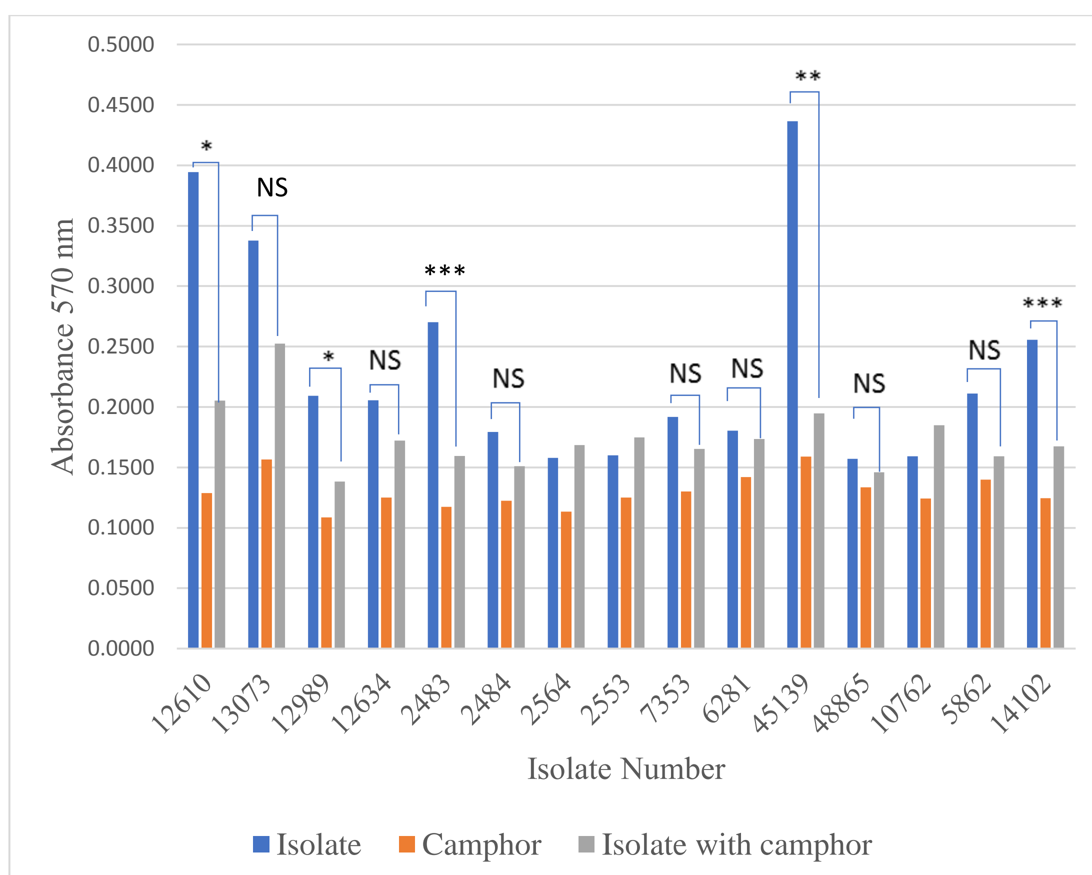


Figure 69. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.072 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

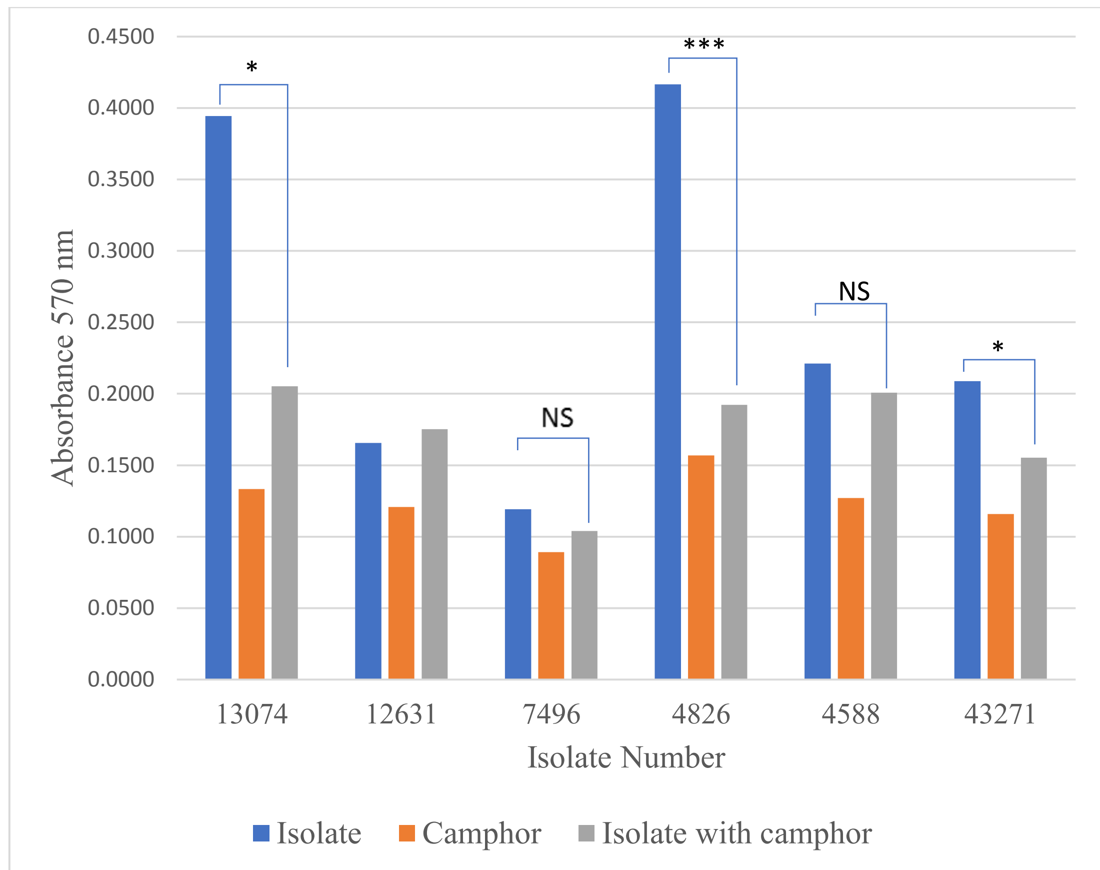


Figure 70. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.072 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 µL of 0.072 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that, methicillin susceptible *S. aureus* strains showed a comparable inhibition in biofilm formation to that observed with 100 µL of 0.072 g/ml camphor solution except for isolates 13073, 6281 and 48865 whose inhibition of biofilm formation was increased with 150 µL of 0.072 g/ml camphor solution as compared to 100 µL of 0.072 g/ml camphor solution (Table 32.a and Figure 71). Also, iolate 45139 had a more significant inhibition in biofilm formation at 150 µL of 0.072 g/ml camphor solution as compared to 100 µL of 0.072 g/ml camphor solution. Isolates 2564, 2553

and 10762 which lacked an inhibition in biofilm formation with 100 μL of 0.072 g/ml camphor solution showed a similar lack in inhibition of biofilm formation with 150 μL of 0.072 g/ml camphor solution. Isolate 2484 lacked inhibition of biofilm formation with 150 μL of 0.072 g/ml camphor solution. On the other hand, for the methicillin resistant *S. aureus* strains, it was shown that 150 μL of 0.072 g/ml camphor solution had an increased effect on inhibition of biofilm formation of isolate 4588 as compared to 100 μL of 0.072 g/ml camphor solution, while 150 μL of 0.072 g/ml camphor solution had a less significant effect on the inhibition of biofilm formation of isolates 13074, 4826 and 43271 as compared to 100 μL of 0.072 g/ml camphor solution (Figure 72). The absence or insignificant inhibition in biofilm formation of isolates 12631 and 7496 with 150 μL of 0.072 g/ml camphor solution was comparable to that observed with 100 μL of 0.072 g/ml camphor solution.

Table 32. Effect of 150 μL volume of camphor solution of concentration 0.072 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.072 g/ml camphor solution	$O.D_{570\ nm}$ of 150 μL of 0.072 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.3279	0.1520	0.1527	53.42
2	13073	0.3779	0.1611	0.1941	48.64
3	12989	0.2092	0.1213	0.1565	25.18
4	12634	0.2263	0.1286	0.1791	20.83
5	2483	0.2542	0.1233	0.1659	34.75
6	2484	0.1705	0.1172	0.1955	
7	2564	0.1612	0.1234	0.1670	
8	2553	0.1632	0.1129	0.1657	
9	7353	0.1833	0.1218	0.1686	8.06
10	6281	0.1806	0.1358	0.1528	15.36
11	45139	0.3934	0.1222	0.1369	65.19
12	48865	0.1838	0.1510	0.1464	20.37
13	10762	0.1520	0.1305	0.1601	
14	5862	0.2374	0.1316	0.1501	36.79
15	14102	0.2313	0.1320	0.1848	20.11

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.072 g/ml camphor solution	$O.D_{570\ nm}$ of 150 µL of 0.072 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.3279	0.1520	0.1527	53.42
2	12631	0.1802	0.1238	0.1711	5.04
3	7496	0.1143	0.0841	0.1047	8.34
4	4826	0.3838	0.1297	0.1572	59.04
5	4588	0.2103	0.1304	0.1683	19.97
6	43271	0.1724	0.1223	0.1336	22.46

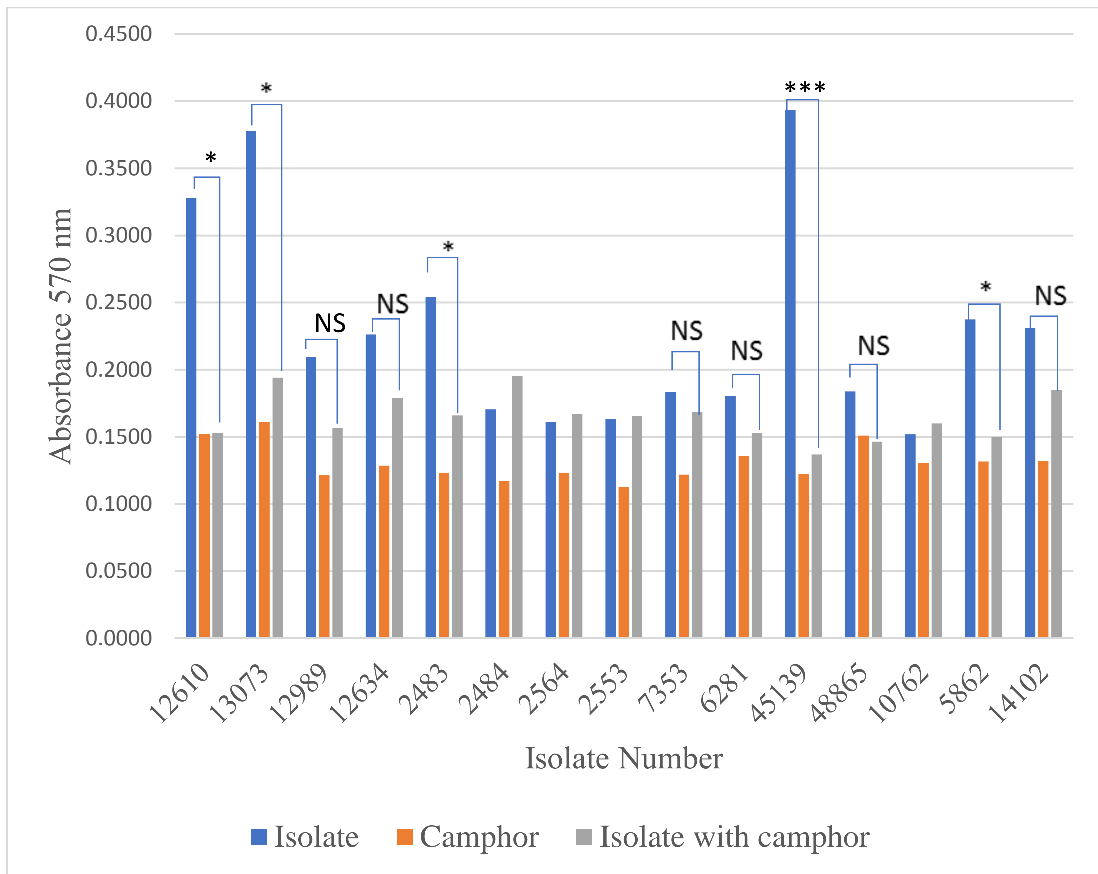


Figure 71. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.072 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

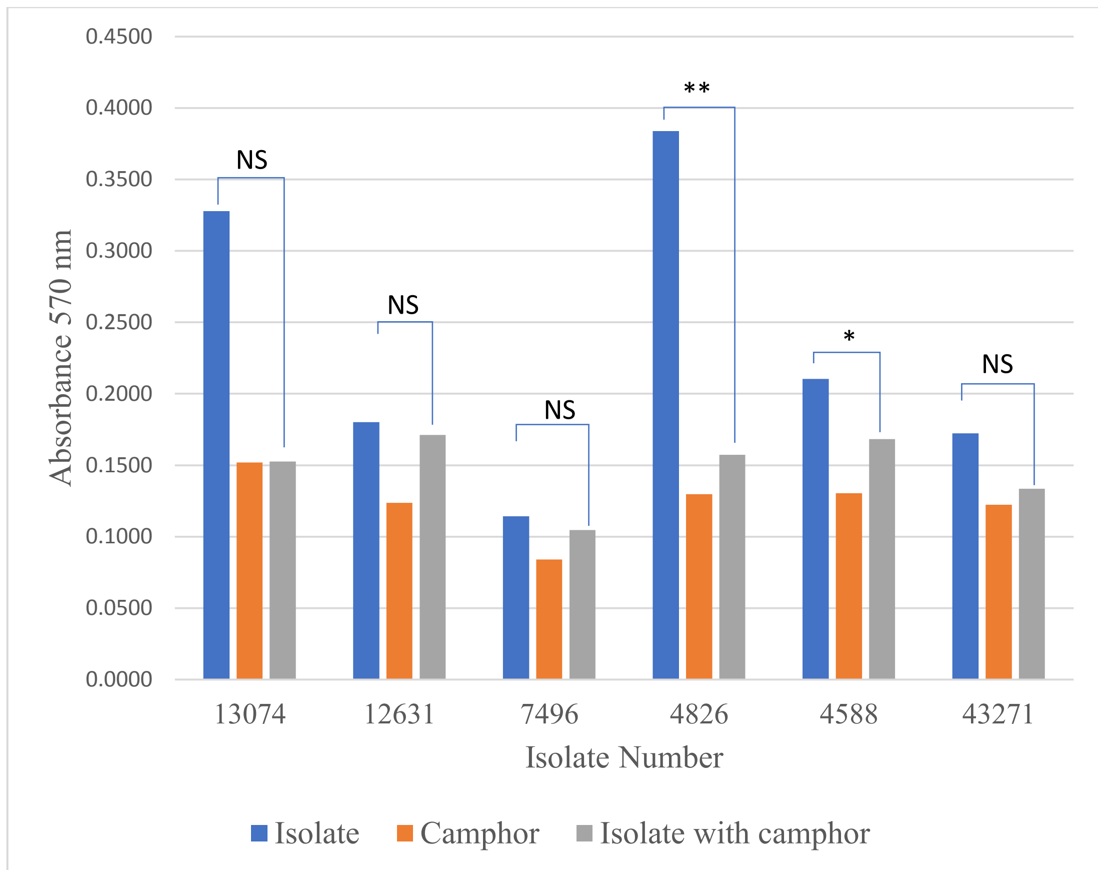


Figure 72. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.072 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.2.3. The effect of camphor solution of concentration 0.143 g/ml, reflecting a concentration ten times higher than the concentration of camphor in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 µL of 0.143 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that increasing the concentration of camphor solution to 0.143 g/ml had a more significant effect on inhibition of biofilm formation of few methicillin susceptible *S. aureus* strains where isolates 5862, 10762, 48865 and 7353 had a more significant inhibition in their biofilm formation (Table 33.a and Figure 73). Isolates

12989, 2484, 2564 and 6281 lacked an effect on inhibition in biofilm formation with 0.143 g/ml camphor solution. On the other hand, only three methicillin resistant *S. aureus* isolates which are 12631, 7496 and 4826 showed inhibition in biofilm formation with 0.143 g/ml camphor. Isolates 12631 and 7496 had an increased inhibition in biofilm formation with 0.143 g/ml while 4826 had a reduced inhibition in biofilm formation as compared to 100 µL of 0.072 g/ml (Table 33.b and Figure 74).

Table 33. Effect of 100 μL volume of camphor solution of concentration 0.143 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.143 g/ml camphor solution	$O.D_{570\ nm}$ of 100 μL of 0.143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.1392	0.1063	0.1215	12.72
2	13073	0.2835	0.1612	0.1646	41.96
3	12989	0.1931	0.1781	0.2436	
4	12634	0.1669	0.1325	0.1241	25.68
5	2483	0.2310	0.2584	0.1397	39.55
6	2484	0.2022	0.1597	0.2276	
7	2564	0.1997	0.1902	0.2535	
8	2553	0.1438	0.1737	0.1206	16.16
9	7353	0.2272	0.1585	0.1166	48.67
10	6281	0.1493	0.2106	0.2185	
11	45139	0.1873	0.1430	0.1516	19.03
12	48865	0.2387	0.1321	0.1176	50.72
13	10762	0.2076	0.1556	0.1626	21.66
14	5862	0.1709	0.1177	0.1211	29.11
15	14102	0.1731	0.2396	0.1522	12.12

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 100 µL of 0.143 g/ml camphor solution	O.D570 nm of 100 µL of 0.143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.1345	0.3293	0.1303	3.15
2	12631	0.2087	0.1170	0.1597	23.45
3	7496	0.1315	0.0906	0.0908	30.95
4	4826	0.2007	0.1524	0.1615	19.51
5	4588	0.2213	0.2281	0.2760	
6	43271	0.1818	0.2372	0.2620	

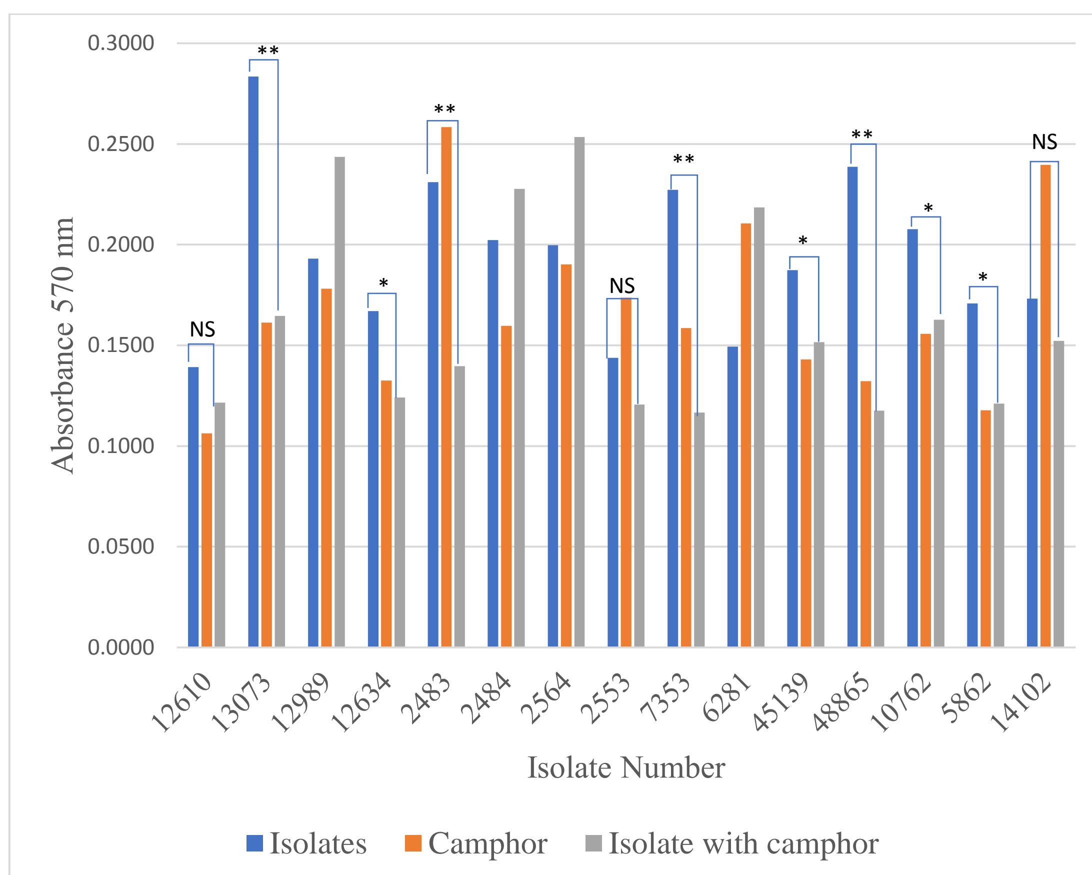


Figure 73. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.143 g/ml camphor solution (0.01 < p ≤ 0.05 (*), 0.001 < p ≤ 0.01 (**), p ≤ 0.001 (***))

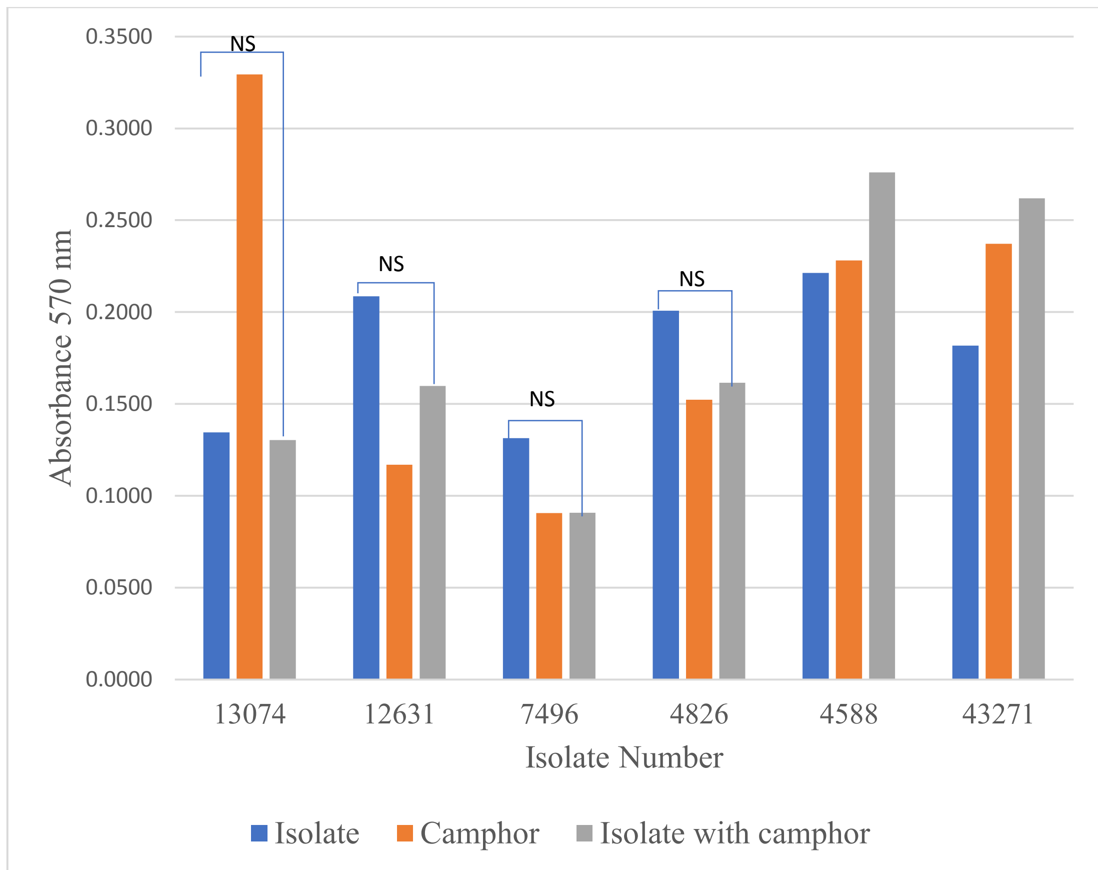


Figure 74. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

The effect of 150 µL of 0.143 g/ml camphor solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 150 µL of 0.143 g/ml camphor solution which contained 0.022 g of camphor, had a weaker effect on inhibition of biofilm formation of methicillin susceptible *S. aureus* strains as compared to 100 µL of 0.143 g/ml camphor solution and to lower concentrations of camphor solution where seven MSSA isolates out of 15 exhibited no inhibition in their biofilm formation with 150 µL of 0.143 g/ml camphor solution (Table 34.a and Figure 75). On the other hand, methicillin resistant *S. aureus* strains exhibited varied inhibition effect than that observed with 100 µL of

0.143 g/ml camphor solution where biofilm formation of isolates 13074 and 12631 was not inhibited with 150 μL of 0.143 g/ml camphor solution while biofilm formation of isolates 4588 and 43271 was not inhibited with 100 μL of 0.143 g/ml camphor solution (Table 34.b and Figure 76).

Table 34. Effect of 150 μL volume of camphor solution of concentration 0.143 g/ml on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.143 g/ml camphor solution	$O.D_{570\ nm}$ of 150 μL of 0.143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	12610	0.1381	0.3745	0.3945	
2	13073	0.3021	0.0962	0.1608	46.76
3	12989	0.1716	0.2700	0.0895	47.88
4	12634	0.1708	0.3125	0.1528	10.57
5	2483	0.1812	0.2070	0.3582	
6	2484	0.2036	0.1658	0.1550	23.86
7	2564	0.1782	0.0932	0.1341	24.77
8	2553	0.1580	0.1721	0.3888	
9	7353	0.1978	0.3010	0.2627	
10	6281	0.1598	0.1800	0.2176	
11	45139	0.1682	0.1593	0.2277	
12	48865	0.1937	0.2631	0.1885	2.69
13	10762	0.1647	0.1514	0.1082	34.30
14	5862	0.1843	0.1701	0.2486	
15	14102	0.1999	0.2245	0.1474	26.30

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.143 g/ml camphor solution	$O.D_{570\ nm}$ of 150 µL of 0.143 g/ml camphor solution with bacterium	% inhibition of biofilm formation
1	13074	0.1164	0.4052	0.1216	
2	12631	0.1775	0.4311	0.4905	
3	7496	0.1231	0.1029	0.0954	22.51
4	4826	0.2127	0.3462	0.1856	12.74
5	4588	0.1961	0.1048	0.1385	29.36
6	43271	0.1861	0.1220	0.1743	6.29

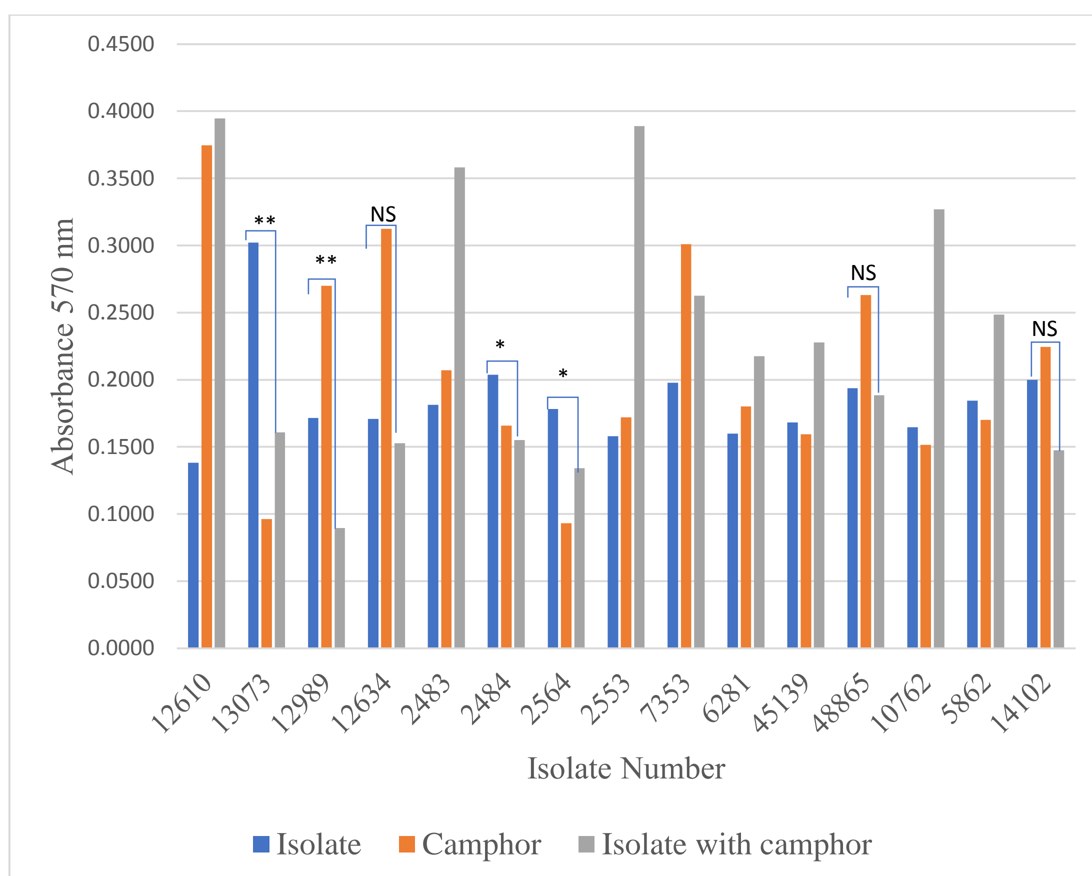


Figure 75. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

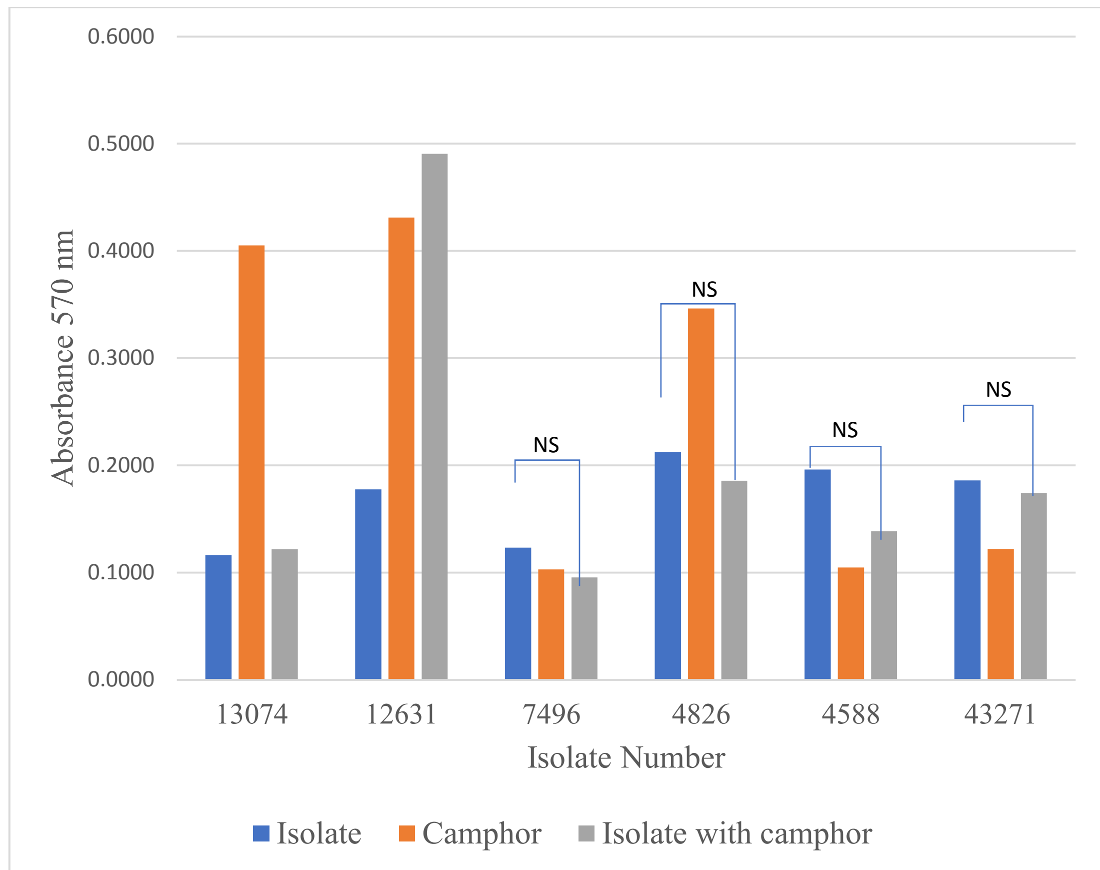


Figure 76. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 μL of 0.143 g/ml camphor solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.3. The effect of 1,8-cineole (Eucalyptol) on inhibition of *Staphylococcus aureus* biofilm formation

3.8.3.1. The effect of 1,8-cineole (Eucalyptol) solution of concentration 0.0273 g/ml, reflecting a concentration equivalent to the concentration of 1,8-cineole in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

In order to determine the effect of 1,8-cineole which is one of the major components of *Rosmarinus officinalis* on inhibition of *S. aureus* biofilm formation, the twenty-one *S. aureus* isolates were incubated with different volumes of 1,8- cineole at different concentrations

The effect of 100 µL of 0.0273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 µL of 0.0273 g/ml 1,8-cineole solution which contained 273 x 10^{-5} g of 1,8-cineole did not possess any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 35, Figure 77 and Figure 78).

Table 35. Effect of 100 µL volume of 0.0273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.0273 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 100 µL of 0.0273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	12610	0.1297	0.1288	0.4560	
2	13073	0.3097	0.1228	0.4602	
3	12989	0.2448	0.2011	0.3854	
4	12634	0.1796	0.2831	0.4077	
5	2483	0.2651	0.1693	0.4267	
6	2484	0.2418	0.1475	0.5286	
7	2564	0.3829	0.2890	0.9186	
8	2553	0.3595	0.2789	0.4897	
9	7353	0.2001	0.1052	0.2783	
10	6281	0.1500	0.1282	0.2683	
11	45139	0.4038	0.2149	0.8312	
12	48865	0.2209	0.2730	0.9228	
13	10762	0.3218	0.1814	0.5711	
14	5862	0.1330	0.1181	0.2680	
15	14102	0.2431	0.2661	0.5936	

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 100 µL of 0.0273 g/ml 1,8-cineole solution	O.D570 nm of 100 µL of 0.0273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.1723	0.2803	0.4591	
2	12631	0.2550	0.1201	0.4792	
3	7496	0.1709	0.3621	0.3756	
4	4826	0.1526	0.0877	0.2423	
5	4588	0.1619	0.2415	0.3395	
6	43271	0.1441	0.3241	0.4175	

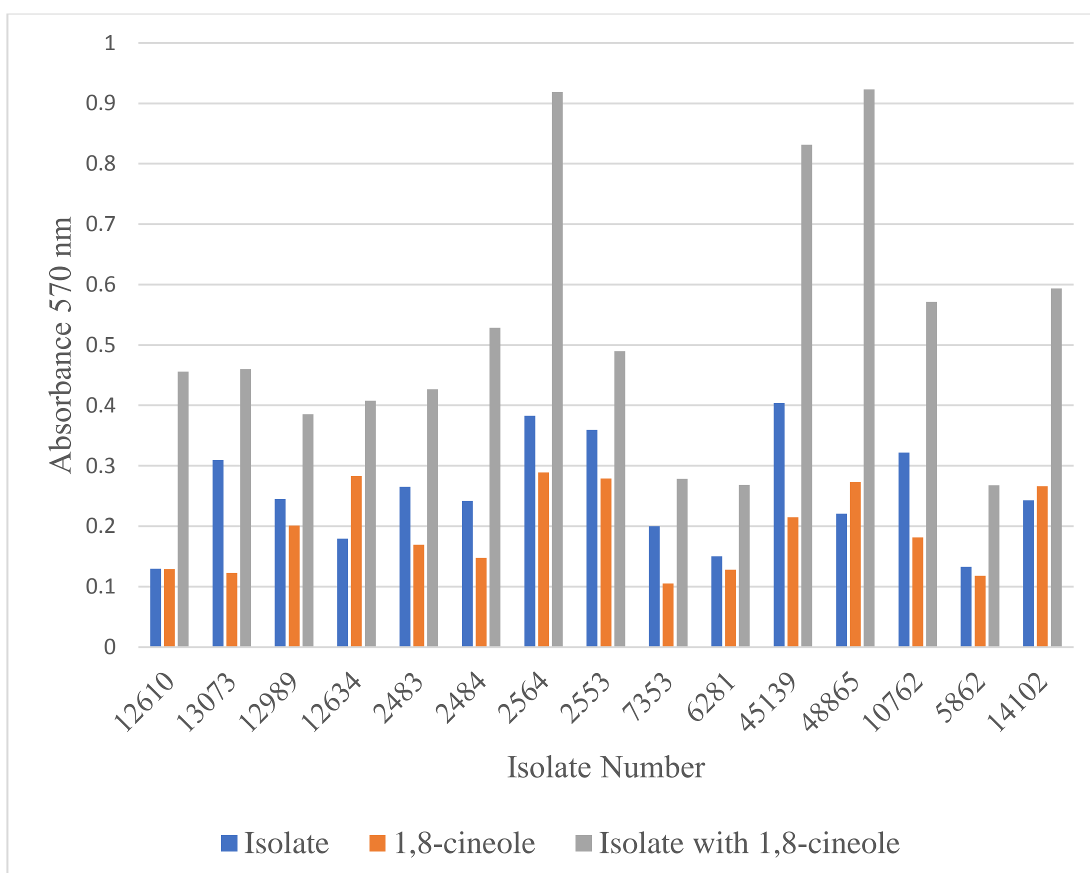


Figure 77. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.0273 g/ml 1,8-cineole solution

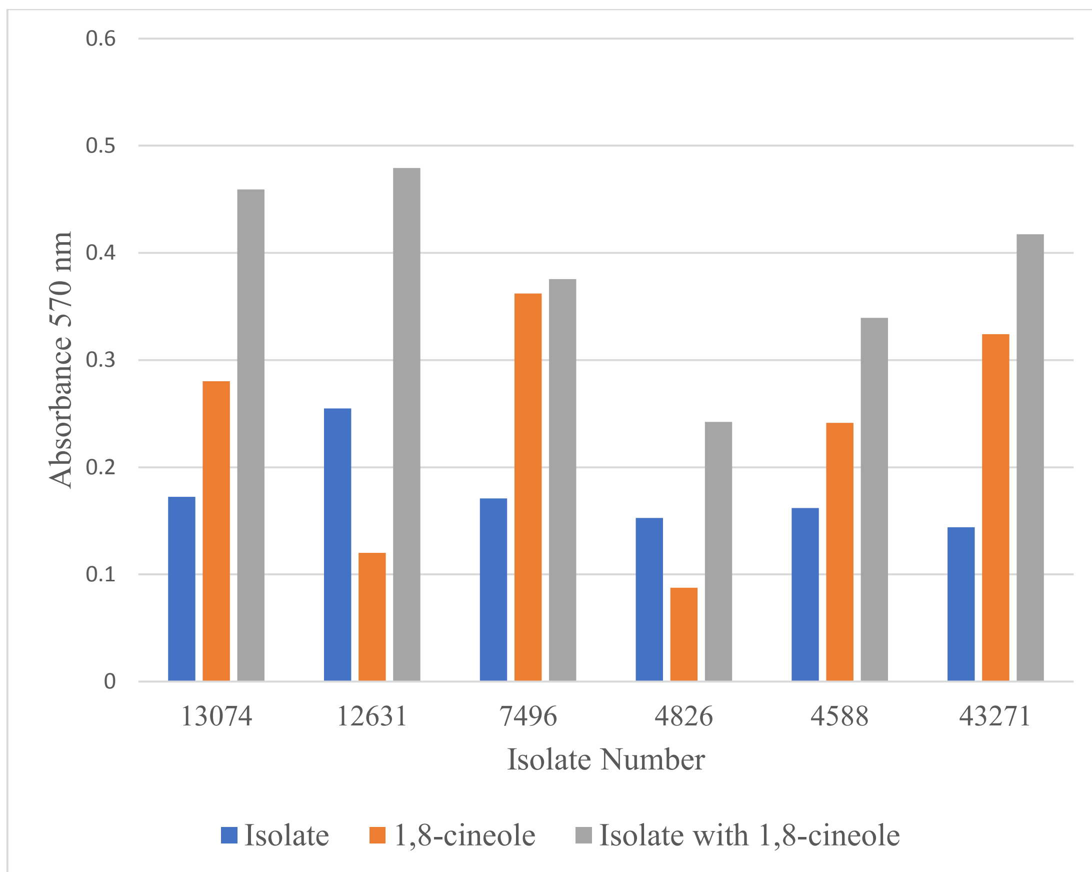


Figure 78. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.0273 g/ml 1,8-cineole solution

The effect of 150 µL of 0.0273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 150 µL of 0.0273 g/ml 1,8-cineole solution which contained 41 x 10^{-4} g of 1,8-cineole did not possess any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 36, Figure 79 and Figure 80).

Table 36. Effect of 150 μL volume of 0.0273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strain

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.0273 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 150 μL of 0.0273 g/ml 1,8-cineole solution with bacterium	% inhibition Of biofilm formation
1	12610	0.1366	0.3466	0.4483	
2	13073	0.2287	0.4085	0.4715	
3	12989	0.2027	0.3299	0.4776	
4	12634	0.1680	0.4618	0.2058	
5	2483	0.2721	0.3143	0.6538	
6	2484	0.2219	0.3554	0.7327	
7	2564	0.3610	0.6821	0.4863	
8	2553	0.3058	0.4056	0.2742	
9	7353	0.2132	0.3998	0.9400	
10	6281	0.1537	0.3081	0.5341	
11	45139	0.6866	0.7181	0.7106	
12	48865	0.3508	0.5845	0.4702	
13	10762	0.2576	0.4212	0.6230	
14	5862	0.1266	0.3079	0.3687	
15	14102	0.2542	0.2782	0.3493	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 µL of 0.0273 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 150 µL of 0.0273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.1553	0.6317	0.1814	
2	12631	0.2380	0.4804	0.9565	
3	7496	0.1599	0.3383	0.2156	
4	4826	0.1480	0.2681	0.3807	
5	4588	0.1571	0.3663	0.1530	
6	43271	0.1436	0.2965	0.2433	

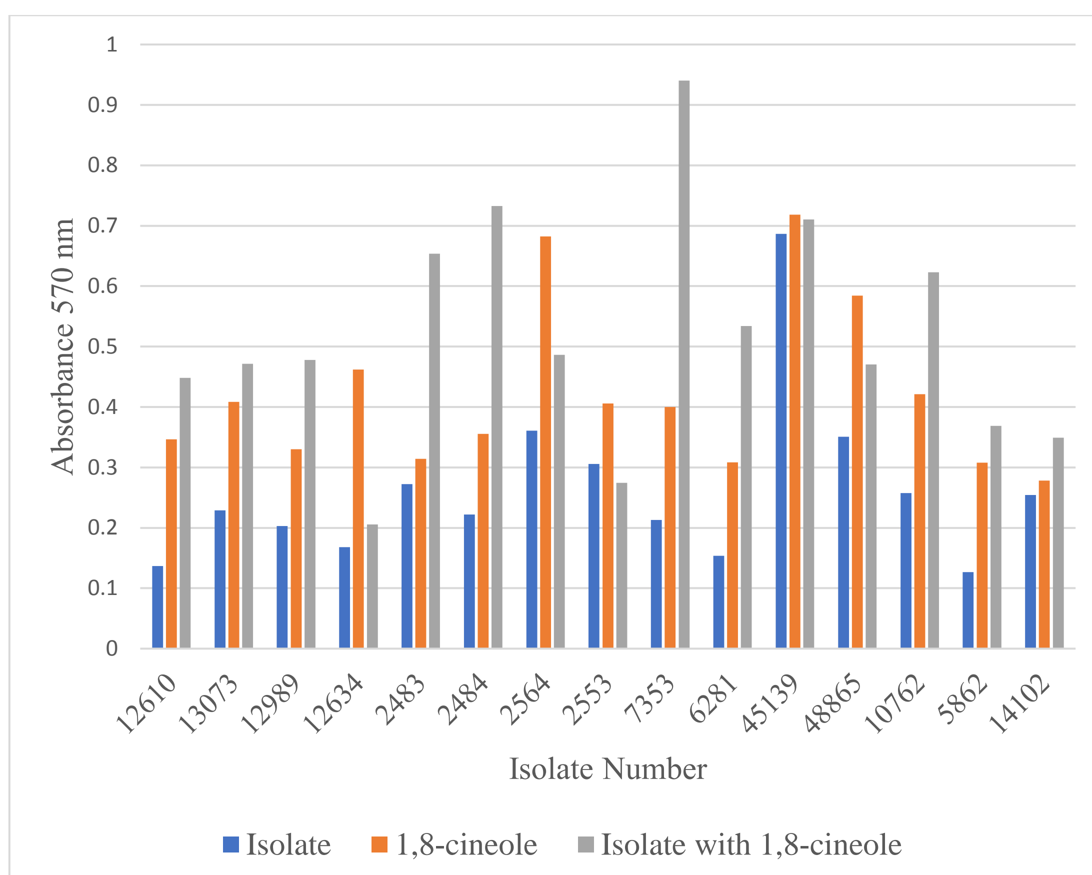


Figure 79. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.0273 g/ml 1,8-cineole solution

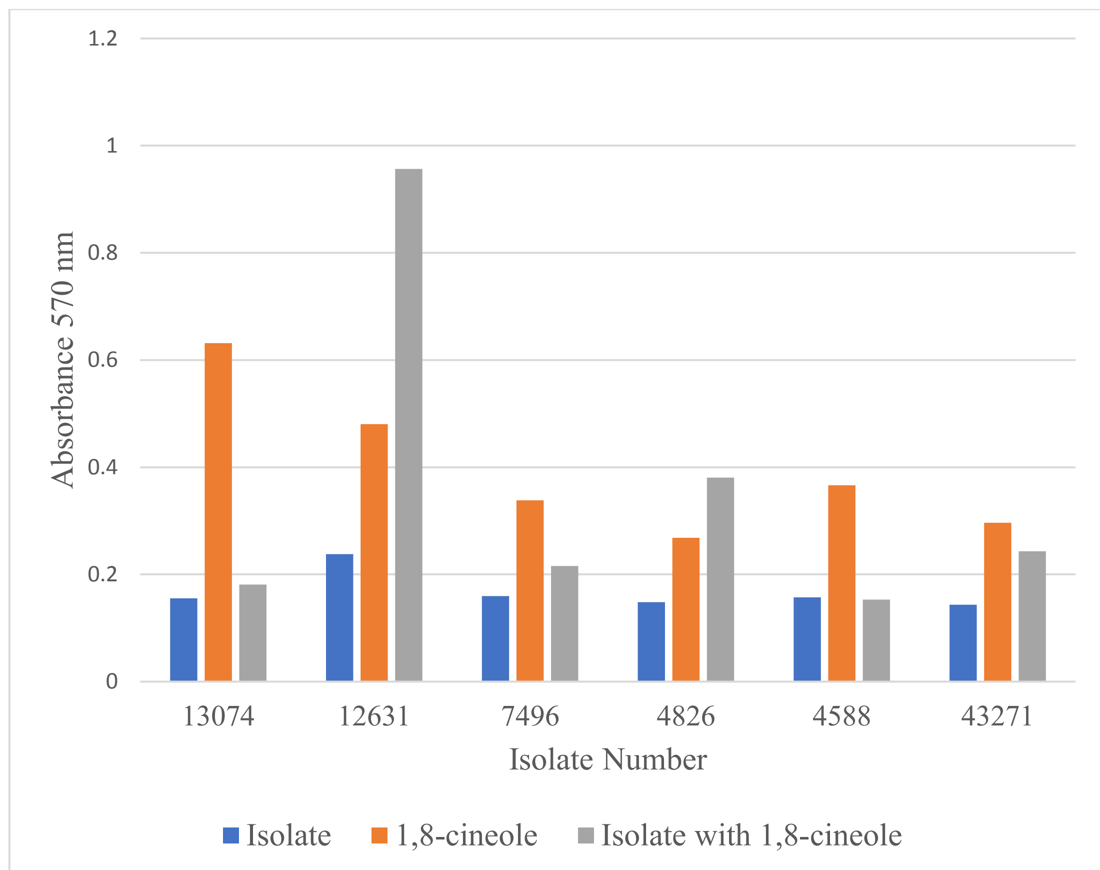


Figure 80. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 μL of 0.0273 g/ml 1,8-cineole solution

3.8.3.2. The effect of 1,8-cineole (Eucalyptol) solution of concentration 0.135 g/ml, reflecting a concentration five times higher than the concentration of 1,8-cineole in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 μL of 0.135 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 μL of 0.135 g/ml 1,8-cineole solution which contained 135 $x10^{-4}$ g of 1,8-cineole, did not have any effect on the inhibition of biofilm formation of all but one methicillin susceptible *S. aureus* strain which is isolate number 12989. Isolate 12989 displayed a 29.66 % inhibition of biofilm formation with a concentration of 1,8-cineole five times higher than the concentration of 1,8-cineole in

the *Rosmarinus officinalis* methanol extract (Table 37.a and Figure 81). However, increasing the concentration of 1,8-cineole to a concentration five times higher than the concentration of 1,8-cineole in the *Rosmarinus officinalis* methanol extract did not exert any effect on the inhibition of biofilm formation of the methicillin resistant *S. aureus* strains (Table 37.b and Figure 82).

Table 37. Effect of 100 µL volume of 0.135 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.135 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 100 µL of 0.135 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	12610	0.2404	0.6827	0.4343	
2	13073	0.3829	0.6655	0.3862	
3	12989	0.7885	0.8991	0.5546	29.66
4	12634	0.2152	0.6585	0.3997	
5	2483	0.1944	0.2070	0.7354	
6	2484	0.1974	0.3141	0.8238	
7	2564	0.2149	0.5972	0.4425	
8	2553	0.1718	0.2880	0.4335	
9	7353	0.1928	0.2584	0.4453	
10	6281	0.1767	0.4259	0.5304	
11	45139	0.4564	0.3250	0.6125	
12	48865	0.2806	0.3554	0.6710	
13	10762	0.2054	0.3142	0.6209	
14	5862	0.1716	0.4513	0.7643	
15	14102	0.2149	0.3770	0.7097	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 μL of 0.135 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 100 μL of 0.135 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.2342	0.6783	0.4129	
2	12631	0.2260	0.7873	0.3451	
3	7496	0.1255	0.6009	0.2155	
4	4826	0.2691	0.3432	0.4867	
5	4588	0.1954	0.2640	0.3786	
6	43271	0.2122	0.4215	0.6132	

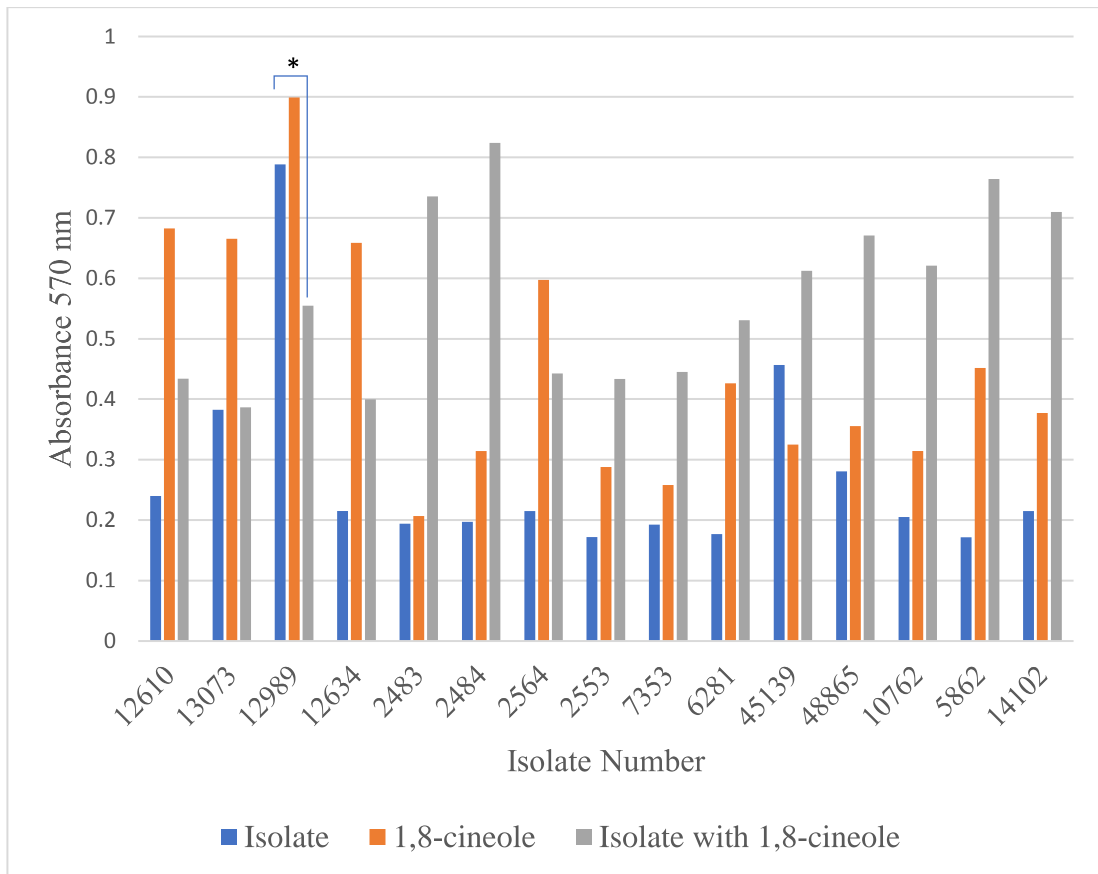


Figure 81. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.135 g/ml 1,8-cineole solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

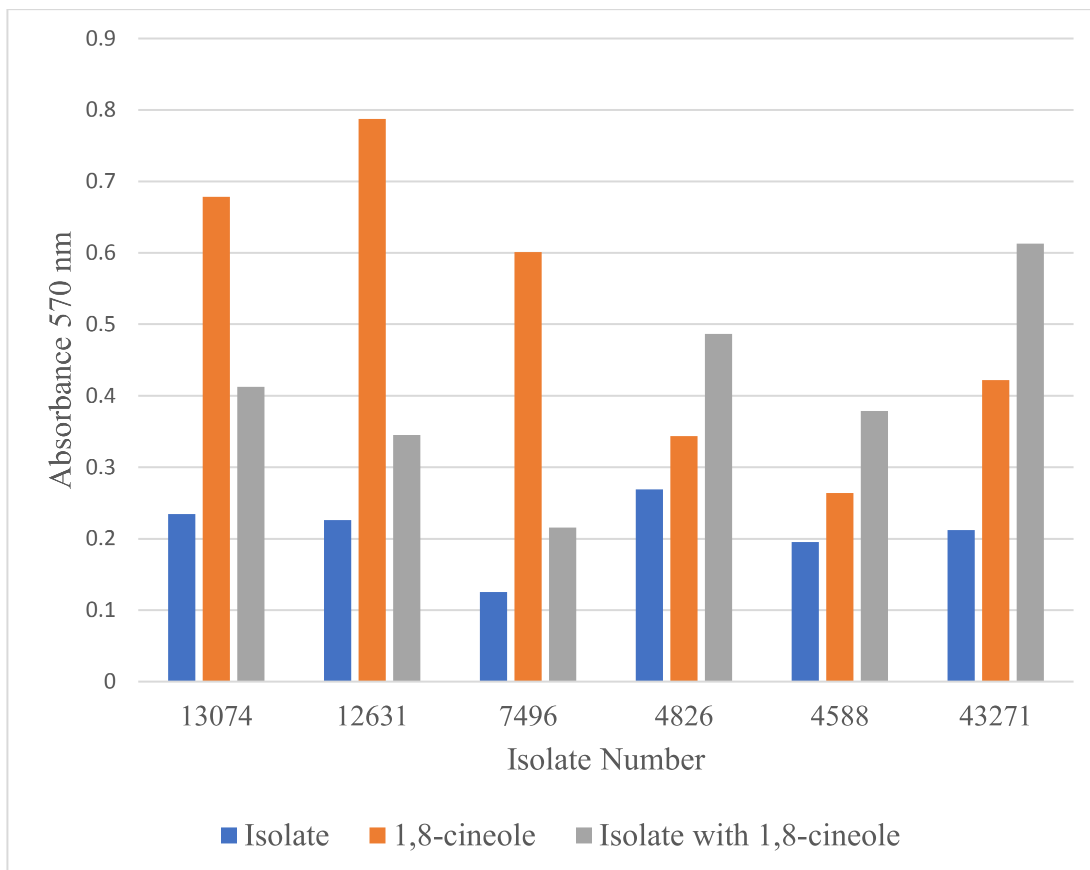


Figure 82. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 μL of 0.135 g/ml 1,8-cineole solution

The effect of 150 μL of 0.135 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 150 μL of 0.135 g/ml 1,8-cineole solution which contained 0.02 g of 1,8-cineole, did not possess any effect on inhibition of biofilm formation of all but two methicillin susceptible *S aureus* strains: isolates 12989 and 12610. Isolate 12989 which previously exhibited a 29.66 % inhibition in biofilm formation once exposed to 100 μL of 0.135 g/ml 1,8-cineole, exhibited 31.98 % inhibition of biofilm formation once with 150 μL of 0.135 g/ml 1,8-cineole (Table 38.a and Figure 83). On the other hand, 150 μL of 0.135 g/ml 1,8-cineole did not exert an effect on inhibition of biofilm formation of all methicillin resistant *S. aureus* strains except for isolate 7496

which showed an insignificant inhibition in biofilm formation with 6.64 % inhibition (Table 38.b and Figure 84).

Table 38. Effect of 150 μL volume of 0.135 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 150 μL of 0.135 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 150 μL of 0.135 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	12610	0.2879	0.8360	0.2685	6.74
2	13073	0.3474	0.8409	0.5096	
3	12989	0.7274	1.1944	0.4948	31.98
4	12634	0.1954	0.9441	0.5094	
5	2483	0.1927	0.1712	0.5878	
6	2484	0.1801	0.2931	0.6425	
7	2564	0.1985	0.6841	0.3565	
8	2553	0.2146	0.3615	0.6508	
9	7353	0.1822	0.2873	0.5499	
10	6281	0.1939	0.4569	0.5495	
11	45139	0.4288	0.3387	0.6874	
12	48865	0.2987	0.3809	0.9045	
13	10762	0.1656	0.3375	0.9083	
14	5862	0.2636	0.3651	0.6684	
15	14102	0.2038	0.3572	0.7786	

b.

Isolate Number		O.D570 nm of bacterial isolate	O.D570 nm of 150 µL 0.135 g/ml 1,8-cineole solution	O.D570 nm of 150 µL of 0.135 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.2591	0.8630	0.4759	
2	12631	0.1961	0.8061	0.3955	
3	7496	0.1219	0.1181	0.1138	6.64
4	4826	0.2916	0.3003	0.5998	
5	4588	0.1704	0.3010	0.5098	
6	43271	0.1859	0.4998	1.0334	

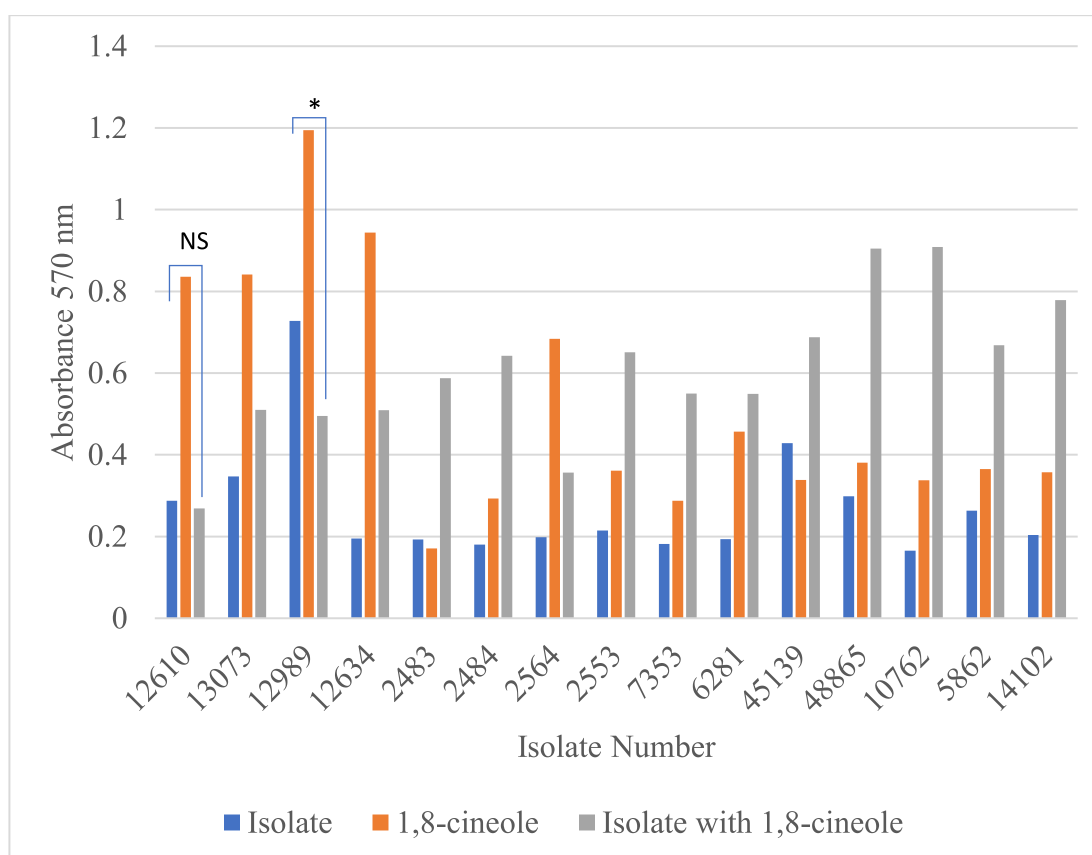


Figure 83. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.135 g/ml 1,8-cineole solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

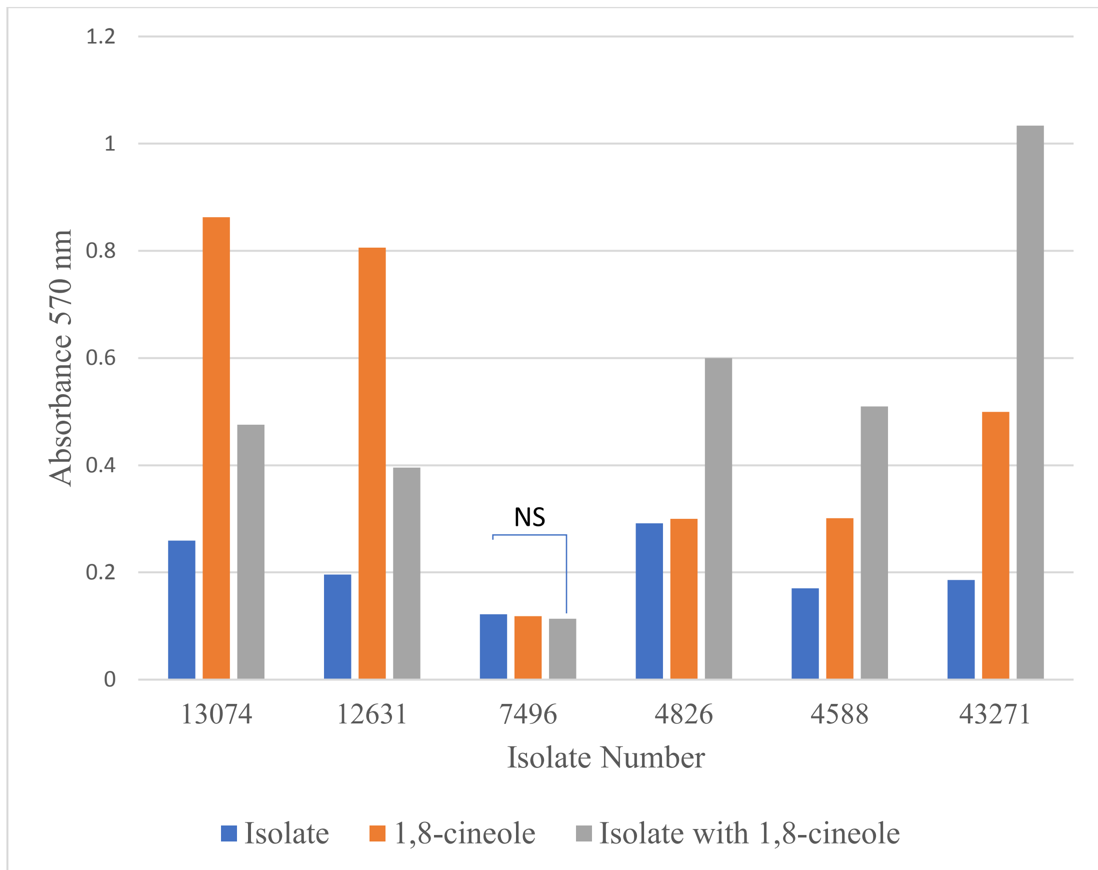


Figure 84. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.135 g/ml 1,8-cineole solution ($0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**), $p \leq 0.001$ (***))

3.8.3.3. The effect of 1,8-cineole (Eucalyptol) solution of concentration 0.273 g/ml, reflecting a concentration ten times higher than the concentration of 1,8-cineole in *Rosmarinus officinalis* methanolic extract, on inhibition of *Staphylococcus aureus* biofilm formation

The effect of 100 µL of 0.273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 100 µL of 0.273 g/ml 1,8-cineole solution which contained 0.0273 g of 1,8-cineole, did not have any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains even at a

concentration ten times higher than the concentration of 1,8-cineole in the *Rosmarinus officinalis* methanol extract (Table 39, Figure 85 and Figure 86).

Table 39. Effect of 100 µL volume of the 0.273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains

a.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.273 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 100 µL of 0.273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	12610	0.2107	1.3397	0.9036	
2	13073	0.5438	1.5478	1.2131	
3	12989	0.4399	1.5364	1.0711	
4	12634	0.2271	1.2520	1.0070	
5	2483	0.1772	1.0651	1.3099	
6	2484	0.2062	0.9260	1.2608	
7	2564	0.1700	1.1170	1.2756	
8	2553	0.1680	1.0930	1.2565	
9	7353	0.2054	1.0611	1.2661	
10	6281	0.1695	1.0323	1.2133	
11	45139	0.6608	0.9186	1.2132	
12	48865	0.2104	1.0838	1.3676	
13	10762	0.1673	1.1525	1.2804	
14	5862	0.1761	0.7919	1.0501	
15	14102	0.1824	1.1065	1.2388	

b.

Isolate Number		$O.D_{570\ nm}$ of bacterial isolate	$O.D_{570\ nm}$ of 100 µL of 0.273 g/ml 1,8-cineole solution	$O.D_{570\ nm}$ of 100 µL of 0.273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.4476	1.4382	1.1608	
2	12631	0.2288	1.4702	1.0100	
3	7496	0.1341	0.2937	0.2576	
4	4826	0.3293	1.0046	1.1591	
5	4588	0.1683	0.7878	1.0107	
6	43271	0.2177	1.0148	1.0491	

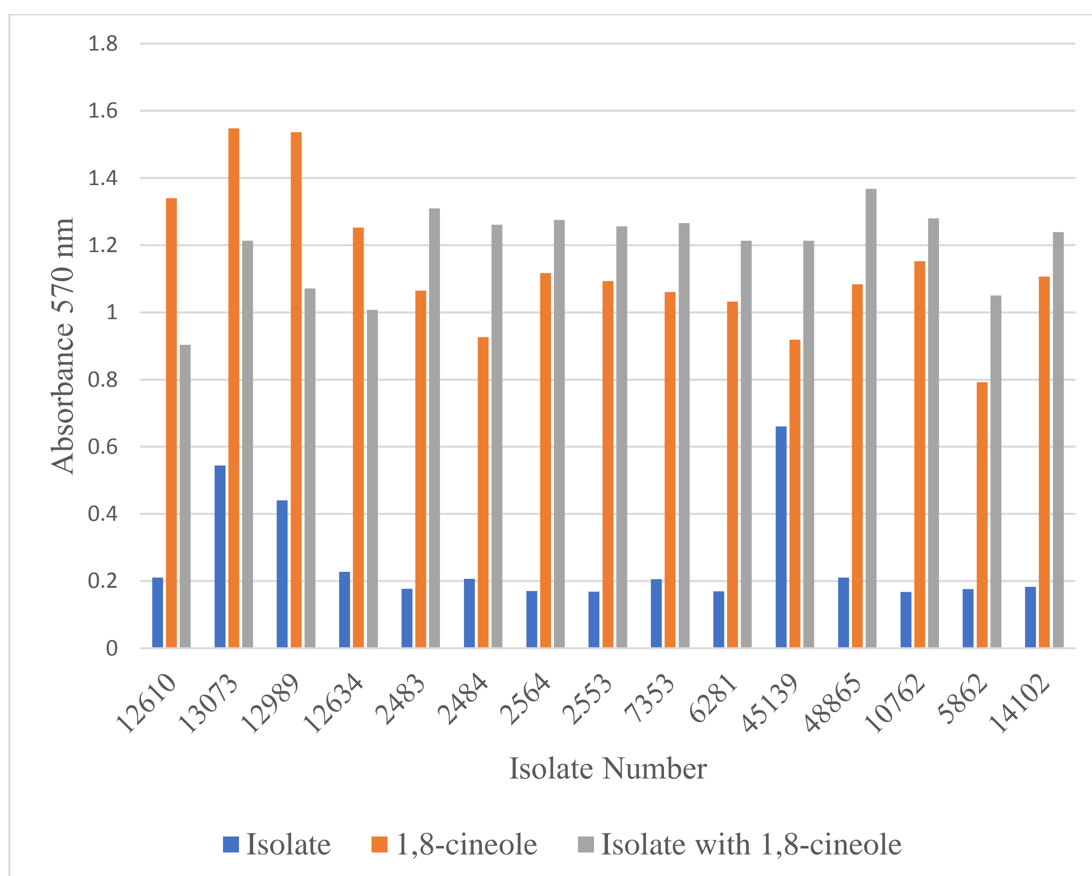


Figure 85. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 100 µL of 0.273 g/ml 1,8-cineole solution

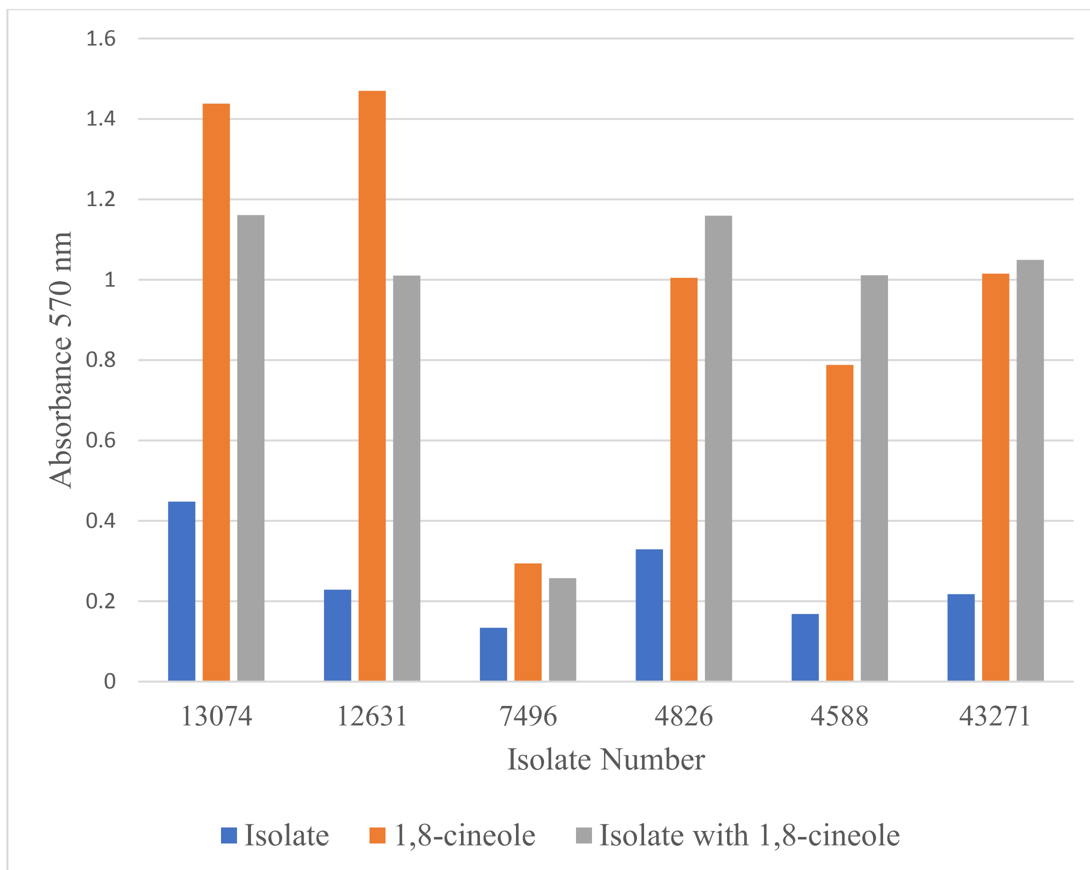


Figure 86. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 100 µL of 0.273 g/ml 1,8-cineole solution

The effect of 150 µL of 0.273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of Staphylococcus aureus biofilm formation

The results showed that 150 µL of 0.273 g/ml 1,8-cineole solution which contained 0.041 g of 1,8-cineole, did not possess any effect on inhibition of biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains (Table 40 and Figure 87 and Figure 88).

Table 40. Effect of 150 μL volume of the 0.273 g/ml 1,8-cineole (Eucalyptol) solution on inhibition of *Staphylococcus aureus* biofilm formation. a. Effect on MSSA strains b. Effect on MRSA strains.

a.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 150 μL of 0.273 g/ml 1,8-cineole solution	O.D$_{570\ nm}$ of 150 μL of 0.273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	12610	0.2817	1.5208	1.2620	
2	13073	0.3478	1.4295	1.3133	
3	12989	0.6388	1.3372	1.2882	
4	12634	0.2115	1.3455	1.2601	
5	2483	0.1967	1.0465	1.2852	
6	2484	0.1909	0.8270	1.1804	
7	2564	0.1916	0.9317	1.2480	
8	2553	0.1837	1.0660	1.2096	
9	7353	0.1897	1.0224	1.1589	
10	6281	0.1655	1.0782	1.1074	
11	45139	0.4182	1.0213	1.2326	
12	48865	0.2437	1.1515	1.2845	
13	10762	0.1841	1.0657	1.3076	
14	5862	0.2092	1.0756	1.2202	
15	14102	0.1982	0.9268	1.0820	

b.

Isolate Number		O.D$_{570\ nm}$ of bacterial isolate	O.D$_{570\ nm}$ of 150 µL of 0.273 g/ml 1,8-cineole solution	O.D$_{570\ nm}$ of 150 µL of 0.273 g/ml 1,8-cineole solution with bacterium	% inhibition of biofilm formation
1	13074	0.3263	1.2369	1.0388	
2	12631	0.2299	1.2643	1.2840	
3	7496	0.1317	1.0107	0.4201	
4	4826	0.2927	1.1019	1.1849	
5	4588	0.1825	1.0596	1.2296	
6	43271	0.1959	1.0540	1.2057	

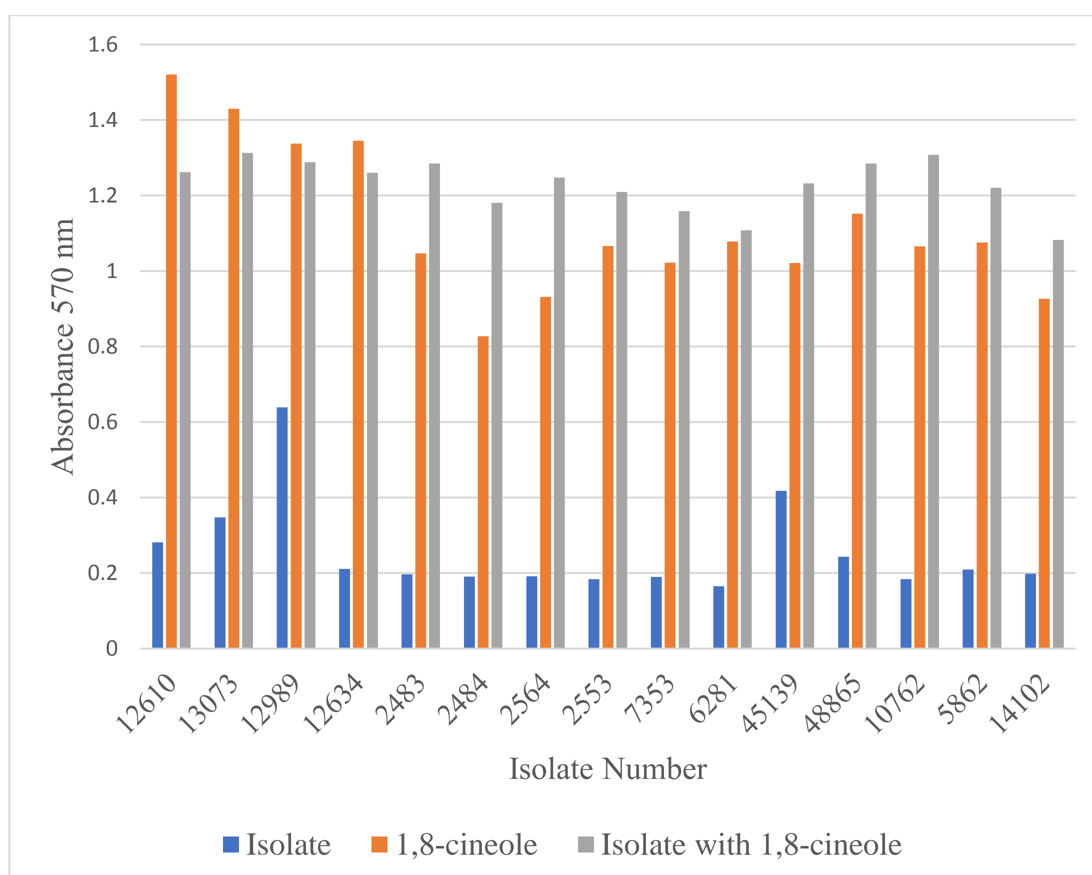


Figure 87. The variation of biofilm formation of methicillin susceptible *Staphylococcus aureus* in presence of 150 µL of 0.273 g/ml 1,8-cineole solution

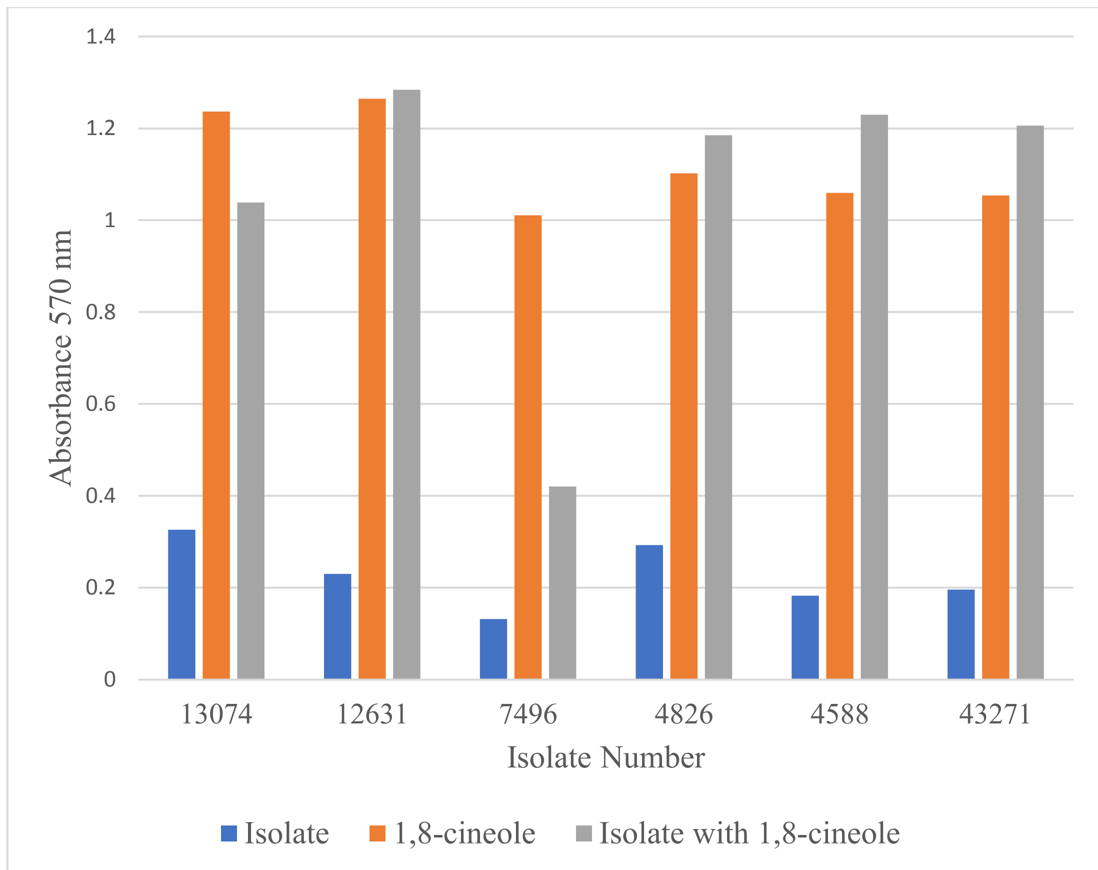


Figure 88. The variation of biofilm formation of methicillin resistant *Staphylococcus aureus* in presence of 150 µL of 0.273 g/ml 1,8-cineole solution

Chapter IV
Discussion

Origanum syriacum is a plant of the Mediterranean region yet, it is most abundantly present in Lebanon and very commonly used. This experimental work reassured the role of *Origanum syriacum* methanolic extract as an antibacterial agent where it was capable of inhibiting the growth of our *S. aureus* isolates. Our experimental work showed that at a concentration of 0.1 g/ml of *Origanum syriacum* methanol extract and higher volumes of 150 μL and up to 300 μL, *Origanum syriacum* methanol extract was capable of inhibiting the growth of both methicillin susceptible and methicillin resistant *S. aureus* strains. This was in accordance with a study in the literature showing that the essential oils of *Origanum syriacum* possessed an antibacterial effect on *Staphylococcus aureus* (Gendy et al., 2015). Interestingly, it was also shown that *Origanum syriacum* methanol extract possessed antibacterial effect against Gram negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa, Campylobacter jejuni* and *Klebsiella pneumonia* (Al-Judaibi, 2015).

However, the target of our experimental work was to detect the ability of the plant to inhibit the biofilm formation of the Gram-positive *Staphylococcus aureus* isolates tested. The results of our experiment, as indicated previously, revealed the remarkable role of *Origanum syriacum* as an inhibitor of biofilm formation of *S. aureus* isolates, that were still viable at the concentrations used. Our work revealed the antibiofilm effect of *Origanum syriacum* methanol extract at 0.02 g/ml concentration which is a concentration five times lower than the concentration at which the antibacterial effect of *Origanum syriacum* methanol extract was detected. In addition, the antibiofilm effect of *Origanum syriacum* was detected at a concentration of 0.01 g/ml which is a

concentration ten times lower than the concentration at which the antibacterial effect of *Origanum syriacum* methanol extract was detected. The results revealed that for the 0.02 g/ml methanol extract both tested volumes which are 50 μL and 100 μL exhibited a significant effect on inhibition of biofilm formation of methicillin susceptible *S. aureus* isolates. However, the higher volume of 100 μL of 0.02 g/ml exhibited a more significant effect on inhibition of biofilm formation of the methicillin resistant *S. aureus* strains. Interestingly, the more diluted *Origanum syriacum* methanol extract of concentration 0.01 g/ml still exhibited an inhibitory effect on biofilm formation of *S. aureus* isolates yet this inhibition of biofilm formation of *S. aureus* isolates was weaker than the inhibition observed at the higher concentration of *Origanum syriacum* methanol extract especially against methicillin resistant *S. aureus* isolates. It is important to note that the higher volume of 100 μL of 0.01 g/ml *Origanum syriacum* methanol extract, which contained 0.001 g of the active components of the *Origanum syriacum* methanol extract which is an equal amount of active component present in 50 μL of 0.02 g/ml *Origanum syriacum* methanol extract produced a similar inhibition in biofilm formation confirming the inhibitory effect of the *Origanum syriacum* methanolic extract on the biofilm formation of both methicillin susceptible and methicillin resistant *S. aureus* strains. These results were in accordance with similar experiments in the literature conducted on a close member of the *Origanum* genus which is *Origanum vulgare* where it was shown that the essential oils of *Origanum vulgare* at sub-MIC of 200, 100, and 50 ng/ml succeeded in reducing sessile bacterial cells (Schillaci et al., 2013).

Interestingly, the inhibitory effect on biofilm formation varied between MSSA strains and MRSA strains, yet this variation was not random. There were certain *S. aureus* isolates that showed a weak or no inhibition of biofilm formation as compared to the

rest of the isolates under the two different concentrations of *Origanum syriacum* methanol extract. In reference to the pulse field gel electrophoresis data analysis, it was revealed that the MSSA isolates 2564, 12634 and 13073 belonged to the same subclade which indicated a genetic similarity between them, and which explains the comparable effect of *Origanum syriacum* methanol extract on their biofilm formation. Similarly, the MRSA strains 7496, 43271, 4826 and 13074 were also found to be genetically related to one another particularly isolates 43271 and 4826 which were the closest to one another and exhibited a comparable inhibition in biofilm formation with *Origanum syriacum* methanol extract. It is worth mentioning that isolate 43271 was collected from a health care center in South Lebanon while isolate 4826 was collected from a hospital in Beirut indicating the carriage and spread of this strain in specific from one region to another over Lebanon.

Thymol, one of major chemical components of *Origanum syriacum* methanolic extract, did not have a notable effect on the inhibition of *S. aureus* biofilm formation. Thymol is a natural monoterpene present in thyme species, so it is an aromatic polar compound that is capable of dissolving in polar organic solvents such as methanol and is partially hydrophilic capable of forming hydrogen bonds (National Center for Biotechnology Information, 2019). The experimental results revealed that thymol did not have an inhibitory effect on the biofilm formation of *S. aureus* strains. At a concentration of 0.0247 g/ml of thymol solution dissolved in methanol, thymol was not capable of inhibiting biofilm formation of any of the *S. aureus* isolates tested whether methicillin susceptible or methicillin resistant. This concentration of 0.0247 g/ml reflected the concentration of thymol in the prepared *Origanum syriacum* methanol extract. It is important to note that, even when the concentration of thymol

solution was increased by five folds to 0.124 g/ml and by ten folds to 0.247 g/ml, the biofilm forming ability of *S. aureus* isolate was not altered.

The effect of carvacrol, the second major component of *Origanum syriacum* methanolic extract on the inhibition of *S. aureus* biofilm formation was also assessed. Carvacrol is derived from the oil of thyme, so it is a natural monoterpene and is a derivative of cymene (National Center for Biotechnology Information, 2019). Similar to thymol, carvacrol is an aromatic compound capable of dissolving in organic polar solvents like methanol and partially hydrophilic capable of hydrogen bonding through its hydroxyl group. The experimental results revealed that carvacrol did not have the ability to inhibit the biofilm formation of *S. aureus* strains. At a concentration of 0.0176 g/ml of carvacrol solution dissolved in methanol, carvacrol was not capable of inhibiting the biofilm formation of any of the *S. aureus* isolates tested whether methicillin susceptible or methicillin resistant. This concentration of 0.0176 g/ml reflected the concentration of carvacrol in the prepared *Origanum syriacum* methanol extract. It is important to note that, even when the concentration of carvacrol solution was increased by five folds to 0.088 g/ml and by ten folds to 0.176 g/ml, the biofilm forming ability of *S. aureus* isolates remained unaltered. In an alternative study in the literature, both thymol and carvacrol were shown to possess an antibiofilm effect, but against Gram-negative bacterium which is *Pseudomonas aeruginosa* (El et al., 2011). The ability of thymol and carvacrol to inhibit biofilm formation in the indicated study by El et al. (2011) was attributed to amphipathic property of thymol and carvacrol which as mentioned previously are aromatic compounds capable with their hydrophobic chain to interact with the hydrophobic lipid rich outer membrane of the Gram-negative bacterial cell wall at sub-growth inhibitory concentrations. This interaction reduced bacterial adhesion to abiotic surfaces, as well as, biotic surfaces

hence, interfering with initiation of biofilm formation. As mentioned previously as well, thymol and carvacrol possess hydrophilic properties attributed by their hydroxyl group. This property allowed thymol and carvacrol to diffuse through the polar matrices. However, the lack of inhibition of biofilm formation of *Staphylococcus aureus* by thymol and carvacrol may be attributed to the inability of those two compounds to efficiently interfere with the thick peptidoglycan layer of *S. aureus* cell wall or interact with the adhesion surface because of the lower hydrophobicity of the peptidoglycan as compared to the lipid outer membrane of Gram negative bacteria. In addition, it may be hypothesized that thymol and carvacrol were not capable to efficiently interfere with the interactions between the polysaccharide intercellular adhesion (ICA) encoded by the *ica* locus in *S. aureus* genome and the abiotic surface to which it adhered even at high concentrations. Moreover, it might have been that thymol and carvacrol diffused into the biofilm matrix, but were degraded by the enzymes existing within the matrix and hence, they were not capable of inhibiting the biofilm formation. It is also suggested that certain physiological changes may have led to a poor diffusion of thymol and carvacrol because of steric hindrance into the biofilm matrix which may affect their ability to inhibit the biofilm formation of *S. aureus* isolates.

The second natural product used in this study is *Rosmarinus officinalis*. *Rosmarinus officinalis* which is another herb of the Meditteranean region known as Rosemary, could be cultivated domestically and could exist in wild forms (Sasikumar, 2004). This study reconfirmed the role of the methanolic extract of *Rosmarinus officinalis* as an antibacterial agent, where it was capable of inhibiting the growth of the tested *S. aureus* isolates. Based on our experimental work it was shown that at a concentration of 0.1 g/ml and at high volumes of 200 μL and 300 μL, *Rosmarinus officinalis*

methanol extract was capable of inhibiting the growth of both methicillin susceptible and methicillin resistant *S. aureus* strains. These results were in accordance with data mentioned previously from the literature revealing the antibacterial effect of *Rosmarinus* against Gram-positive bacteria such as *Staphylococcus aureus* and Gram-negative bacteria such as *Pseudomonas aeruginosa* (Ait-Ouazzou et al., 2011; Assaf, et al., 2016).

It is important to note, however, that in comparison with the antibacterial effect of *Origanum syriacum, Rosmarinus officinalis* possessed a weaker antibacterial effect and that the effect was observed at higher volumes of the methanolic extract as compared to the volumes at which the antibacterial effect of *Origanum syriacum* was observed.

The main focus of the above study was to assess the effect of *Rosmarinus officinalis* methanol extract on the inhibition of the biofilm forming ability of *Staphylococcus aureus* isolates. The results revealed that for the 0.02 g/ml methanol extract both tested volumes which were 100 μL and 150 μL exhibited a significant effect on the inhibition of biofilm formation of the methicillin susceptible *S. aureus* isolates. Similarly, 0.02 g/ml *Rosmarinus officinalis* methanol extract at both tested volumes, 100 μL and 150 μL, was effective in inhibiting the biofilm formation of methicillin resistant *S. aureus* strains. Yet, the higher volume of 150 μL was more capable of significantly inhibiting the biofilm forming ability of five out of the six MRSA isolates as compared to the volume of 100 μL which inhibited biofilm formation of four MRSA isolates only. Interestingly, at a lower concentration of 0.01 g/ml, *Rosmarinus officinalis* methanol extract still exhibited an inhibitory effect on the biofilm forming ability of methicillin susceptible *S. aureus* isolates similar to that observed at the higher concentration of 0.02 g/ml *Rosmarinus officinalis* methanol extract. This inhibition of biofilm

formation of *S. aureus* isolates at 0.01 g/ml of *Rosmarinus officinalis* methanol extract was weaker than the inhibition observed at the higher concentration of 0.02 g/ml of *Rosmarinus officinalis* methanol extract against methicillin resistant *S. aureus* isolates. Importantly, there was no remarkable variation in inhibition of the biofilm forming ability of the isolates between the two volumes of 100 μL and 150 μL of 0.01 g/ml *Rosmarinus officinalis* methanolic extract. In the literature, it was shown that *Rosmarinus officinalis* essential oils have the ability to disrupt the hydrophobic glucans of a Gram-positive bacterium, *Streptococcus sobrinus* associated with oral biofilms; while in another study, it was shown that impairment in the synthesis of glucans disrupted biofilm formation (Ge et al., 2016; Kouidhi, Al Qurashi, & Chaieb, 2015). This suggested that the effect of *Rosmarinus officinalis* on the inhibition of *S. aureus* biofilm formation may be through the disruption of glucans which is required for proper adhesion and initiation of biofilm formation by bacterial cells. In a separate study, it was shown that *Rosmarinus officinalis* ethanolic extract inhibited the biofilm formation of *S. aureus* at concentrations of 0.032 mg/ml which is lower than the concentration of the methanolic extract of *Rosmarinus officinalis* that we utilized (Quave et al., 2008). However, another separate study revealed that extraction of *Rosmarinus officinalis* with a different organic solvent which is ethyl acetate revealed an effect on inhibition of *S. aureus* biofilm formation at a concentration of 0.125 mg/ml (Costa et al., 2015). In order to further validate the results of our study which revealed the effect of *Rosmarinus officinalis* methanolic extract on inhibition of *S. aureus* biofilm formation, those results were compared to the effect of *Rosmarinus officinalis* extracted with another organic solvent which is ethylene glycol and dissolved in DMEM on inhibition on *S. aureus* biofilm formation. The effective concentration of *Rosmarinus officinalis* extracted with ethylene glycol and dissolved

in DMEM on inhibiting *S. aureus* biofilm formation was 200 mg/ml which is ten folds highr than the concentration of *Rosmarinus officinalis* methanolic extract utilized in our study (de Oliveira et al., 2017).

It is important to note, however, that the inhibitory effect of *Rosmarinus officinalis* methanol extract on the forming ability of the biofilm of *S. aureus* isolates was independent of the genetic relatedness between the *S. aureus* strains. The methicillin susceptible strains, which showed a weak or lack of inhibition of biofilm formation with *Rosmarinus officinalis* methanol extract were not close genetic relatives. Yet, it is worth mentioning that the two MRSA isolates, 7496 and 43271 which were relatively related to one another, both showed a similar weak or absence in inhibition of the biofilm formation once exposed to *Rosmarinus officinalis* methanol extract. However, the more closely related MRSA isolates 43271 and 4826 displayed a varying inhibition in biofilm forming ability with *Rosmarinus officinalis* methanol extract.

The effect of one of the major chemical components of *Rosmarinus officinalis* methanolic extract: α-pinene, on the inhibition of the *S. aureus* biofilm formation was assessed. α-pinene is a natural terpene present in coniferous trees such as pine trees and in plants such as rosemary (*Rosmarinus officinalis)* (National Center for Biotechnology Information, 2019). It is an alkene with a six membered ring structure that is capable of alternating its stereochemical conformation and which is soluble in organic solvents such as alcohol. The experimental results revealed that α-pinene was capable of inhibiting biofilm formation of approximately all the methicillin susceptible as well as methicillin resistant *S. aureus* strains at the three tested concentrations of 0.0194 g/ml, 0.097 g/ml and 0.194 g/ml α-pinene solution. Yet, the inhibitory effect of α-pinene solution on *S. aureus* strains at lower concentration of 0.0194 g/ml as well as at 0.097 g/ml was the most significant. This inhibitory effect on biofilm formation

was reduced at 150 μL of the highest tested concentration of 0.194 g/ml of α-pinene solution. The strength of the inhibitory effect of α-pinene solution varied between one isolate and the other at each concentration. The most significant results were observed with 150 μL of 0.0194 g/ml α-pinene solution which is a concentration equivalent to the concentration of α-pinene in the *Rosmarinus officinalis* methanolic extract. In reference to the literature, studies conducted on α-pinene revealed a role for α-pinene in inhibition of biofilm formation of *Bacillus cereus*, a Gram-positive bacterium and *Escherichia coli,* a Gram-negative bacterium (Kerekes et al., 2013). In addition, α-pinene was shown to be capable of inhibiting biofilm formation of fungi such as the yeast *Pichia anomala* (Kerekes et al., 2013). The work by Kerekes et al., (2013) studied the three-dimensional structure of the biofilm after being exposed to α-pinene and showed that cells treated with α-pinene partially adhered to the abiotic surface as compared to the untreated cells which showed microcolony formation which is a stage beyond irreversible attachment and more into biofilm maturation. In the literature, it was indicated that the presence of a double bond in a chemical component enhanced its inhibitory effect on bacterial cells (Dorman & Deans, 2000). As mentioned previously, α-pinene is an alkene so this chemical property of α-pinene may have contributed to its inhibitory effect on bacterial cells and their biofilm forming ability. In addition, it was indicated that the target of terpenoids such as α-pinene was the cell wall of the bacterial cells (Dorman & Deans, 2000). This suggested that, the antibiofilm effect of α-pinene at sub-inhibitory concentration may be through disrupting the proper adhesion of the bacterial cells to a surface, which would prevent the irreversible attachment of bacterial cells to that surface, thus preventing microcolony formation and biofilm maturation. It is important to note, however, that one of the patterns detected between the effect of α-pinene on the isolates and their

genetic relatedness was the effect of α-pinene on the two genetically closely related isolates 7353 and 10762 which showed a similar inhibition of biofilm formation under the varying tested concentrations of α-pinene.

The effect of another major component of *Rosmarinus officinalis* methanolic extract: camphor, on the inhibition of *S. aureus* biofilm formation was also assessed. Camphor is a terpenoid that can exist as a white crystalline powder that is present in the bark of woody trees, as well as, in certain herbs such as *Rosmarinus officinalis* (National Center for Biotechnology Information, 2019). The experimental results revealed that camphor was an effective inhibitor of biofilm formation of *S. aureus* strains both methicillin susceptible and resistant. This inhibition was most effective at the lowest tested concentration of camphor which was 0.0143 g/ml, equivalent to the camphor concentration in *Rosmarinus officinalis* methanolic extract. It remained effective at the higher concentrations of 0.072 g/ml and 0.143 g/ml, yet this effect was lower at those higher concentrations as compared to the initial lowest concentration 0.0143 g/ml. It is worth mentioning that 150 μL of 0.0143 g/ml of camphor solution was more effective against methicillin susceptible *S. aureus* strains while 100 μL of 0.0143 g/ml was more effective against methicillin resistant *S. aureus* strains. In the literature, it was reported that camphor possessed antibiofilm properties not only against bacterial cells but also against fungi, particularly, the fungal pathogen, *Candida albicans.* It was shown that camphor inhibited the stage of hyphal formation by *Candida albicans* through downregulation of genes involved in progression to hyphal stage which are *ECE1, ECE2, RBT1* and *EED1* as indicated by transcriptomic analysis by qRT-PCR which is a factor thought to have prevented biofilm formation of *Candida albicans* (Manoharan, Lee, & Lee, 2017).

Interestingly, once camphor was synthetically coupled to di-selenides which are selenium containing derivatives involved in a variety of redox reactions in living organisms, the camphor-di-selenide exhibited a remarkable antibiofilm activity against *S. aureus* as well as another Gram-positive bacterium which is *Staphylococcus epidermidis* (Sancineto et al., 2016). In addition, in a separate study on an ethanolic extract whose major component was camphor, the antibiofilm effect was observed against the Gram-negative bacterium *Escherichia coli* (Al-Bakri, Othman, & Afifi, 2010). The literature reports that compounds possessing an unsaturated hexane ring which is the case with camphor have a stronger effect against bacterial cells (Dorman & Deans, 2000). This is a chemical property of camphor that may explain its antibiofilm effect against *Staphylococcus aureus* strains at sub-inhibitory concentrations. It is important to note, however, that the effect of camphor against the *S. aureus* isolates was not random, as it was noted that in the majority of the times isolates that were closely related to one another based on the genetic analysis exhibited low or no alteration in biofilm formation at the varying concentrations of camphor. It was also observed that isolates 48865, 2564, 12634 and 13073 which belonged to one subclade were not significantly affected by camphor under most of the tested experimental conditions.

Furthermore, a third major component of *Rosmarinus officinalis* methanolic extract: 1,8-cineole (Eucalyptol), was also tested for an effect on the inhibition of *S. aureus* biofilm formation. 1,8-cineole is a cyclohexanol compound and a monoterpene known also as Eucalyptol and found mainly in the Eucalyptus tree as well as in herbs like *Rosmarinus officinalis* (National Center for Biotechnology Information, 2019). The experimental results revealed that 1,8-cineole did not have an inhibitory effect on the biofilm formation of *S. aureus* strains. At a concentration of 0.0273 g/ml of 1,8-cineole

solution dissolved in methanol, 1,8-cineole was not capable of inhibiting biofilm formation of any of the *S. aureus* isolates tested whether methicillin susceptible or methicillin resistant. This concentration of 0.0273 g/ml reflected the concentration of 1,8-cineole in the prepared *Rosmarinus officinalis* methanol extract. However, it is important to note that, when the concentration of 1,8-cineole solution was increased by five folds to 0.135 g/ml, the biofilm formation of only two methicillin susceptible *S. aureus* isolates was altered. Those two isolates were isolate 12989 and isolate 12610. In addition, 0.135 g/ml of 1,8-cineole had a slight effect on inhibiting the biofilm forming ability of one methicillin resistant *S. aureus* isolate which is 7496. It is important to note that this effect of 1,8-cineole on 12989 and 12610 was not random because those two isolates were the closest in relatedness to one another according to the dendrogram. Once the concentration of 1,8-cineole was increased by ten folds to 0.273 g/ml, the biofilm formation of *S. aureus* isolates remained unaltered. However, it was reported in the literature that 1,8-cineole possessed an antibiofilm effect against the uropathogen *Proteus mirabilis.* It was shown that 1,8-cineole, as a major component in Eucalyptos oil, inhibited the biofilm forming ability of *Proteus mirabilis* on a catheter. In addition, it was reported in a separate work that 1,8-cineole possessed an antibiofilm effect against the Gram-positive multi drug resistant *Enterococcus faecalis* (Correa et al., 2019). The lack of inhibitory effect of 1,8-cineole on biofilm formation of the *S. aureus* isolates, in the above study, may require re-assessment of biofilm formation of these *S. aureus* isolates with different concentrations of 1,8-cineole, higher and lower than those tested.

In general, the methanolic extracts of the two natural products *Origanum syriacum* and *Rosmarinus officinalis* were more effective than their major chemical components in inhibiting the *S. aureus* biofilm forming ability. For that reason, further studies need

to be conducted in order to further assess the effect of the major chemical components experimented in this study on inhibiting the biofilm forming ability of *S. aureus.* This assessment can be achieved through testing higher and lower concentrations of each of the tested compounds. In addition, altering the solvent might be a means to evaluate the ability of the tested compounds to inhibit the biofilm forming ability of *S. aureus* isolates as changing the solvent might render the compounds more reactive and thus, more effective in inhibiting the biofilm formation of the bacteria tested. Moreover, in order to further assess the effect of the methanolic extracts of *Origanum syriacum* and *Rosmarinus officinalis,* they can be tested against multiresistant gram positive and gram negative bacteria.

Chapter V
Conclusion

It is well known that treating infections caused by sessile bacterial cells embedded in a biofilm matrix is much harder than treating planktonic bacterial cells. The findings of this study, which point towards preventing bacterial adhesion and biofilm maturation, are a very significant advance towards avoiding the difficulties accompanying the treatment of various infections caused by pathogenic organisms. This study which highlighted the significance of *Origanum syriacum* and *Rosmarinus officinalis* as effective inhibitors of *Staphylococcus aureus* biofilm formation, points towards reassessing the importance of traditional medicine in achieving an easily accessible and effective means for prevention of bacterial infections. In addition, this study suggests a possible role for α -pinene and camphor as natural alternatives for coating medical devices that may usually be contaminated with bacterial biofilms such as urinary catheters, pacemakers, prosthetic devices and mechanical heart valves. Further studies must be conducted to establish the important role of such chemicals in preventing infections caused by dangerous pathogens.

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